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OPHTHALMIC DISEASE

Using Mendelian randomization to evaluate the causal relationship between serum C-reactive protein levels and age-related macular degeneration

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Abstract

Serum C-reactive protein (CRP), an important inflammatory marker, has been associated with age-related macular degeneration (AMD) in observational studies; however, the findings are inconsistent. It remains unclear whether the association between circulating CRP levels and AMD is causal. We used two-sample Mendelian randomization (MR) to evaluate the potential causal relationship between serum CRP levels and AMD risk. We derived genetic instruments for serum CRP levels in 418,642 participants of European ancestry from UK Biobank, and then conducted a genome-wide association study for 12,711 advanced AMD cases and 14,590 controls of European descent from the International AMD Genomics Consortium. Genetic variants which predicted elevated serum CRP levels were associated with advanced AMD (odds ratio [OR] for per standard deviation increase in serum CRP levels: 1.31, 95% confidence interval [CI]: 1.19–1.44, $P = 5.2 \times 10^{-8}$). The OR for the increase in advanced AMD risk when moving from low (< 3 mg/L) to high (> 3 mg/L) CRP levels is 1.29 (95% CI: 1.17–1.41). Our results were unchanged in sensitivity analyses using MR models which make different modelling assumptions. Our findings were broadly similar across the different forms of AMD (intermediate AMD, choroidal neovascularization, and geographic atrophy). We used multivariable MR to adjust for the effects of other potential AMD risk factors including smoking, body mass index, blood pressure and cholesterol; this did not alter our findings. Our study provides strong genetic evidence that higher circulating CRP levels lead to increases in risk for all forms of AMD. These findings highlight the potential utility for using circulating CRP as a biomarker in future trials aimed at modulating AMD risk via systemic therapies.

Keywords C-reactive protein · Age-related macular degeneration · Mendelian randomization · Causal effect

Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible central vision loss among the elderly population in the Western world [1–5]. The etiology of AMD is not yet well understood; however, several hypotheses focus on the pathogenic pathways related to genetic predisposition, inflammation, complement, lipid, and oxidative stress [1, 6–8]. In support of this, genome-wide association studies (GWAS) have identified a variety of complement pathway related genes, such as complement factor H (*CFH*), factor I (*CFI*), and the complement components *C*2, *C*3, and *C*9 [6, 9]. The presence of complement and inflammatory reactions in drusen, the hallmark lesions of AMD, suggests the important role of inflammation in AMD pathogenesis.

C-reactive protein (CRP) is the most studied systemic marker of inflammation [10], and could induce proinflammatory responses and the progression of AMD [8]. Drugs

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targeted to CRP that alleviate inflammatory responses have been postulated to prevent the progression of AMD [7, 8]. However, observational studies have shown mixed conclusions on the association between circulating CRP levels and the risk of AMD [11–18]. Previous genetic studies have found no evidence of association between genetic variants in the *CRP* gene and AMD risk [19–22]. However, these genetic variants at the *CRP* locus only account for a relatively small proportion of the variability of circulating CRP levels, and more robust instruments for quantifying the genetic contribution to circulating CRP are needed [22]. Therefore, it remains unclear whether elevated circulating CRP levels are causally related to AMD risk.

Mendelian randomization (MR) is an instrumental-variable based approach to investigate the causal relationships between risk factors and outcomes via the use of genetic instruments (single nucleotide polymorphisms [SNPs] being most commonly used) [23, 24]. In MR analysis, as genetic instruments are distributed randomly at conception, the genetically predicted circulating CRP levels are unlikely to be related to confounders of AMD risks or consequentially influenced by AMD disease status through reverse causality. Therefore, the study design of MR is akin to a natural analogue of traditional RCT where unmeasured confounding are randomized across both the genetically predisposed (circulating CRP-increasing allele carriers) and unaffected group (reference; non-effect allele carriers) [23]. In this study, we investigate the causal relationship between genetically predicted circulating CRP levels and AMD risk, which would provide therapeutic implications for the prevention and treatment of AMD.

