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Integrating Metabolomics, Genomics, and Disease Pathways in Age-Related Macular Degeneration

The EYE-RISK Consortium

İlhan E. Acar, MSc,[1] Laura Lores-Motta, PhD,[1] Johanna M. Colijn, MD, MSc,[2,3] Magda A. Meester-Smoor, PhD,[2,3] Timo Verzijden, MSc,[2,3] Audrey Cougnard-Gregoire, PhD,[4] Soufiane Ajana, PhD,[4] Benedicte M.J. Merle, PhD,[4] Anita de Breuk, MD, MSc,[1] Thomas J. Heesterbeek, MD, MSc,[1] Erik van den Akker, PhD,[5,6] Mohamed R. Daha, PhD,[7] Birte Claes,[8] Daniel Pauleikhoff, MD, PhD,[9] Hans-Werner Hense, MD, PhD,[8] Cornelia M. van Duijn, PhD,[3,10] Sascha Fauser, MD, PhD,[11,12] Carel B. Hoyng, MD, PhD,[1] Cécile Delcourt, PhD,[4] Caroline C.W. Klaver, MD, PhD,[1,2,3] Tessel E. Galesloot, PhD,[13] Anneke I. den Hollander, PhD,[1] for the EYE-RISK Consortium

Purpose: The current study aimed to identify metabolites associated with age-related macular degeneration (AMD) by performing the largest metabolome association analysis in AMD to date, as well as aiming to determine the effect of AMD-associated genetic variants on metabolite levels and investigate associations between the identified metabolites and activity of the complement system, one of the main AMD-associated disease pathways.

Design: Case-control association analysis of metabolomics data.

Participants: Five European cohorts consisting of 2267 AMD patients and 4266 control participants.

Methods: Metabolomics was performed using a high-throughput proton nuclear magnetic resonance metabolomics platform, which allows quantification of 146 metabolite measurements and 79 derivative values. Metabolome–AMD associations were studied using univariate logistic regression analyses. The effect of 52 AMD-associated genetic variants on the identified metabolites was investigated using linear regression. In addition, associations between the identified metabolites and activity of the complement pathway (defined by the C3d-to-C3 ratio) were investigated using linear regression.

Main Outcome Measures: Metabolites associated with AMD.

Results: We identified 60 metabolites that were associated significantly with AMD, including increased levels of large and extra-large high-density lipoprotein (HDL) subclasses and decreased levels of very low-density lipoprotein (VLDL), amino acids, and citrate. Of 52 AMD-associated genetic variants, 7 variants were associated significantly with 34 of the identified metabolites. The strongest associations were identified for genetic variants located in or near genes involved in lipid metabolism (*ABCA1*, *CETP*, *APOE*, and *LIPC*) with metabolites belonging to the large and extra-large HDL subclasses. Also, 57 of 60 metabolites were associated significantly with complement activation levels, independent of AMD status. Increased large and extra-large HDL levels and decreased VLDL and amino acid levels were associated with increased complement activation.

Conclusions: Lipoprotein levels were associated with AMD-associated genetic variants, whereas decreased essential amino acids may point to nutritional deficiencies in AMD. We observed strong associations between the vast majority of the AMD-associated metabolites and systemic complement activation levels, independent of AMD status. This may indicate biological interactions between the main AMD disease pathways and suggests that multiple pathways may need to be targeted simultaneously for successful treatment of AMD. *Ophthalmology 2020;■:1–17 © 2020 by 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) is a multifactorial disease caused by a combination of genetic and environmental factors.[1–4] AMD has a strong genetic component, explaining up to 70% of the variance in disease risk, but lifestyle habits such as smoking and diet also influence AMD risk.[5–7] The largest genome-wide association study (GWAS) to date identified 52 genetic variants at 34 loci that are associated significantly with AMD.[2] These variants were calculated to account for 27.2% of AMD variability, thus explaining more than half of the genomic heritability (estimated to be 46.7% in the same study). Genes at these AMD loci group into 3 main biological pathways: the complement system, lipid metabolism, and extracellular matrix remodeling.[2] An important role of the complement system in AMD has also been established by proteomics studies, which identified components of the complement system in drusen.[8,9] In addition, systemic measurements of complement activation products, such as C3a, C3d, and C5a, have consistently been found to be elevated in AMD.[10]

Several studies have investigated systemic measurements of lipid metabolism in AMD, such as dietary fatty acids, cholesterol, and lipoprotein levels, but results have not always been consistent among studies.[10] A recent meta-analysis of more than 30 000 individuals from 14 cohorts investigated the association of cholesterol measurements and AMD risk.[4] Elevated high-density lipoprotein (HDL) cholesterol levels were identified in AMD, which is opposite to the effect that has been established in cardiovascular diseases.[11] It has been postulated that size and composition of lipoprotein particles may be more reliable biomarkers of cardiovascular disease as compared with general cholesterol level measurements.[12] An exploratory analysis of lipoprotein subfractions in the Rotterdam Study (RS) identified increased extra-large HDL to be associated with AMD.[4] A more in-depth analysis of lipoprotein subfractions using larger sample sizes is needed to further delineate the associations of lipoprotein particle size and composition in AMD.

High-throughput systemic measurements of lipids and other metabolites (metabolomics) can aid in delineating biomarkers and pathways that contribute to disease pathophysiologic characteristics. To date, only a small number of metabolomics studies have been performed in AMD, with limited sample sizes.[13–19] Associations with glycerophospholipids and amino acids have been identified in some of these studies but results of the studies are difficult to compare because of different study designs and metabolomics approaches.[13–20]

In the current study, we aimed to perform the largest metabolomics study in AMD to date using data collected from 2307 AMD patients and 4294 control individuals from 5 cohorts. In addition, we aimed to determine the effect of AMD-associated genetic variants on metabolite levels and investigate associations between the identified metabolites and activity of the complement system, one of the main AMD-associated disease pathways.

Methods

Study Populations

Five cohorts were included in this study: the European Genetic Database (EUGENDA), consisting of 1780 participants at the Radboud University Medical Center in Nijmegen, The Netherlands (EUGENDA-Nijmegen); 1534 participants at the University of Cologne, Cologne, Germany (EUGENDA-Cologne)[21]; the Antioxydants, Lipides Essentiels, Nutrition et Maladies Oculaires (ALIENOR) study visit 2, consisting of 537 participants from Three-City Study, France[3,22]; the Münster Age and Retina Study (MARS) visit 3, consisting of 482 participants from Münster, Germany[23,24]; 2640 participants from the RS visit 4[25,26]; and 76 participants from the Combined Ophthalmic Research Rotterdam Biobank (CORRBI) collected in Rotterdam, The Netherlands. All cohorts were from similar populations of European ancestry. The samples from the ALIENOR cohort were fasting at the time of blood sample collection, whereas the samples of the other cohorts were collected in a nonfasting state. All EDTA plasma and serum samples were collected and centrifuged according to standard protocols and frozen at −80° C within 1 hour.

Only individuals 50 years of age or older were included in the analysis (Table S1, available at www.aaojournal.org). Individuals with any stage of AMD were defined as patients, and individuals without AMD were defined as control participants. Neither the patients nor the control participants were selected because of the presence or absence of other diseases. AMD classification was done separately within each cohort using international classification systems (File S1, available at www.aaojournal.org). More detailed descriptions of the cohorts, data collection, AMD classification, and sampling procedures are provided in Supplemental File 1 and the references provided in this file.

All studies were approved by the appropriate ethical committees (Commissie Mensgebonden Onderzoek [CMO] Arnhem-Nijmegen for EUGENDA-Nijmegen, Ethics Commission of Cologne University's Faculty of Medicine for EUGENDA-Cologne, Medical Ethics Committee of the Erasmus Medical Center for RS and CORRBI, Ethical Committee of Bordeaux for ALIENOR, and Ethics Committee of the Muenster University Hospital for MARS cohort), and all participants provided written informed consent. The study was performed in accordance with the tenets of the Declaration of Helsinki (7th revision).

Metabolomics Measurements

For EUGENDA, ALIENOR, and RS, EDTA plasma samples were available, whereas serum samples were available for MARS. For CORRBI, both plasma and serum samples were available. All samples were analyzed with a high-throughput proton nuclear magnetic resonance (NMR) metabolomics platform (Nightingale Health, Ltd, Helsinki, Finland). This platform provides a total of 225 measurements, which includes ratios and percentages. To maximize the number of unique measurements in this study, we removed the markers that were mentioned as ratio or percentage on the biomarker list of the platform itself (blood biomarker list located at: https://nightingalehealth.com/biomarkers; accessed March 27, 2020). This resulted in inclusion of 146 metabolite measurements in plasma and 3 additional metabolites in serum. Metabolites measured on this platform include amino acids, glycolysis measures, ketone bodies, inflammation-related measurements, fatty acids, and lipoprotein subclasses. Details on the platform and procedures used for quantification of metabolites

were described previously.[27] Abbreviations and group names of the metabolites can be found in Table S2 (available at www.aaojournal.org). Samples from the EUGENDA-Nijmegen, EUGENDA-Cologne, ALIENOR, MARS, and CORRBI cohorts were shipped and measured at Nightingale Health in 1 batch (Nightingale version 2016). Samples from the RS cohort were shipped and measured previously (Nightingale version 2014).