Methods

Study design

To investigate the causal relationship between serum CRP levels and AMD risk, we applied the two-sample MR framework [25], an approach to make causal inference using GWAS summary statistics for exposure and outcome from separate GWASs. We conducted a GWAS for serum CRP levels in the UK Biobank (UKBB) cohort to obtain genetic instruments for measured circulating CRP levels. We then conducted a series of GWAS analyses for advanced AMD and other AMD subtypes using the individual level data from the International AMD Genomics Consortium (IAMDGC). To assess sample overlap between UKBB and IAMDGC datasets, we ran LD score regression between CRP GWAS in UK Biobank and advanced AMD GWAS in IAMDGC dataset. The intercept of genetic covariance is 0.0058 (standard error 0.0104), which indicated that the intercept is approximately zero and there is little or no sample overlap between the two datasets.

The UK Biobank study was approved by the National Research Ethics Service Committee North West—Haydock, all participants provided informed written consent, and all study procedures were performed in accordance with the World Medical Association Declaration of Helsinki ethical principles for medical research. In the International AMD Genomics Consortium, all groups collected data according to Declaration of Helsinki principles. Study participants provided informed consent, and protocols were reviewed and approved by the local ethics committees.

Genetic instruments for serum C-reactive protein levels

The UKBB is a large-scale population-based cohort study of half a million people aged between 40 and 69 years living in the United Kingdom [26]. The serum CRP levels were available for 469,881 individuals (UKBB data field 30,710, http://biobank.ctsu.ox.ac.uk/crystal/field.cgi?id=30710) as part of the recent UKBB release (2019 release) for serum biochemistry data, and were measured using immunoturbidimetric method (high sensitivity analysis on a Beckman Coulter AU5800). The reportable range of high sensitivity serum CRP is from 0.08 to 80 mg/L (mean and standard deviation: 2.60 ± 4.34 mg/L, Supplementary Table 1). We included 418,642 participants of white British ancestry in the following serum CRP GWAS analysis (Supplementary Figure 1). We calculated the average values of serum CRP levels for individuals that underwent two assessments. We applied a rank-based inverse-normal transformation to serum CRP levels.

For the serum CRP GWAS in UKBB, we conducted a linear mixed model under an additive genetic model implemented via the BOLT-LMM software (version 2.3) [27]. The model was adjusted for sex, age and the first ten principal components (PCs). We selected independent genome-wide significant variants as genetic instruments for serum CRP levels using the following criteria: (1) *P* value on serum CRP $< 5 \times 10^{-8}$; (2) linkage disequilibrium (LD) between SNPs $r^2 < 0.01$; and (3) the SNPs being present in the AMD GWAS summary statistics (described below). The LD-clumping procedure was performed using PLINK (version 1.9) [28].

In our sensitivity analyses, we used the following methods to derive the genetic instruments: (1) in UKBB, we removed 16,946 (4%) participants with circulating CRP levels > 10 mg/L (e.g. due to a serious infection in the participant) and adjusted for body mass index (BMI, data field 21,001) in the association models; (2) we used previously reported circulating CRP variants (44 SNPs in our AMD GWAS summary statistics described below); [29] (3) to

evaluate the potential pleiotropic effects of CRP genetic instruments, we also ran a series of GWASs for other potential AMD risk factors including smoking, BMI, systolic blood pressure (SBP), high-density lipoprotein cholesterol (HDL-C), and glycated haemoglobin (HbA1c) in UKBB (Supplementary Table 1). In the causal inference of CRP levels on the risk of AMD, we adjusted for these risk factors by a multivariable MR analysis (see statistical analysis section, below).

Age-related macular degeneration dataset

The International Age-related Macular Degeneration Genomics Consortium (IAMDGC) dataset is the largest European GWAS focusing on AMD susceptibility (16,144 advanced AMD cases and 17,832 controls) [6]. The full description of the study design, phenotype definition, and genetic data were described previously [6]. Briefly, in IAMDGC, data were gathered from 26 studies with each including (a) advanced AMD cases with choroidal neovascularization (CNV) and/or geographic atrophy (GA) in at least one eye and age at first diagnosis more than 50 years old; (b) intermediate AMD cases with pigmentary changes in the retinal pigment epithelium or more than five macular drusen greater than 63 µm in diameter and age at first diagnosis more than 50 years old; or (c) controls without known advanced or intermediate AMD [6]. The individual level AMD phenotype data and genetic data are available in the database of Genotypes and Phenotypes (dbGaP, study accession: phs001039.v1.p1) [6]. We downloaded the imputation data for 35,358 participants. The imputation was based on the 1000 Genomes Project reference panel (1000 Genomes Project Phase I, version 3) using Minimac [6, 30]. SNPs with imputation quality score > 0.3 and MAF > 0.01 were retained for association analysis. In our association analysis, we removed non-European ancestry participants based on the first two principal components inferred ancestry [6]. Finally, we included 12,711 advanced AMD cases (8544 CNV, 2656 GA, and 1511 mixed AMD [both of CNV and GA] cases), 5336 intermediate AMD cases, and 14,590 controls in our analysis (Supplementary Table 2). We ran GWAS analyses for 12,711 advanced AMD cases and other AMD subtypes with 14,590 controls in PLINK software (version v2.00a1LM) adjusting for sex, age, and the first ten PCs.

Statistical analysis

To assess the power of our MR analyses, we used the mRnd (http://cnsgenomics.com/shiny/mRnd/) method to evaluate power for different AMD subtypes [31]. We conducted two-sample MR for circulating CRP levels and AMD risk using inverse-variance weighted (IVW) method as the main analysis [32, 33]. We verified the estimates using the MR weighted median and MR-Egger methods to allow violations of MR assumptions [34, 35]. Specifically, the weighted median MR method allows genetic variants representing over 50% of the weight in the MR analysis are valid instruments, while MR-Egger method can detect and correct for the bias due to directional pleiotropy (pleiotropic effects of genetic instruments do not average to zero) [36, 37]. The intercept from MR-Egger method was used to assess directional pleiotropy (i.e. intercept P value < 0.05) [36]. Although pleiotropy is concerning, if the pleiotropic effects are equally to be positive or negative (no directional pleiotropy), the overall estimate would be unbiased [37]. We also used the funnel plot and MR-PRESSO method to evaluate bias from outliers and assess the heterogeneity of genetic instruments [36, 38]. To further assess potential pleiotropic effects of related risk factors, we conducted a multivariable MR analysis [39, 40, 41]. In univariate MR analysis, the causal effect of a risk factor (CRP level) on the outcome (AMD) was assessed via genetic variants that are solely associated with that specific risk factor. The univariate IVW MR method is a weighted linear regression method to regress the effects of genetic instruments on AMD (outcome) against their effects on CRP level (exposure), with a forced intercept term at zero and weighted by inverse-variance. In multivariable MR analysis, we conducted GWAS for other potential AMD risk factors including smoking, BMI, SBP, HDL-C, and HbA1c. The analytic framework for multivariable MR-IVW method is similar to univariable MR-IVW except regressing on the effects of multiple risk factors in a single regression model [41]. In general, the univariate MR estimates the total effect of the circulating CRP on AMD risk, whereas multivariable MR could estimate the direct causal effect of circulating CRP on AMD risk when conditioned on the presence of other AMD risk factors [41, 42].

We performed MR analyses using R packages MendelianRandomization and TwoSampleMR [36, 43]. All analyses were performed with R (version 3.4.1) [44].

Results

Genetic instruments and statistical power

In our UKBB circulating CRP GWAS, we identified 526 independent genome-wide significant SNPs as genetic instruments (Supplementary Table 3), which explained 13% of the variance of circulating CRP levels (Supplementary Figure 2). Our MR analyses yield adequate power to detect moderate effect sizes (e.g. odds ratio [OR] 1.2 per standard deviation increase of circulating CRP levels); our power for advanced AMD, intermediate, GA, CNV, and mixed AMD is 100%, 99%, 91%, 100%, and 75%, respectively (Supplementary Table 4).