Quality Control

Principal component analysis was performed on the metabolite data shared between cohorts to explore potential systematic deviations between the cohorts.[28] Because the CORRBI cohort included metabolite measurements in both serum and EDTA plasma samples for the same patients, deviations of principal components were checked between 2 different sample types. Thereafter, quality control steps were applied to each cohort separately. The EUGENDA cohort was analyzed as 2 independent cohorts based on site of inclusion of participants (i.e., EUGENDA-Nijmegen and EUGENDA-Cologne). For the CORRBI cohort, the results based on the plasma samples were selected for further analysis. Quality control consisted of: (1) exclusion of outliers of metabolite measurements, defined as values that differed more than 5 times the standard deviation (SD) from the mean, which were set as missing values; (2) exclusion of samples with more than 10% missing metabolite measurements; and (3) exclusion of metabolite measurements with more than 10% missing values. Finally, we performed a log transformation of each metabolite measurement. To enable log transformation of 0 values, 1 was added to each measurement. Transformed metabolite values were standardized, resulting in a standard deviation of 1 and mean value of 0.

After the quality control procedures were performed, several different association analyses were performed on the cleaned data. These models are summarized in Figure S1 (available at www.aaojournal.org).

Metabolite Association Analysis

Metabolite−AMD associations were studied using univariate logistic regression analyses. For each cohort, age, gender, body mass index, and lipid medication data were available and evaluated as potential confounders. Selection of confounders was based on comparison of effect estimates of unadjusted and adjusted regression models in β−β plots (Fig S2, available at www.aaojournal.org). This resulted in adjustment for age and gender in all cohorts and additional adjustment for lipid-lowering medication in the RS cohort. The resulting odds ratios (OR) of the logistic regression models express the change in odds for AMD per 1-standard deviation increase of each log-transformed +1 metabolite level.

Cohort-specific metabolome association analysis results were combined in a random effects meta-analysis using the R package 'meta' (R Foundation for Statistical Computing, Vienna, Austria).[29] A random effects model was chosen to account for possible heterogeneity resulting from differences in AMD assessment, AMD severity, and sample collection among cohorts. Metabolite measurements that were available in only 1 or 2 cohorts after quality control were not meta-analyzed. Heterogeneity in the meta-analysis was assessed using the I^2 value. Correction for multiple testing was performed by applying the Benjamini-Hochberg procedure, a false discovery rate (FDR) correction, because of the strong correlations between the measured metabolites.[20,30] The threshold for statistical significance was defined as an FDR-corrected P value (P_{FDR}) of less than 0.05 after FDR correction.

Association Analysis of Age-Related Macular Degeneration-Associated Genetic Variants with Metabolites

For the identified AMD-associated metabolites, a single nucleotide polymorphism (SNP)−metabolite association analysis was performed to investigate the effect of 52 AMD-associated genetic variants, reported in the most recent GWAS by Fritsche et al[2] on metabolite levels. Two of our cohorts (EUGENDA-Nijmegen and EUGENDA-Cologne) were used partially in the study of Fritsche et al. First, the associations of the 52 genetic variants with AMD as presented in primary analyses of Fritsche et al were replicated using data from the 5 cohorts of the current study. Next, the 52 AMD-associated genetic variants were tested in linear regression models for association with the quality-controlled, log-transformed, and standardized metabolite levels that were found to be associated significantly with AMD in the metabolomics analysis. Genetic variants were coded according to an additive model, that is, as 0 if the patient was homozygous for the reference allele, as 1 if the patient was heterozygous, carrying 1 copy of each allele, and as 2 if the patient was homozygous for the alternative allele. For the genetic variants that were not genotyped directly, that is, variants that were not present on the genotyping platforms that were used to measure genotypes in the cohorts, we used statistical imputation with Haplotype Reference Consortium and 1000 Genomes as a reference panel to estimate genotypes for all nondirectly genotyped variants.[31,32] Quality control of the genetic data and imputation can be found in the publications of each cohort (Colijn et al, unpublished data, 2020; de Breuk et al[33] 2020.[2,34–36] For the imputed variants, dosage information was used as genotype to take the uncertainty in genotype imputation into account. Per cohort, the percentage of imputed variants of the 52 genetic variants were: EUGENDA-Nijmegen, 9%; EUGENDA-Cologne, 11%; ALIENOR, 22%; and RS, 23% of the total genotypes within the cohorts. All of the available genetic data of CORRBI were genotyped directly, so none of the 52 genetic variants had to be imputed. Reference and alternative alleles were defined as presented in the AMD GWAS of Fritsche et al.[2] For the cohorts with multiple sequencing platform data, the inclusion preference from high to low was single-molecule molecular inversion probes sequencing, whole-exome sequencing, exome chip direct genotyping, and exome chip imputed genotypes (Colijn et al, unpublished data, 2020). AMD status was used as a covariate in the regression models to account for the potential confounding effect of disease status, along with age and gender. Cohort-specific results were combined in a fixed-effects meta-analysis because the underlying effect of the genetic variants on the metabolites was assumed to be the same in each cohort. The threshold for statistical significance was again defined as $P_{FDR} < 0.05$ after FDR correction.

To identify whether the metabolites are acting as mediators of the association between genetic variants and AMD, for example, if lipoprotein levels can explain the association between a genetic variant and AMD, a mediation analysis was performed by building 2 regression models for each associated metabolite and genetic variant. The first model (model A) was a linear regression model with the AMD-associated genetic variant as predictor of the associated metabolite measurement. The second model (model B) was a logistic regression model with the AMD-associated genetic variant and the associated metabolite predicting AMD status. These models were also adjusted for age and gender. Based on these model duos (models A and B), we calculated the average casual

mediation effects (ACMEs) for each SNP−metabolite duo in each cohort using the "mediate" function of the R package "Mediation." Resulting cohort-specific ACMEs were subsequently meta-analyzed to combine the results across cohorts.

Additionally, we tested whether the genetic variant−AMD associations were mediated by a combination of metabolites instead of only 1, thereby focusing only on those metabolites that showed a statistically significant association with the genetic variant under study. For this, we built a logistic regression model with the genetic variant as predictor, AMD as outcome, and all genetic variant-associated metabolites as covariates (model C). This was carried out for each cohort separately, except for CORRBI cohort because of its low sample number, which was not sufficient to include all associated metabolites in the model for some of the genetic variants. The results were combined in a random effects meta-analysis. We compared effect estimates and *P* values of model C with single variant association models, that is, with the genetic variant as the predictor and AMD status as the outcome.

Association Analysis of Complement Activation with Metabolites

For the identified AMD-associated metabolites, we studied the association between metabolites and activity of the complement pathway. For the EUGENDA cohort, systemic complement activation measurements (defined by the C3d-to-C3 ratio) were available for a total of 3073 individuals because they were used previously to test the effect of genetic variants on complement activation.[37,38] For the metabolite−complement activation association analyses, we analyzed the EUGENDA cohort as 2 independent cohorts based on site of inclusion of participants (i.e., EUGENDA-Nijmegen and EUGENDA-Cologne). After log transformation and standardization of the complement activation levels for these 2 cohorts, a linear regression model was built. In this model, significantly associated metabolites were tested 1 by 1 as independent variables; systemic complement activation was used as a dependent variable; and age, gender, and AMD status were used as covariates. Results from the 2 cohorts were combined in a random effects meta-analysis to investigate the associations between complement activation and significant metabolites. False discovery rate correction for multiple testing was applied because of the strong correlations between the metabolites and a significance threshold of $P_{FDR} < 0.05$. The same linear regression models were also built for AMD patients and healthy control participants separately to compare the effect estimates between patients and control participants.

Results

Study Population and Quality Control

Metabolomics measurements were performed in 7049 plasma and serum samples from 5 cohorts using a high-throughput proton NMR metabolomics platform, which allows the quantification of 146 metabolite measurements in EDTA plasma (Table S2). The maximum number of outliers per metabolite was 13 for EUGENDA-Nijmegen, 9 for EUGENDA-Cologne, 5 for ALIENOR, 1 for CORRBI, and 49 for RS. The number of samples filtered out because of 10% missingness was 11 for EUGENDA-Nijmegen, 6 for EUGENDA-Cologne, 2 for ALIENOR, 0 for CORRBI, and 15 for RS. Measurement level check for 10% missingness resulted in removal of valine in the CORRBI cohort and 3-hydroxybutyrate in both the ALIENOR and CORRBI-plasma cohorts.

Principal component analysis showed a general cluster formed by the measurements of all cohorts, but the MARS cohort clustered outside of this cluster (Fig S3, available at www.aaojournal.org). This was not the result of the difference between plasma and serum measurements, because serum samples from CORRBI clustered along with plasma samples from CORBBI and all other cohorts. Rather, it may indicate a potential difference in quality of the samples.[28] Additionally, we received a quality report of the Nightingale platform measurements mentioning that higher than normal pyruvate and lactate levels were observed for the MARS cohort, indicating that the MARS samples may have been kept at room temperature for too long.[39] To prevent inclusion of low-quality samples in our study, we excluded the MARS cohort from further analysis. Because of exclusion of the MARS cohort, which was the only cohort with only serum samples available, all metabolites included in our study are based on measurements in plasma samples.

After cohort-specific quality control, 146 metabolites (Table S2) and a total of 6533 samples of 2267 AMD patients and 4266 control participants (Table 1) remained for further analysis. The average age of all of the participants in this study was 74 years, and 58% of the remaining participants were women. More detailed information about age, gender, and lipid-altering medication use in the remaining samples can be found in Table S1.

Metabolite Associations with Age-Related Macular Degeneration

Meta-analyses identified 60 metabolite measurements that were associated significantly with AMD ($P_{FDR} < 0.05$; Fig 1; Table S3, available at www.aaojournal.org), which showed high correlations among lipid-related metabolites (Fig S4, available at www.aaojournal.org). For the lipoprotein subclasses, significant associations in particular were observed in the very low-density lipoprotein (VLDL) and HDL subclasses. Significantly associated metabolite measurements also included 3 other lipid and apolipoprotein measurements, 3 fatty acids, 1 glycolysis-related metabolite, and 5 amino acids.