Circulating CRP levels are associated with advanced AMD

The MR scatter plot indicates that higher serum CRP levels were associated with increased risk of advanced AMD (Fig. 1). The overall MR-IVW OR of advanced AMD per standard deviation (SD, 4.34 mg/L) increase in genetically predicted circulating CRP levels was 1.31 (95% confidence interval [CI]: 1.19 to 1.44, $P = 5.2 \times 10^{-8}$, Table 1), which is 1.06 for each one mg/L increase in circulating CRP levels. Another way of interpreting these results is to consider a more clinically relevant change. For example we can consider a change in CRP for those with high (> 3 mg/L) versus low (< 3 mg/L) levels. The estimated odds ratio for the difference between these groups is 1.29 (exp($\log_e$1.31/4.34*4.09); where 4.09 mg/L is the change in CRP between the median level in the high group and the median level in the low group). The estimation between genetically predicted circulating CRP levels and advanced AMD was similar to the results from MR-Egger method (OR = 1.41, 95% CI: 1.22–1.63, $P = 1.9 \times 10^{-6}$) and MR weighted median method (OR = 1.17, 95% CI: 1.00–1.37, $P = 0.046$) with overlapping confidence intervals. We found no evidence of directional pleiotropy effects based on MR-Egger intercept test (intercept −0.003, $P = 0.15$). The MR-PRESSO outlier-corrected result was not meaningfully different from the MR-IVW estimate (OR = 1.17, 95% CI: 1.07–1.29, $P = 8.2 \times 10^{-4}$) and the MR funnel plot showed no evidence of asymmetry (Supplementary Figure 3). To further investigate whether pleiotropy effects distorted our estimates, we also conducted a multivariable Mendelian randomization analysis to adjust for other potential AMD risk factors including: smoking; body BMI; SBP; HDL-C; and HbA1c. The association between circulating CRP levels and advanced AMD was essentially unchanged in multivariable MR analysis (OR = 1.27, 95% CI: 1.14–1.40, $P = 7.1 \times 10^{-6}$). The consistency of total effect and direct effect of CRP levels on AMD risk based on univariate and multivariable MR estimates supported an independent association between circulating CRP levels and the risk of AMD (Table 1).

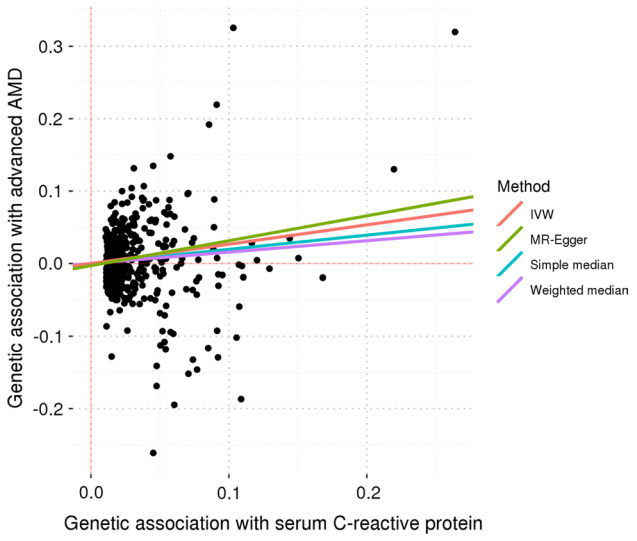

Fig. 1 Serum C-reactive protein-increasing risk variants are associated with increased risk of advanced age-related macular degeneration. The x-axis shows the estimates for the 526 genetic instruments for serum C-reactive protein levels, the y-axis shows the estimates (log odds ratios) of the effects of the same variants on advanced age-related macular degeneration. The Mendelian randomization (MR) inverse-weighted (IVW), MR-Egger, simple median and weighted median method lines are plotted with red, green, blue, and purple lines, respectively

Sensitivity analysis

We constructed genetic instruments for circulating CRP by removing participants with serum CRP > 10 mg/L and adjusting for body mass index (BMI) in circulating CRP GWAS. The average MR-IVW OR of advanced AMD per SD (1.83 mg/L) increase in genetically predicted circulating CRP levels was 1.22 (95% CI: 1.09–1.37, $P = 6.4 \times 10^{-4}$). We also repeated our MR analysis using 44 previously reported circulating CRP variants (independent from the UKBB cohort) as genetic instruments [29], the estimation was similar to our main analysis (OR per unit change in the natural-log-transformed CRP (mg/L) was 1.40, 95% CI: 1.16–1.70, $P = 5.4 \times 10^{-4}$); this shows our results are robust to the particular SNP instruments used, although as expected our power is highest (and consequential our confidence intervals are narrowest) with the full set of genome-wide significant SNPs.

Circulating CRP levels are associated with different AMD subtypes

We then evaluated the relationships between circulating CRP levels and different AMD subtypes (Table 1 and Supplementary Figure 4). The MR-IVW ORs of genetically predicted circulating CRP levels on different AMD subtypes were highly consistent: for intermediate AMD, GA, CNV, and mixed AMD types the ORs were 1.15 (95% CI: 1.04–1.27, $P = 7.1 \times 10^{-3}$), 1.28 (95% CI: 1.11–1.48, $P = 7.3 \times 10^{-4}$), 1.28 (95% CI: 1.15–1.43, $P = 3.5 \times 10^{-6}$), and 1.52 (95% CI: 1.28–1.79, $P = 1.3 \times 10^{-6}$), respectively. Our results indicated circulating CRP levels were associated with each of intermediate AMD, CNV, GA, and mixed AMD types. These results show that the overall findings are unlikely to be driven by a very strong association on specific AMD subtypes, suggesting CRP may be involved in different stages and types of AMD progression.