Of the 42 VLDL-related subclass measurements, 30 were associated inversely with AMD (OR, <1), indicating that AMD patients have decreased VLDL levels compared with control participants. Measurements of all VLDL subclasses were associated with AMD, except for very small VLDL. The most significant association was observed for cholesterol esters in medium VLDL (OR, 0.89; 95% CI, 0.84−0.95; $P_{FDR} = 0.014$; Fig 2). Of 28 HDL-related subclass measurements, 5 very large and 6 large HDL measurements were associated positively with AMD (OR, >1), indicating that AMD patients have increased very large and large HDL levels compared with control participants. The most significant association was observed for phospholipids in very large HDL particles (OR, 1.12; 95% CI, 1.05−1.20; $P_{FDR} = 0.014$; Fig 2). Only 1 medium HDL measurement was associated significantly with AMD, whereas none of the 7 small HDL measurements were associated with AMD. None of the 7 intermediate-density lipoprotein-related and 21 of the LDL-related subclass measurements were associated significantly with AMD (Fig 1).

Of other lipid and apolipoprotein measurements, decreased remnant cholesterol level (remnant-C; OR, 0.90; 95% CI, 0.85−0.96; $P_{FDR} = 0.017$), apolipoprotein B (ApoB; OR, 0.91: 95% CI, 0.86−0.97; $P_{FDR} = 0.022$), and total serum triglycerides (OR, 0.91; 95% CI, 0.86−0.97; $P_{FDR} = 0.020$) were identified in AMD patients compared with control participants (Fig 2). In addition, 3 fatty acids were associated significantly with AMD (Fig 3). Decreased levels of monounsaturated fatty acids (MUFAs), saturated fatty acids (SFA), and total fatty acids

Table 1. Descriptives of the Cohorts*

	Antioxydants, Lipides Essentiels, Nutrition et Maladies Oculaires	European Genetic Database - Nijmegen	European Genetic Database - Cologne	Combined Ophthalmic Research Rotterdam Biobank	Rotterdam Study	Total
Total no.	535	1769	1528	76	2625	6608
Age (yrs), mean ± SD	83.19 ± 4.2	72.18 ± 7.9	72.4 ± 8.3	75.18 ± 9.1	74.98 ± 5.9	74.22 ± 7.9
Female gender, no. (%)	335 (63)	1038 (59)	908 (59)	39 (51)	1515 (58)	3835 (58)
AMD, no. (%)						
Early plus intermediate	97 (18)	379 (21)	339 (22)	11 (14)	395 (15)	1221 (19)
Late	53 (10)	605 (34)	286 (19)	34 (45)	68 (3)	1046 (16)
Any stage	150 (28)	984 (55)	625 (41)	45 (59)	463 (18)	2311 (35)
Control participants, no. (%)	385 (72)	785 (45)	903 (59)	31 (41)	2162 (82)	4266 (65)

*Remaining number of samples after quality control is shown. In our analysis, we used any stage of age-related macular degeneration (AMD) as patients and non-AMD individuals as control participants. All samples were from European ancestry.

(TotFA) were identified in AMD patients compared with control participants, with effect estimates ranging from an OR of 0.92 to 0.93 (95% CI, 0.86−0.98; $P_{FDR} = 0.005-0.014$).

Of 3 glycolysis-related metabolites, a decreased citrate level was identified in AMD patients compared with control participants (OR, 0.88; 95% CI, 0.83−0.94; $P_{FDR} = 0.010$; Fig 4). In addition, decreased levels of 5 amino acids were identified in AMD (Fig 4). The most significant association was observed for phenylalanine (OR, 0.86; 95% CI, 0.81−0.91; $P_{FDR} = 1.54 \times 10^{-4}$), which was highly consistent among the cohorts ($I^2 = 0$). Levels of alanine, isoleucine, leucine, and tyrosine were also decreased significantly in AMD patients compared with control participants, although these measurements showed moderate heterogeneity between the cohorts ($I^2 = 0.5-0.6$; Fig 4). No significant associations with AMD were observed for metabolites related to fluid balance (albumin, creatinine), ketone bodies (acetate, acetoacetate, 3-hydroxybutyrate), and inflammation (glycoprotein acetyls; Fig 1).

Associations of Age-Related Macular Degeneration-Associated Genetic Variants with Metabolites

Associations of 52 AMD-associated genetic variants with AMD and comparison with the results as presented by Fritsche et al[2] are shown in Table S4 (available at www.aaojournal.org). The directions of effects for 50 variants were validated to be similar between our study and that of Fritsche et al. For 2 SNPs, rs2043085 in the *LIPC* gene and rs10033900 in the *CFI* gene, we observed an opposite direction of effect, but both associations were not statistically significant.

For the 60 identified AMD-associated metabolites, a gene−metabolite association analysis was performed to investigate the association of 52 AMD-associated genetic variants, reported in the most recent GWAS by Fritsche et al,[2] with metabolite levels. We identified 98 significant associations, comprising 34 metabolite measurements and 7 SNPs (Fig 5; Table S5, available at www.aaojournal.org). Six of these SNPs are located in or near genes involved in lipid metabolism (*ABCA1*, *CETP*, *APOE*, and *LIPC*). The seventh SNP (rs10781182) resides in the *MIR6130* gene, which encodes a microRNA. The largest number of SNP−metabolite associations were identified in the metabolites belonging to the large and very large HDL subclasses, with a total of 50 associations between different subclasses and variants.

The T allele of rs17231506 in the *CETP* gene, which is associated with an increased risk of AMD, showed the largest number of significant associations (26 in total). The T allele of rs17231506 was associated with increased levels of 13 HDL-related measurements ($\beta > 0$) and decreased levels of 11 VLDL-related measurements ($\beta < 0$). Among these associations, free cholesterol in very large HDL was the most significant association ($P_{FDR} = 1.57 \times 10^{-24}$; β for allele T, 0.20; 95% CI, 0.17−0.24) of all gene−metabolite associations. A second independent variant in the *CETP* gene, rs5817082, of which the CA allele (A insertion) is associated with decreased AMD risk, showed 22 significant associations with similar metabolites as rs17231506, albeit with an opposite direction of effect. The CA allele of rs5817082 was associated with decreased levels of 13 HDL-related measurements ($\beta < 0$) and increased levels of 8 VLDL-related measurements ($\beta > 0$).

Variant rs2043085 in the *LIPC* gene, of which the C allele is associated with an increased risk of AMD (Table S4),[2,40] showed 21 significant associations. The C allele of rs2043085 was associated with decreased levels of 15 HDL-related measurements ($\beta < 0$), decreased levels of 2 small VLDL-related measurements ($\beta < 0$), and decreased levels of 3 fatty acids measurements: MUFA, SFA, and TotFA ($\beta < 0$). A second independent variant in the *LIPC* gene, rs2070895, of which the A allele is associated with a decreased risk of AMD, showed 18 significant associations with similar metabolites as rs2043085, albeit with an opposite direction of effect. The A allele of rs2070895 was associated with increased levels of 14 HDL-related measurements ($\beta > 0$), decreased levels of 2 VLDL-related measurements ($\beta < 0$), and increased levels of 2 fatty acid measurements, SFA and TotFA ($\beta > 0$).

Variant rs429358 in the *APOE* gene, of which the C allele is associated with a decreased risk of AMD, showed 5 significant associations with metabolite levels. The C allele of rs429358 was associated with increased levels ($\beta > 0$) of ApoB, remnant-C, a small VLDL measurement, and cholesterols in very large HDLs, whereas an opposing effect was observed for triglycerides in a medium HDL measurement. Variant rs2740488 in the *ABCA1* gene, of which the C allele is associated with a decreased risk of AMD, was associated with decreased levels of 5 HDL-related

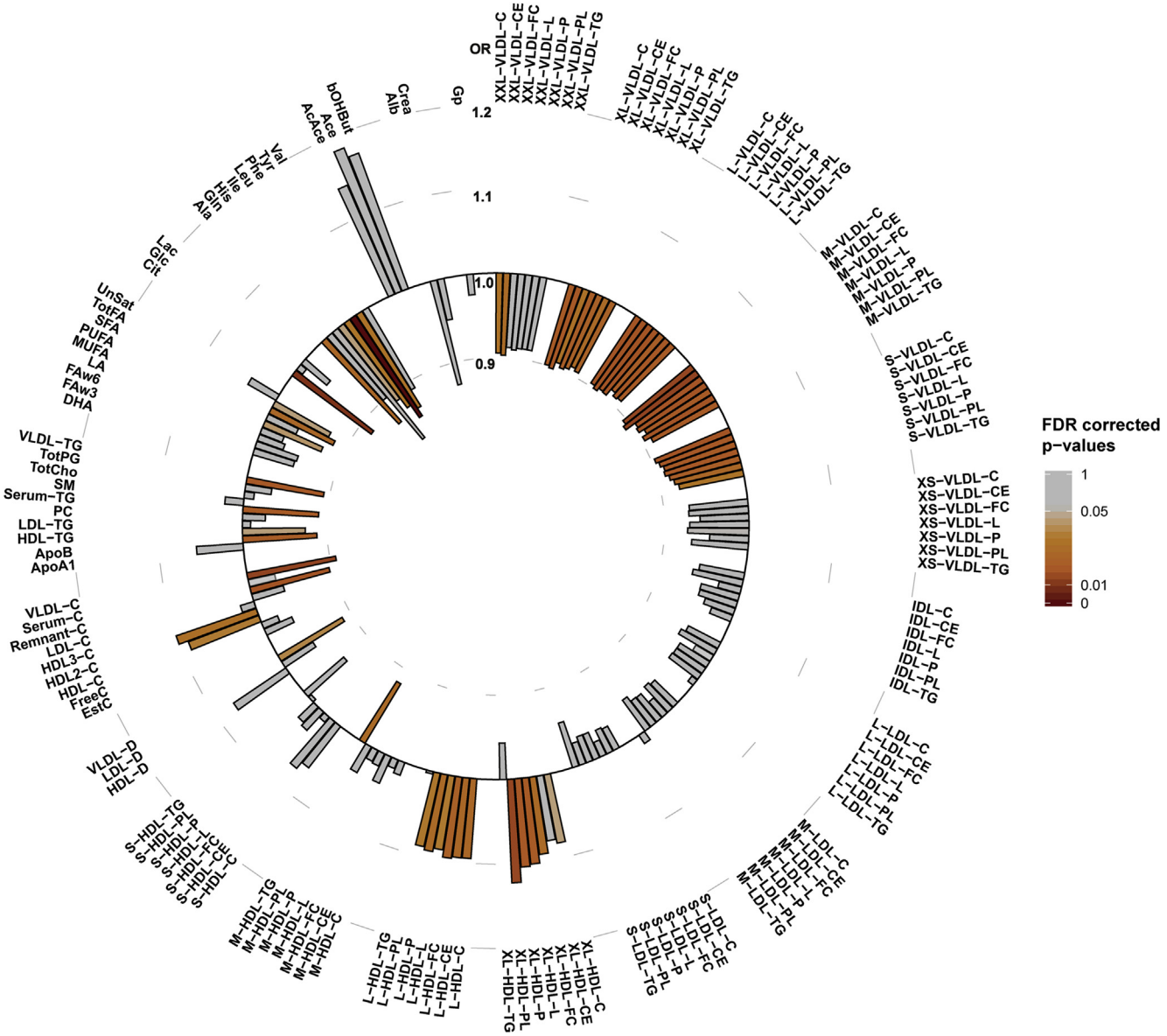


Figure 1. Metabolites associated with age-related macular degeneration (AMD). Effect sizes (odds ratios [ORs]) are indicated with bars, and the black circle indicates the no-effect level (OR, 1). Bars pointing outward of the black circle are associated with increased levels in AMD (OR, >1), whereas bars pointing inward of the black circle are associated with decreased levels in AMD (OR, <1). Colors indicate the false discovery rate-corrected significance level. For abbreviations of the metabolites, see Table S2.

measurements ($\beta < 0$). Variant rs10781182 in the *MIR6130* gene, of which the T allele is associated with an increased risk of AMD, showed a significant association with citrate ($P_{FDR} = 4.52 \times 10^{-2}$; β for allele T, −0.06; 95% CI, −0.10 to 0.02; $I^2 = 0$).

Next, a mediation analysis was performed to examine if the association of the genetics variants with AMD can be explained (i.e., are mediated) by the associated metabolites. Two different approaches were adopted for this. First, each metabolite was evaluated individually for a mediation effect by estimating the ACMEs in every cohort. Meta-analyzed ACMEs revealed no significant mediation effect of any of the metabolites for the SNP−AMD associations (Table S6, available at www.aaojournal.org). Second, all metabolites that were found to be associated with a genetic variant were evaluated jointly for a mediation effect by comparing the effect estimates of the genetic variant on AMD in an unadjusted and metabolite-adjusted model. In case of (partial) mediation, effect estimates will be lower in the adjusted model. However, we observed that effect estimates were increased rather than decreased (Table S7, available at www.aaojournal.org). Consistent with the first analysis using quantified metabolites, this second analysis thus also revealed no mediation effect.

Associations of Complement Activation with Metabolites

Because the complement pathway is one of the main disease pathways in AMD pathogenesis, we next investigated associations between AMD-associated metabolites and activity of the complement system measured as the C3d-to-C3 ratio. The analysis

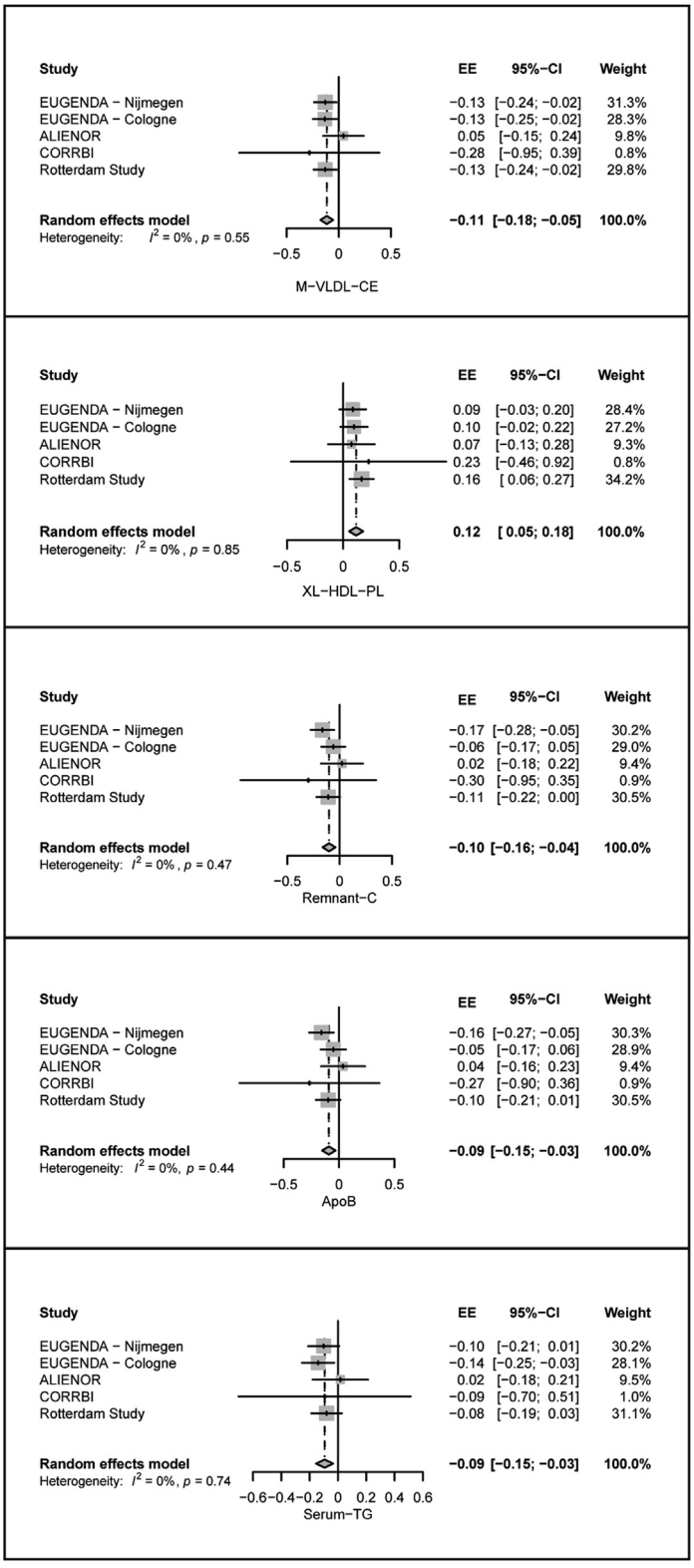


Figure 2. Forest plots showing the most significantly associated very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) subparticles, remnant cholesterol level (remnant-C), apolipoprotein B (ApoB), and triglycerides with age-related macular degeneration (AMD). The effect estimate (EE) shows the β effect estimate, and the 95% confidence interval (CI) shows the 95% CI of this effect estimate. Random effects meta-analysis estimation is shown in the figures. ALIENOR = Antioxydants, Lipides Essentiels, Nutrition et Maladies Oculaires; CORRBI = Combined Ophthalmic Research Rotterdam Biobank; EUGENDA = European Genetic Database; M-VLDL-CE = cholesterol esters in medium VLDL; serum-TG = serum total triglycerides; XL-HDL-PL = phospholipids in very large HDL.