Table 1 Mendelian randomization estimates of the associations between serum C-reactive protein levels and age-related macular degeneration

Trait[a]	Method	OR[b]	95% CI	*P* value
Advanced AMD	IVW	1.31	[1.19, 1.44]	5.2×10^{-8}
	Multivariable MR[c]	1.27	[1.14, 1.40]	7.1×10^{-6}
	Weighted median	1.17	[1.00, 1.37]	0.047
	MR-Egger	1.41	[1.22, 1.63]	1.9×10^{-6}
	(intercept)	−0.003	[−0.007, 0.001]	0.15
Intermediate AMD	IVW	1.15	[1.04, 1.27]	7.1×10^{-3}
	Multivariable MR	1.12	[1.00, 1.24]	0.046
	Weighted median	1.1	[0.92, 1.32]	0.29
	MR-Egger	1.25	[1.08, 1.45]	2.7×10^{-3}
	(intercept)	−0.003	[−0.008, 0.001]	0.11
GA AMD	IVW	1.28	[1.11, 1.48]	7.3×10^{-4}
	Multivariable MR	1.19	[1.02, 1.38]	0.03
	Weighted median	1.07	[0.82, 1.38]	0.63
	MR-Egger	1.21	[0.98, 1.50]	0.08
	(intercept)	0.002	[−0.004, 0.008]	0.49
CNV AMD	IVW	1.28	[1.15, 1.43]	3.5×10^{-6}
	Multivariable MR	1.26	[1.13, 1.42]	5.4×10^{-5}
	Weighted median	1.25	[1.05, 1.48]	0.01
	MR-Egger	1.39	[1.19, 1.62]	3.1×10^{-5}
	(intercept)	−0.003	[−0.007, 0.001]	0.17
Mixed AMD	IVW	1.52	[1.28, 1.79]	1.3×10^{-6}
	Multivariable MR	1.48	[1.23, 1.77]	2.2×10^{-5}
	Weighted median	1.61	[1.17, 2.21]	3.3×10^{-3}
	MR-Egger	2.08	[1.63, 2.67]	6.7×10^{-9}
	(intercept)	−0.01	[−0.02, −0.005]	6.9×10^{-4}
All AMD	IVW	1.26	[1.16, 1.37]	1.1×10^{-7}
	Multivariable MR	1.23	[1.12, 1.34]	1.2×10^{-5}
	Weighted median	1.17	[1.02, 1.34]	0.03
	MR-Egger	1.36	[1.21, 1.55]	9.9×10^{-7}
	(intercept)	−0.003	[−0.007, 0.0004]	0.08

AMD age-related macular degeneration, *CI* confidence interval, *IVW* inverse-variance weighted, *MR* Mendelian randomization, *OR* odds ratio

[a]Different subtypes of age-related macular degeneration: advanced AMD, intermediate AMD, geographic atrophy (GA) AMD, choroidal neovascularization (CNV) AMD, mixed AMD (CNV and GA), and all AMD (both of intermediate AMD and advanced AMD)

[b]The intercepts for MR-Egger are shown on the raw scale rather than the exponential scale

[c]Multivariable Mendelian randomization analysis is a regression-based MR method adjusting here for the effects of smoking, body mass index, systolic blood pressure, high-density lipoprotein cholesterol, and glycated haemoglobin (HbA1c)

Discussion

In this study, we conducted comprehensive MR analyses to investigate the causal relationships between circulating CRP levels and the risk of different AMD subtypes. We found that higher genetically predicted circulating CRP levels were associated with increased risk of advanced AMD and other AMD subtypes. These findings enhance our understanding of the underlying pathological mechanism of AMD and could have clinical utility for identification of high-risk individuals.