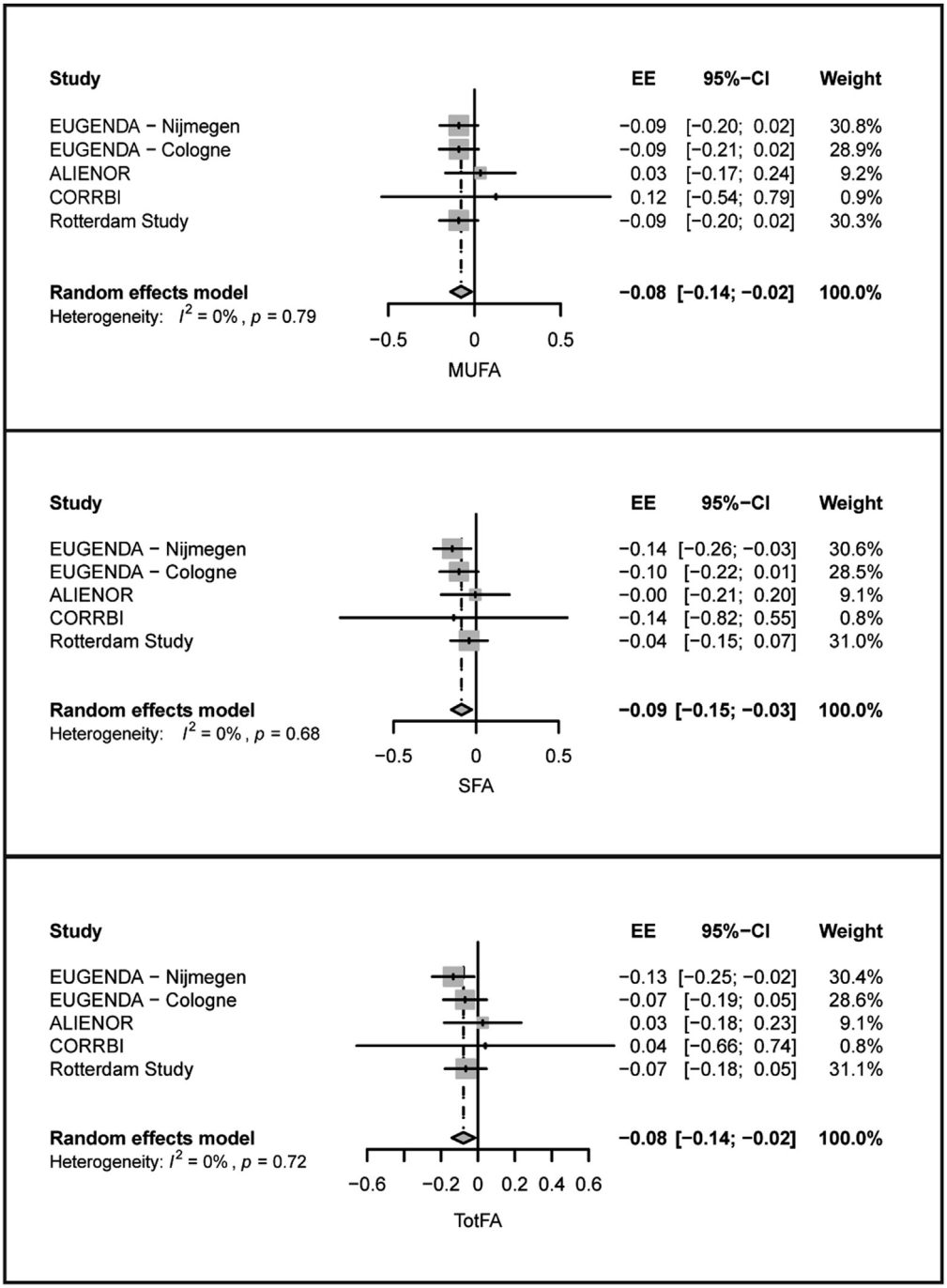


Figure 3. Forest plots showing significantly associated fatty acids. The effect estimate (EE) shows the β effect estimate, and the 95% confidence interval (CI) shows the 95% CI of this effect estimate. Random effects meta-analysis estimation is shown in the figures. ALIENOR = Antioxydants, Lipides Essentiels, Nutrition et Maladies Oculaires; CORRBI = Combined Ophthalmic Research Rotterdam Biobank; EUGENDA = European Genetic Database; MUFA = monounsaturated fatty acids (oleic and palmitoleic); SFA = saturated fatty acids; TotFA = total fatty acids.

was performed on AMD patients and control participants of the EUGENDA cohorts and was adjusted for AMD status. Of 60 significantly associated metabolite measurements, 57 were found to be associated significantly with complement activation (Table S8, available at www.aaojournal.org). Effect estimates of significant associations were similar for AMD patients and control participants when stratifying instead of adjusting for AMD status.

For all AMD-associated VLDL measurements, decreased VLDL levels were associated with increased complement activity ($\beta < 0$). Conversely, increased levels of all large and very large HDL subclasses showed a significant association with increased levels of C3d or C3 ($\beta > 0$). For other lipid and apolipoprotein measurements, decreased remnant-C, ApoB, and total serum triglycerides were associated with increased complement activation ($\beta < 0$). In addition, of the AMD-associated fatty acid

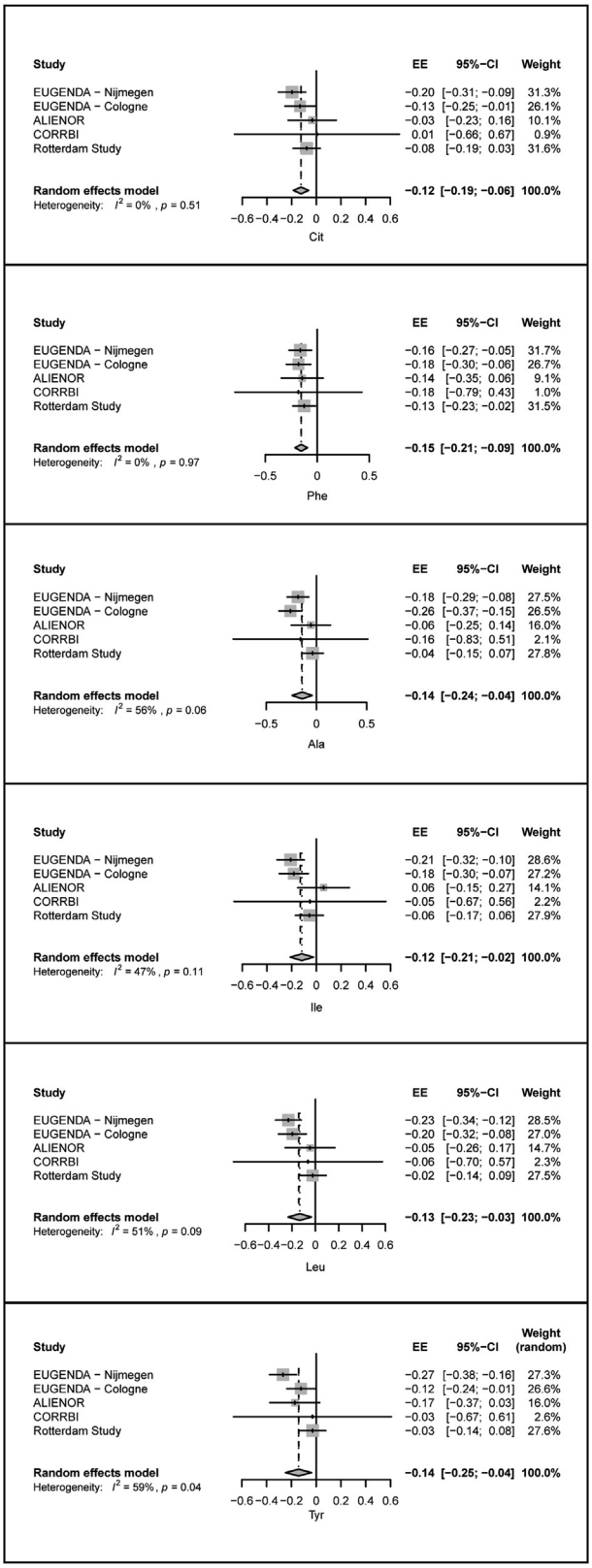


Figure 4. Forest plots showing significantly associated amino acids and citrate. The effect estimate (EE) shows the β effect estimate, and the 95% confidence interval (CI) shows the 95% CI of this effect estimate. Random effects meta-analysis estimation is shown in the figures. Ala = alanine; ALIENOR = Antioxydants, Lipides Essentiels, Nutrition et Maladies Oculaires; Cit = citrate; CORRBI = Combined Ophthalmic Research Rotterdam Biobank; EUGENDA = European Genetic Database; Ile = isoleucine; Leu = leucine; Phe = phenylalanine; Tyr = tyrosine.

measurements, decreased MUFA, SFA, and TotFA levels were associated with increased complement activation ($\beta < 0$).

Of 5 amino acids that were associated with AMD status, decreased levels of alanine, isoleucine, and leucine were associated significantly with increased complement activation levels ($\beta < 0$). These 3 amino acids showed less significance in terms of association compared with the associations of the other metabolites, and the associations were heterogeneous among the cohorts ($I^2 = 0.54-0.74$). Phenylalanine, tyrosine, and citrate were not associated significantly with complement activation levels.

Discussion

In the current study, we performed the largest metabolomics analysis in AMD to date, including 2307 AMD patients and 4294 control individuals from 5 cohorts. Using an NMR-based platform, 146 metabolite measurements were quantified in plasma. Overall, we identified 60 metabolite measurements that were associated significantly with AMD. Among the significant associations were HDL and VLDL lipoprotein particles, other lipids and apolipoproteins (remnant C, ApoB, serum triglycerides), fatty acids (MUFA, SFA, and TotFA), amino acids (alanine, isoleucine, leucine, phenylalanine, and tyrosine), and citrate (Fig 6A). Several lipid-related metabolites were correlated highly with each other (Fig S4, available at www.aaojournal.org).

High-density lipoprotein levels were increased in AMD, whereas VLDL levels were decreased in AMD. These findings are in agreement with previous findings, because increased HDL levels were also recently reported for AMD in a large meta-analysis of 30 000 individuals from European cohorts.[4] Our current study showed that particle size and composition of lipoproteins are relevant: associations were identified with very large and large HDL but not with medium and small HDL. For VLDL, we identified associations with all subparticles except for very small VLDL, whereas medium VLDL was most significant. In dementia, increased large HDL levels have also been associated with an increased risk, whereas small VLDL levels are associated with a decreased risk of dementia.[41] Intriguingly, the associations in AMD and dementia are opposite to what is observed in cardiovascular disease (CVD). Increased HDL levels are associated with a decreased risk of CVD, whereas increased VLDL levels are associated with an increased risk of CVD.[11,42] Lipoprotein associations in CVD extend to all VLDL, intermediate-density lipoprotein, LDL, and HDL subparticles, although the strongest effect is observed for large HDL.[43] The different associations among lipoprotein subparticle sizes support the notion that the composition of lipoproteins may be important, and this can affect their functional role.[44] Further research into the composition of HDL and VLDL subparticles among different diseases is warranted to understand the meaning of these associations.[12]

Decreased MUFA, SFA, and TotFA levels were identified in AMD patients compared with control participants in the current metabolomics study. Conversely, increased MUFA and SFA have been associated with an increased risk of CVD.[43] Previous epidemiologic studies found inconsistent associations of MUFA and SFA with AMD, and many results were not statistically significant.[12] Among MUFAs, oleic acid, a main constituent of olive oil, may have a protective effect on AMD, whereas other MUFAs may exert a detrimental effect.[45] Therefore, more detailed analyses of specific fatty acids are needed to determine their effect on AMD risk. The NMR-based platform used in this study does not perform such a detailed analysis; moreover, fatty acid measurements in red blood cell membranes may reflect longer-term dietary intakes than plasma measurements, which are subject to daily variations.[46]

We detected decreased amino acid (alanine, isoleucine, leucine, phenylalanine, and tyrosine) and citrate levels in AMD patients compared with control participants. A recent metabolomics study in urine samples also showed depleted citrate and amino acid levels in AMD, which was suggested to reflect an enhancement in energy requirement in the disease.[47] Decreased amino acid levels (valine, isoleucine, leucine) have also been reported in dementia.[41] Valine, isoleucine, leucine, and phenylalanine are essential amino acids, and circulating levels are determined largely by dietary intake.[48] Thus, reduced levels of these essential amino acids may indicate subclinical nutritional deficiencies in dementia and AMD. In addition, branched amino acids (valine, isoleucine, leucine) are associated with muscle mass,[49] which may reflect reduced physical activity in dementia and AMD. Moreover, studies have reported that certain bacteria of the gut microbiome can covert phenylalanine and tyrosine to other derivative metabolites, possibly leading to reduced uptake and consequently reduced plasma levels of these amino acids.[50,51] Future studies on the role of the gut microbiome in AMD would therefore be of interest. Notably, the same 5 amino acids (alanine, isoleucine, leucine, phenylalanine, and tyrosine) that were associated with AMD in our study were found to be increased in CVD, suggesting that not only lipoproteins and fatty acids but also amino acid metabolism exert opposite effects in AMD and CVD.[43] Inverse associations have also been reported for genetic risk for AMD as opposed to CVD: individuals with increased genetic risk for CVD and related traits have a lower genetic risk for AMD.[40] Therefore, pleiotropic effects seem to underlie AMD and CVD, reflected by opposite effects of genetic risk as well as metabolite measurements. Notably, some epidemiologic studies have suggested CVD as a potential risk factor for AMD, but conflicting results have been reported among studies. A large meta-analysis showed that the associations between AMD and CVD remain inconclusive.[52]

Identification of altered metabolite levels in plasma points toward systemic effects in AMD and suggests that the identified metabolites represent promising biomarkers for AMD. Analysis of the identified metabolites in prospective cohorts over time is recommended to determine their value as early predictors of AMD or biomarkers for disease progression. Such follow-up studies will need to explore whether a combined group of metabolites has added value to predict disease progression before such biomarkers can be implemented into clinical practice. Epidemiologic and intervention studies have reported that nutritional and

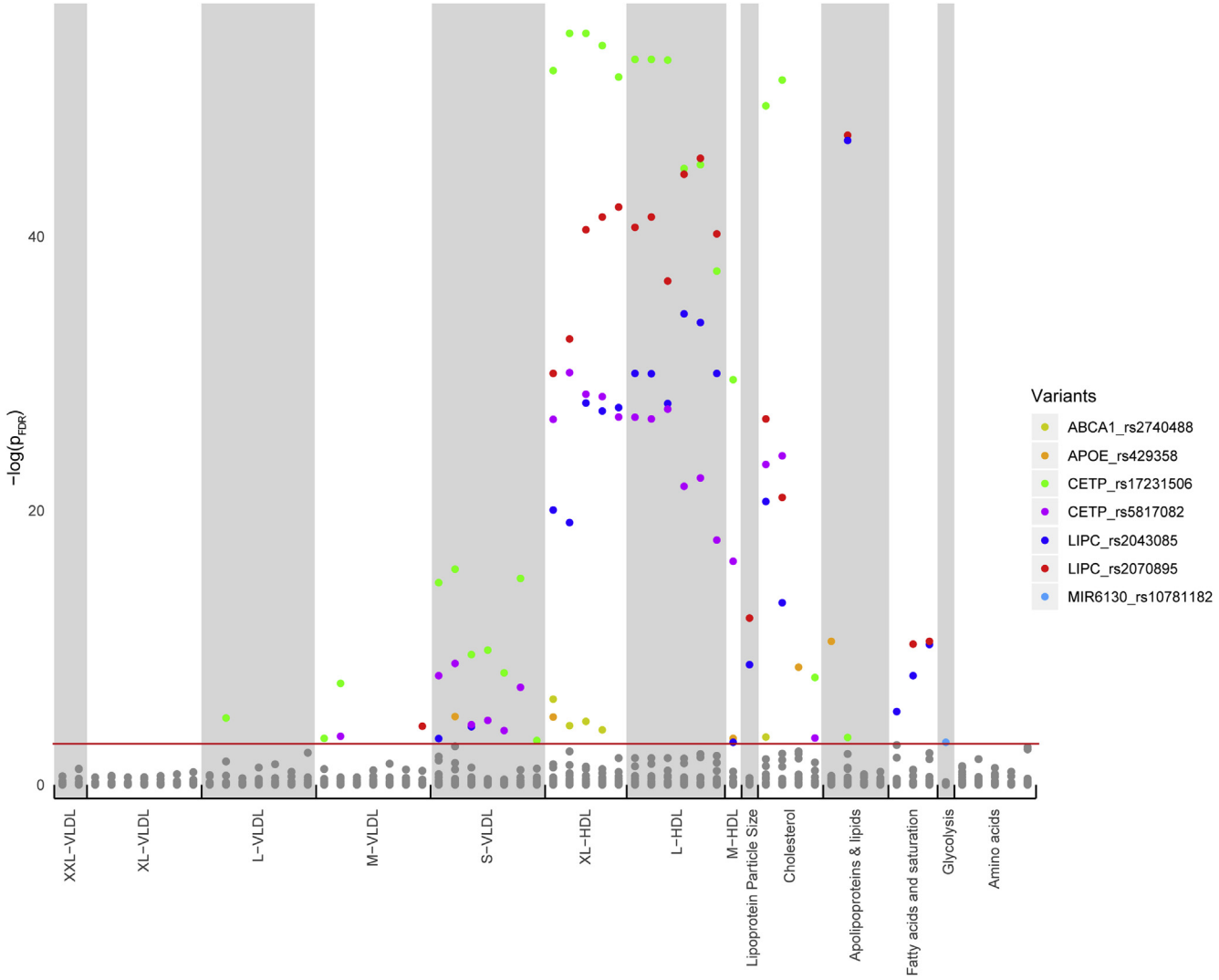
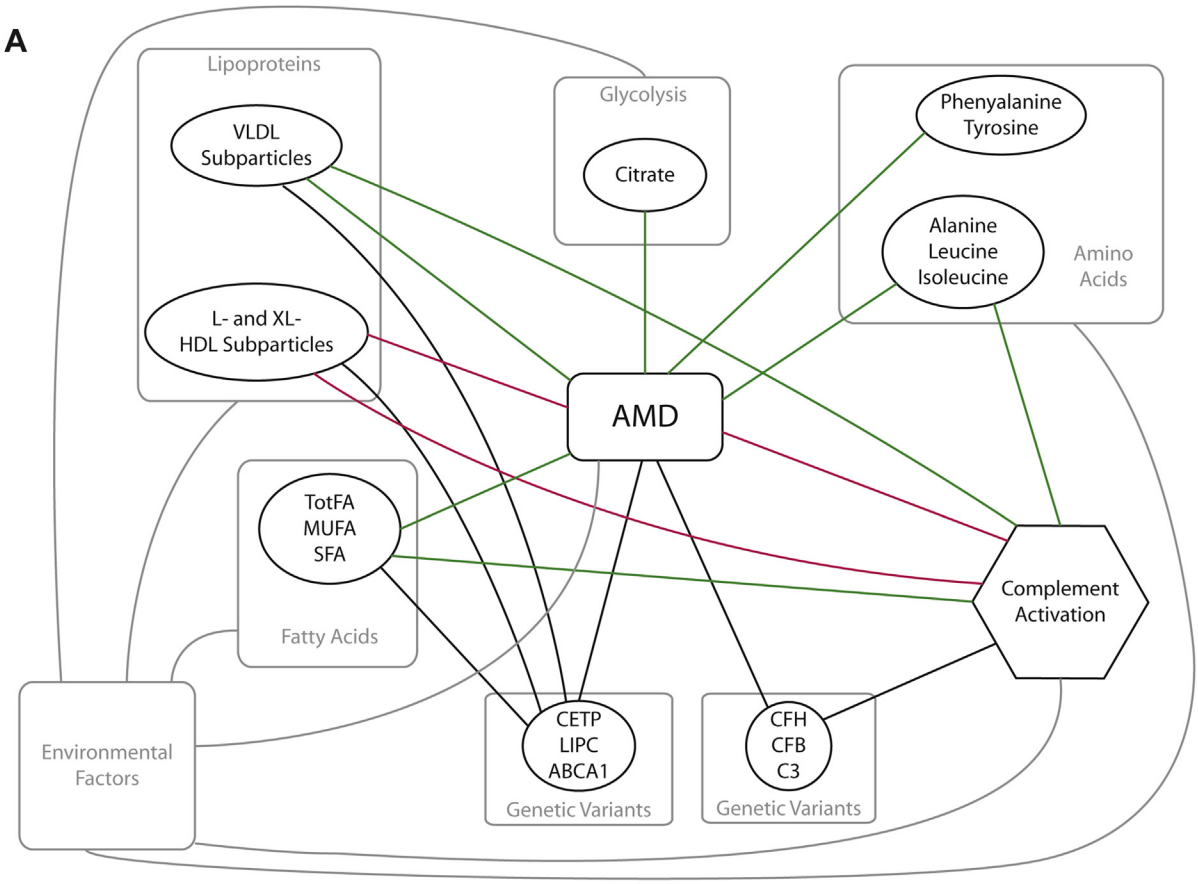