Our study corroborates results from previous observational studies and meta-analysis showing elevated circulating CRP is a risk factor for AMD [11, 12, 15–17]. A meta-analysis showed that the OR for higher circulating CRP level (CRP > 3 mg/L vs < 1 mg/L) was 2.19 (95% CI: 1.38–3.47) for advanced AMD; the OR was 1.31 (95% CI: 1.04–1.65) for combined early and late AMD [16]. Another pooled analysis of five cohorts also indicated that elevated CRP levels (CRP > 3 mg/L vs < 1 mg/L) increased the risk of overall incident AMD (OR = 1.49; 95% CI: 1.06–2.08) and neovascular AMD (OR = 1.84; 95% CI: 1.14–2.98) [17]. In

our MR analysis, the OR is also higher for advanced AMD (OR = 1.31; 95% CI: 1.19–1.44) compared with only intermediate AMD (OR = 1.15; 95% CI: 1.04–1.27) albeit with overlapping CIs. These results may indicate circulating CRP levels have a larger effect on advanced AMD than early or intermediate AMD. However, some observational studies failed to obtain evidence for the association between circulating CRP levels and AMD risk [13, 14]. The inconsistent results from observational studies may be due to selection bias of AMD subtype composition (small proportion of advanced AMD cases), small sample size, and sub-optimal study designs (i.e. susceptible to confounding for cross sectional or case-control designs) [13, 14]. The key advantage of Mendelian randomization analysis is that the causal inference drawn through genetic instruments is less likely to be susceptible to confounding and reverse causation. As an ancillary analysis we used reverse-direction MR to examine the effect of AMD on circulating CRP levels but found no effect (MR-IVW *P* value 0.56) [23].

Several studies investigated the association of genetic variants in *CRP* gene and AMD risk, but found no evidence for an association [19–22]. Although the variants in *CRP* gene that were used in these studies are associated with circulating CRP levels, these SNPs in aggregation only explain a relatively small proportion of the variance in circulating levels of CRP ($r^2 < 2\%$), hindering power for a proper MR analysis [22]. Moreover, the sample sizes for AMD cases and controls in their studies were relatively small. In our MR analysis, we conducted the largest GWAS for circulating CRP levels to date, and the lead 526 circulating CRP levels related SNPs explained 13% of the variance. In our sensitivity analysis, we also used a 44 SNP set (explaining about 7% of the variance; our results were similar, although as expected confidence intervals were considerably wider [29].

A concern in MR analysis is the possibility of pleiotropic effects of genetic instruments [37]. It is possible that a subset of our CRP variants might have been associated with AMD risk through measured or unmeasured confounders, which may violate one of the MR assumption [23]. To address this concern, our sensitivity MR analysis performed using the MR-Egger and weighted median methods results in similar conclusion showing that our findings were robust [24, 45]. We found no evidence of directional pleiotropy based on MR-Egger intercept test and the MR funnel plot showed no evidence of asymmetry. We also used a multivariable Mendelian randomization analysis to adjust for potential AMD risk factors including smoking, BMI, SBP, HDL-C, and HbA1c. The associations between circulating CRP levels and advanced AMD or other AMD subtypes in the multivariable model were similar to those estimated from the main analysis. The sensitivity analysis to construct genetic instruments for circulating CRP by removing participants with serum CRP > 10 mg/L and adjusting for BMI or using 44 previously reported circulating CRP variants also showed similar results to the main analysis. These results indicate the finding of an association between circulating CRP and AMD risk is unlikely to be driven by horizontal pleiotropy effects.

There are several limitations in our study. In the GWASs of circulating CRP and AMD, we only included European ancestry participants, thus it is unclear whether our results are also applicable to people not of European ancestry. The generalizability of the association between circulating CRP levels and AMD risk in other ethnic groups would require further investigation. Secondly, we estimated the overall population-averaged effect of elevated circulating CRP levels and AMD risk assuming linearity, and did not attempt to dissect potential non-linear relationships between circulating CRP levels and AMD risk. Thirdly, the MR findings reflect the change in AMD risk due to a genetically predisposed (lifetime) change in circulating CRP levels, hence the short-term effect of increasing of circulating CRP levels on AMD risk is unknown.

In conclusion, genetically predicted elevated circulating CRP levels were associated with increased risk of AMD. Our study provided strong evidence for a causal effect of inflammation as proxied via higher circulating CRP concentrations on AMD risk, regardless of AMD disease subtypes. Further studies are warranted to investigate the clinical utility of serum CRP levels in combination with the other AMD predictors for identification of high-risk individuals and therapeutic treatment in preventing AMD.

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Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.

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