Figure 5. Dot plot showing associations between metabolites and age-related macular degeneration (AMD)-associated genetic variants. This plot shows the association results of AMD-associated single nucleotide polymorphisms, as defined by Fritsche et al,[2] with the metabolite levels that were significant associated with AMD in our meta-analysis. The y-axis shows log-transformed false discovery rate-corrected *P* values ($-\log(P_{FDR})$). HDL = high-density lipoprotein; L = large; M = medium; S = small; VLDL = very low-density lipoprotein; XL = very large; XXL = very very large.

lifestyle factors contribute to the development of AMD, and several nutrients such as lutein, zeaxanthin, vitamins C and E, zinc, and fatty acids may help to reduce the risk of AMD.[12] Our study suggested that investigation into the effect of dietary interventions in AMD may be extended to other nutrients, such as essential amino acids, although we cannot exclude that these associations may be related to reduced physical activity or an altered gut microbiome rather than reduced dietary intake.

Most AMD-associated metabolites identified in this study are influenced by both environmental and genetic factors, but the relative contribution differs between metabolites. Although the identified amino acids to a large extent are driven by environmental factors, HDL levels have a strong genetic component (heritability is estimated to be 70%).[48] In the current study, we investigated the association of 52 AMD-associated genetic variants with metabolite levels. Associations were detected with variants in the *CETP*, *LIPC*, *ABCA1*, and *APOE* genes, encoding components of the HDL metabolism pathway: cholesteryl ester transfer protein; lipase C, hepatic type; adenosine triphosphate-binding cassette transporter A1; and apolipoprotein E. Cholesteryl ester transfer protein exchanges cholesteryl esters and triglycerides between HDL and other lipoproteins and thereby influences HDL particle size. Lipase C, hepatic type, hydrolyses triglycerides and phospholipids in lipoproteins, partly converts very large VLDL and intermediate-density lipoprotein to LDL and plays a role in altering the HDL content. Adenosine triphosphate-binding cassette transporter A1 forms a cellular cholesterol efflux pump leading to formation of nascent HDL. Apolipoprotein E facilitates cholesterol uptake by HDL.[4] Most of the AMD-associated variants in the *CETP*, *LIPC*, *ABCA1*, and *APOE* genes are intronic or intergenic,

A



B

Genetic Variant	Reference Allele	Effect Allele	Association with			
			AMD	L- and XL- HDL	VLDL	Fatty Acids
CETP rs17231506	C	T	+++	+++	-	0
CETP rs5817082	C	CA	---	---	+	0
LIPC rs2043085	T	C	+++	---	-	--
LIPC rs2070895	G	A	---	+++	-	+++
ABCA1 rs2740488	A	C	---	-	0	0
APOE rs429358	T	C	---	+	+	0

Figure 6. Overview of metabolomics, genetic variants, and complement activation in age-related macular degeneration (AMD). **A**, Diagram showing an increase in metabolite levels or AMD risk with red lines and a decrease in metabolite levels or AMD risk with green lines, according to the effect estimates. Environmental factors are known to affect metabolites,[48] AMD, and complement activation,[38] depicted with gray lines. Black lines show associations with different directions of effect, which are shown in more detail in (**B**). **B**, Table in which, for each genetic variant that was associated with a lipoprotein subparticle and fatty acid measurement in their related metabolite groups, effects are shown as plus for effect estimate of more than 0 and minus for effect estimate of less than 0. In cases where there were no associations with the group, it is shown as 0 in the table. The number of plus or minus signs indicates the significance of the *P* value, where $0.05 \geq P > 0.001$ is shown as + or −, $0.001 \geq P > 0.0001$ is shown as ++ or − −, and $0.0001 \geq P$ is shown as +++ or − − −. AMD = age-related macular degeneration; HDL = high-density lipoprotein; L = large; MUFA = monounsaturated fatty acid; SFA = saturated fatty acid; TotFA = total fatty acid; VLDL = very low-density lipoprotein; XL = very large.

and the functional effects of these variants are not clear, with the exception of the rs429358 variant (*APOE* e4 allele) in *APOE* leading to the amino acid change Cys156Arg, which alters the lipoprotein binding properties of *APOE*.[12] In our analysis, the strongest effect estimates were detected for large and very large HDL levels, which were mainly associated with variants in the *CETP* (rs17231506, rs5817082) and *LIPC* (rs2043085, rs2070895) genes, and weaker associations were identified in the *ABCA1* (rs2740488) and *APOE* (rs429358) genes. Genetic variants in *CETP*, *LIPC*, and *APOE* were also associated with VLDL, although these associations were weaker than those observed for large and very large HDL. Fatty acids (MUFA, SFA, and TotFA) were associated with variants in *LIPC*, but no associations were detected with variants in *CETP*, *ABCA1*, and *APOE*. For most SNP−HDL associations, we observed the expected direction of effect, where AMD risk-conferring alleles were associated with increased large and very large HDL levels, whereas protective AMD alleles were associated with decreased HDL levels (Fig 6B). The exceptions are the variants in the *LIPC* gene. The C allele of *LIPC* variant rs2043085 is associated with an increased risk of AMD, whereas it is associated with decreased large and very large HDL levels. Similarly, the A allele of *LIPC* variant rs2070895 is associated with a decreased risk of AMD, whereas it is associated with increased large and very large HDL levels. The inverse effect of LIPC variants on AMD and HDL levels was also noted in other studies, which concluded that not all mechanisms for increased circulating HDL concentration increase AMD risk uniformly.[4,53] A potential explanation is that the variants may have a local effect on the lipid metabolism in the retina, and the association with circulating HDL is the result of the effect on other tissues that express these genes and are known to regulate circulating HDL levels, like the liver.[12] In the current study, we performed a mediation analysis to investigate whether AMD-associated genetic variants exert their effect on AMD via the identified metabolites. We did not find evidence for mediation effects, suggesting that the genetic variants do not influence AMD via the tested metabolites. A possible explanation is that the genetic variants influence AMD disease pathways locally, such as the local lipid metabolism in the retina. This finding suggests that the systemic metabolites identified in this study represent relevant biomarkers of local processes, but further investigation is needed to evaluate whether systemic lipids and lipoproteins also have a direct effect on the pathophysiologic characteristics of AMD.

Genetic variants in genes of the complement system are well-established determinants of AMD risk, with the strongest associations identified at the complement factor H (*CFH*) locus. In this study, we did not observe any associations of AMD-associated genetic variants in the complement system with AMD-associated metabolites. It is known that AMD is associated with increased systemic complement activation, which in this study was defined as the C3d-to-C3 ratio.[54] Notably, we observed strong associations of systemic complement activation measurements with AMD-associated metabolites, including lipoprotein subfractions (large and very large HDL and VLDL), other lipids and apolipoproteins (remnant-C, ApoB, and triglycerides), fatty acids (MUFA, SFA, and TotFA), and amino acids (leucine, isoleucine, and alanine), and these associations were independent of AMD status. The direction of effect of the complement−metabolite associations were in alignment with the direction of effect of AMD−metabolite associations. Increased large and very large HDL levels were associated with increased complement activation, and both HDL levels and complement activation were increased in AMD patients compared with control participants. However, decreased VLDL, remnant-C, ApoB, triglycerides, MUFA, SFA, TotFA, isoleucine, leucine, and alanine levels were associated with increased complement activation, and these metabolites were decreased in AMD patients compared with control participants (Fig 6). Although these statistical associations do not prove causality, they may indicate biological interactions between the main AMD disease pathways: lipid metabolism and complement activation. A potential interaction between HDL and the complement system was shown in proteomic studies that demonstrated that HDL lipoprotein particles contain various complement components.[44] Notably, large HDL subparticles were shown to contain complement factor H (CFH), whereas small HDL and medium HDL subparticles did not. Although Zhang et al[44] propose that the increased CFH concentration would provide large HDL subparticles with anti-inflammatory properties, our study suggested the opposite: increased large HDL levels were associated with increased systemic complement activity. Perhaps increased uptake of CFH by large HDL subparticles leads to reduced levels of circulating unbound CFH, subsequently causing increased activity of the complement system. Recent studies have also shown that dietary intake of fatty acids is associated with complement system activity.[55,56] The biological mechanisms of whether and how the AMD-associated metabolites interact with the complement system should be investigated in future studies. Besides biological studies, future studies could also apply statistical approaches such as Mendelian randomization to study causal mechanisms among metabolites, disease pathways, and AMD.

Our study has several strengths and weaknesses. A strength of our study is the analysis of metabolite measurements in 6533 samples, which makes it the largest metabolomics study performed so far in AMD. Most of the identified metabolite associations were identified consistently among cohorts. The large sample size of our study allowed a thorough analysis of genetic associations for the identified metabolites. In addition, the availability of complement activation measurements for the EUGENDA cohort enabled us to study associations among the different AMD disease pathways.

Our study also has some weaknesses. We used an NMR-based targeted metabolomics platform, and it must be noted that this platform involves a limited number of measurements compared with mass spectrometry-based approaches. However, the platform has been used by a large number of epidemiologic studies, and the measurements provided are direct quantifications, which are consistent between batches

and studies. Next, AMD classifications slightly differed among cohorts (Supplemental File 1). To study differences in metabolite associations among AMD stages, harmonization of the AMD classifications should be performed in a follow-up study. We also acknowledge that systemic (blood) measurements may not reflect the local disease mechanisms in AMD. The samples from ALIENOR were fasting samples, whereas the samples of the other cohorts were collected in a nonfasting state. In Figures 2 through 4 and Table S3, the association results for each cohort are shown to provide more information on the effects in fasting samples. Lipid-altering medication was used as a covariate in the analyses of the RS, whereas no confounding effect of lipid-lowering medication was observed in the other cohorts. However, we cannot exclude that there may be residual confounding because of inaccurate documentation of lipid-altering medication through self-reported questionnaire data. It must also be noted that because of being restricted to European samples within our study, the findings may be different for different ethnical backgrounds.

In conclusion, we identified 60 metabolites that were associated with AMD, including HDL and VLDL lipoprotein particles, fatty acids (TotFA, MUFA, and SFA), amino acids (alanine, isoleucine, leucine, phenylalanine, and tyrosine), and citrate. Strong associations between the vast majority of the AMD-associated metabolites and complement activation may indicate biological interactions between the main AMD pathways, and multiple disease pathways may need to be targeted for successful treatment of AMD.

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Footnotes and Financial Disclosures

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[1] Department of Ophthalmology, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, Nijmegen, The Netherlands.

[2] Department of Ophthalmology, Erasmus University Medical Center, Rotterdam, The Netherlands.

[3] Department of Epidemiology, Erasmus University Medical Center, Rotterdam, The Netherlands.

[4] Université de Bordeaux, Inserm, Bordeaux Population Health Research Center, UMR 1219, Team LEHA, Bordeaux, France.

[5] Department of Biomedical Data Sciences, Leiden University Medical Center, Leiden, The Netherlands.

[6] Pattern Recognition and Bioinformatics, Delft University of Technology, Delft, The Netherlands.

[7] Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands.

[8] Institute for Epidemiology and Social Medicine, University of Muenster, Muenster, Germany.

[9] Augenzentrum, St. Franziskus Hospital, Münster, Germany.

[10] Nuffield Department of Population Health (NDPH), University of Oxford, Oxford, United Kingdom.

[11] Department of Ophthalmology, University Hospital of Cologne, Cologne, Germany.

[12] Roche Pharma Research and Early Development, F. Hoffmann-La Roche, Ltd., Basel, Switzerland.

[13] Radboud Institute for Health Sciences, Radboud University Medical Center, Nijmegen, The Netherlands.

For all metabolite abbreviations please see Table S2 (available at www.aaojournal.org).

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Members of the Eye-Risk Consortium: Blanca Arango-Gonzalez, MD (Centre for Ophthalmology, Institute for Ophthalmic Research, Eberhard Karls University Tübingen, University Clinic Tübingen, Tübingen, Germany); Angela Armento, PhD (Centre for Ophthalmology, Institute for Ophthalmic Research, Eberhard Karls University Tübingen, University Clinic Tübingen, Tübingen, Germany); Franz Badura (PRO RETINA Deutschland e.V.); Vaibhav Bhatia, PhD (Department of Regeneration and Cell Therapy, Andalusian Molecular Biology and Regenerative Medicine Centre (CABIMER), Seville, Spain); Shomi S. Bhattacharya, PhD (Department of Regeneration and Cell Therapy, Andalusian Molecular Biology and Regenerative Medicine Centre (CABIMER), Seville, Spain); Marc Biarnés, PhD (Barcelona Macula Foundation, Barcelona, Spain); Anna Borrell, MSc (Barcelona Macula Foundation, Barcelona, Spain); Sofia M. Calado, PhD (Department of Regeneration and Cell Therapy, Andalusian Molecular Biology and Regenerative Medicine Centre (CABIMER), Seville, Spain); Sascha Dammeier, PhD (Centre for Ophthalmology, Institute for Ophthalmic Research, Eberhard Karls University Tübingen, University Clinic Tübingen, Tübingen, Germany); Berta De la Cerda, PhD (Department of Regeneration and Cell Therapy, Andalusian Molecular Biology and Regenerative Medicine Centre (CABIMER), Seville, Spain); Francisco J. Diaz-Corrales, MD, PhD (Department of Regeneration and Cell Therapy, Andalusian Molecular Biology and Regenerative Medicine Centre (CABIMER), Seville, Spain); Sigrid Diether, PhD (Centre for Ophthalmology, Institute for Ophthalmic Research, Eberhard Karls University Tübingen, University Clinic Tübingen, Tübingen, Germany); Eszter Emri, PhD (Centre for Experimental Medicine, Queen's University Belfast, Belfast, United Kingdom); Tanja Endermann, PhD (Assay Development, AYOXXA Biosystems GmbH, Cologne, Germany); Lucia L. Ferraro, MD (Barcelona Macula Foundation, Barcelona, Spain); Míriam Garcia, OD, MSc (Barcelona Macula Foundation, Barcelona, Spain); Sabina Honisch, PhD (Centre for Ophthalmology, Institute for Ophthalmic Research, Eberhard Karls University Tübingen, University Clinic Tübingen, Tübingen, Germany); Ellen Kilger, PhD (Centre for Ophthalmology, Institute for Ophthalmic Research, Eberhard Karls University Tübingen, University Clinic Tübingen, Tübingen, Germany); Elod Kortvely, PhD (Roche Innovation Center Basel); Claire Lastrucci, PhD (Centre for Genomic Regulation, Barcelona, Spain); Hanno Langen, PhD (Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd., Basel, Switzerland); Imre Lengyel, PhD (Centre for Experimental Medicine, Queen's University Belfast, Belfast, United Kingdom); Phil Luthert, PhD (Institute of Ophthalmology, University College London, London, United Kingdom); Jordi Monés, MD, PhD (Barcelona Macula Foundation, Barcelona, Spain); Everson Nogoceke, PhD (Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd., Basel, Switzerland); Tunde Peto, MD, PhD (Centre for Public Health, Queen's University Belfast, Belfast, United Kingdom); Frances M. Pool, PhD (Ocular Biology, UCL Institute of Ophthalmology, London, United Kingdom); Eduardo Rodriguez-Bocanegra, MSc (Barcelona Macula Foundation, Barcelona, Spain); Luis Serrano, PhD (Centre for Genomic Regulation, Barcelona, Spain); Jose Sousa, PhD (Advanced Informatics CTU, Queen's University Belfast, Belfast, United Kingdom); Eric Thee, MD (Department of Epidemiology, Erasmus Medical Center, Rotterdam, Netherlands, Department of Ophthalmology, Erasmus Medical Center, Rotterdam, Netherlands); Marius Ueffing, PhD (Centre for Ophthalmology, Institute for Ophthalmic Research, Eberhard Karls University Tübingen, University Clinic Tübingen, Tübingen, Germany, Department of Ophthalmology, University Medical Centre Tübingen, Tübingen, Germany); Karl U. Ulrich Bartz-Schmidt, MD (Centre for Ophthalmology, Institute for Ophthalmic Research, Eberhard Karls University Tübingen, University Clinic Tübingen, Tübingen, Germany, Department of Ophthalmology, University Medical Centre Tübingen, Tübingen, Germany); and Markus Zumbansen, PhD (Research and Development, AYOXXA Biosystems GmbH, Cologne, Germany).

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Author Contributions:

Conception and design: Acar, Lores-Motta, van den Akker, Galesloot, den Hollander

Analysis and interpretation: Acar, Lores-Motta, Delcourt, Klaver, Galesloot, den Hollander

Data collection: Acar, Lores-Motta, Colijn, Meester-Smoor, Verzijden, Cougnard-Gregoire, Ajana, Merle, de Breuk, Heesterbeek, Daha, Claes, Pauleikhoff, Hense, van Duijn, Fauser, Hoyng, Delcourt, Klaver, Galesloot, den Hollander

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Abbreviations and Acronyms:
ACME = average casual effect estimates; **ALIENOR** = Antioxydants, Lipides Essentiels, Nutrition et Maladies Oculaires; **AMD** = age-related macular degeneration; **ApoB** = apolipoprotein B; **CI** = confidence interval; **CORRBI** = Combined Ophthalmic Research Rotterdam Biobank; **CVD** = cardiovascular disease; **EUGENDA** = European Genetic Database; **FDR** = false discovery rate; **GWAS** = genome-wide association study; **HDL** = high-density lipoprotein; **MARS** = Münster Age and Retina Study; **MUFA** = monounsaturated fatty acid; **NMR** = nuclear magnetic resonance; **OR** = odds ratio; **PCA** = principal component analysis; P_{FDR} = false discovery rate-corrected *P* value; **remnant-C** = remnant cholesterol level; **RS** = Rotterdam Study; **SD** = standard deviation; **SFA** = saturated fatty acid; **SNP** = single nucleotide polymorphism; **TotFA** = total fatty acid; **VLDL** = very low-density lipoprotein.

Correspondence:
Anneke I. den Hollander, PhD, Department of Ophthalmology 409, Radboud University Medical Center, Nijmegen, the Netherlands. E-mail: Anneke.denhollander@radboudumc.nl.