Genetically Determined Levels of Circulating Cytokines and Risk of Stroke: Role of Monocyte Chemoattractant Protein-1

Running title: *Georgakis et al.; MCP-1 levels and stroke: Mendelian randomization*

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ABSTRACT

Background—Cytokines and growth factors have been implicated in the initiation and propagation of vascular disease. Observational studies have shown associations of their circulating levels with stroke. Our objective was to explore whether genetically determined circulating levels of cytokines and growth factors are associated with stroke and its etiologic subtypes by conducting a two-sample Mendelian randomization (MR) study.

Methods—Genetic instruments for 41 cytokines and growth factors were obtained from a genome-wide association study (GWAS) of 8,293 healthy adults. Their associations with stroke and stroke subtypes were evaluated in the MEGASTROKE GWAS dataset (67,162 cases; 454,450 controls) applying inverse-variance-weighted meta-analysis, weighted-median analysis, MR-Egger regression, and multivariable MR. The UK Biobank cohort was used as an independent validation sample (4,985 cases; 364,434 controls). Genetic instruments for monocyte chemoattractant protein-1 (MCP-1/CCL2) were further tested for association with etiologically related vascular traits using publicly available GWAS data.

Results—Genetic predisposition to higher MCP-1 levels was associated with increased risk of any stroke (OR per 1-SD increase: 1.06, 95% CI: 1.02-1.09, p=0.0009), any ischemic stroke (OR: 1.06, 95% CI: 1.02-1.10, p=0.002), large artery stroke (OR: 1.19, 95% CI: 1.09-1.30, p=0.0002) and cardioembolic stroke (OR: 1.14, 95% CI: 1.06-1.23, p=0.0004), but not with small vessel stroke or intracerebral hemorrhage. The results were stable in sensitivity analyses and remained significant after adjustment for cardiovascular risk factors. Analyses in the UK Biobank showed similar effect sizes for available phenotypes (any stroke: OR: 1.08, 95% CI: 0.99-1.17, p=0.09; any ischemic stroke: OR: 1.07, 95% CI: 0.97-1.18, p=0.17). Genetically determined higher MCP-1 levels were further associated with coronary artery disease (OR: 1.04, 95% CI: 1.00-1.08, p=0.04) and myocardial infarction (OR: 1.05, 95% CI: 1.01-1.09,

p=0.02), but not with atrial fibrillation. A meta-analysis of observational studies showed higher circulating MCP-1 levels in stroke patients compared to controls.

Conclusions—Genetic predisposition to elevated circulating levels of MCP-1 is associated with increased risk of stroke, particularly with large artery stroke and cardioembolic stroke. Whether targeting MCP-1 or its receptors can lower stroke incidence requires further study.

Key Words: MCP-1; CCL2; inflammation; cytokines; atherosclerosis; stroke; Mendelian randomization; genetics, human

CLINICAL PERSPECTIVE

What is new?

- Genetic predisposition to higher circulating levels of monocyte chemoattractant protein-1 (MCP-1/CCL2) was associated with the risk of stroke
- Associations were also found for etiologic stroke subtypes, specifically large artery stroke and cardioembolic stroke
- Genetically determined levels of MCP-1 also associated with the risk of the related phenotypes of coronary artery disease and myocardial infarction

What are the clinical implications?

- The results suggest that interventions aimed at targeting MCP-1 or its downstream effectors may be a promising strategy for lowering stroke risk

INTRODUCTION

Stroke is the leading cause of long-term disability and the second most common cause of death world-wide[1, 2] with a growing burden on global health.[3] Inflammatory mechanisms have been implicated in stroke and etiologic stroke subtypes,[4-7] and specifically demonstrated for large artery atherosclerotic stroke.[4, 5] Cytokines and growth factors regulate the inflammatory response[4] and thus may serve as targets for cardiovascular disease prevention.[8] Indeed, the CANTOS trial recently demonstrated the potential of targeting specific inflammatory cytokines in reducing vascular endpoints.[9]

Few studies have investigated associations between circulating levels of inflammatory cytokines and risk of stroke. Levels of IL-1β and IL-6 were found to be associated with incident and recurrent ischemic stroke.[4] However, these associations derived from observational studies preclude conclusions about causal relationships because of possible confounding and reverse causation.[10] Also, associations with etiologic stroke subtypes were not investigated in depth.[4] Hence, the potential causative role of individual cytokines in determining stroke risk remains elusive. Developing meaningful strategies for stroke prevention will require defining these relationships.[11]

Mendelian randomization (MR) aims to overcome the limitations of conventional epidemiologic studies with respect to confounding and reverse causation. By using genetic variants as instrumental variables for a trait, MR enables an investigation of causal effects.[12, 13] A recent genome-wide association study (GWAS) in 8,293 healthy subjects of Finnish ancestry identified multiple common genetic variants that influence circulating levels of 41 cytokines and growth factors (referred to hereafter as 'cytokines' for simplicity),[14] thus providing comprehensive data on genetic determinants of circulating inflammatory biomarkers.[14]

Here, by leveraging data from this recent GWAS on cytokines[14] and the largest GWAS meta-analysis on stroke and stroke subtypes to date,[15] we implemented a two-sample MR study to: (i) explore the associations between genetic predisposition to higher or lower circulating cytokine levels with risk of any stroke; (ii) evaluate specific associations with ischemic stroke and its major etiologic subtypes (large artery stroke, cardioembolic stroke, and small vessel stroke), as well as with intracerebral hemorrhage; (iii) validate these findings in UK Biobank as an independent cohort; (iv) compare the MR effects to effect estimates derived from meta-analyses of observational studies and (v) examine the association with etiologically related vascular outcomes including coronary artery disease (CAD), myocardial infarction (MI), and atrial fibrillation (AF).

METHODS

Study design and data sources

The overall design of this study is displayed in **Figure 1**. **Supplemental Table 1** summarizes our data sources for this MR study. The genetic instruments were taken from publicly available summary statistics.[14] For each of the 41 cytokines (full list provided in **Supplemental Table 2**) we selected single nucleotide polymorphisms (SNPs) associated with its circulating levels at a significance threshold of a false discovery rate (FDR) <5%.[16] To avoid bias by selection of false positive instruments, we performed additional analyses using a genome-wide threshold of significance (p <5x10^{-8}). After extracting the summary statistics for significant SNPs, we pruned all SNPs in linkage disequilibrium (LD; r^2 <0.1 in the European 1000G reference panel) retaining SNPs with the lowest p-value as independents instrument. We identified 698 SNPs not in LD to be significantly associated with circulating cytokine levels; 615 of them were also available in the MEGASTROKE dataset. To avoid use of pleiotropic instruments we excluded 126 SNPs that were associated with levels of more than

one cytokine[17] leaving 489 SNPs as the final instruments. These instruments related to the circulating levels of 23 cytokines, whereas for 18 cytokines no SNPs associated with their circulating levels at a significance level of FDR <5% could be identified.

The primary outcomes for this study were any stroke, any ischemic stroke, etiologic ischemic stroke subtypes defined by TOAST criteria (large artery stroke, cardioembolic stroke, and small vessel stroke),[18] and intracerebral hemorrhage. We extracted effect estimates for the associations of the selected instruments with any stroke, any ischemic stroke and its subtypes from the MEGASTROKE multi-ancestry GWAS dataset (67,162 cases; 454,450 controls).[15] Sensitivity analyses restricted to individuals of European ancestry (40,528 cases; 445,396 controls) were conducted, to minimize ancestral mismatch with the Finnish population used for the discovery GWAS on cytokines.[14] For intracerebral hemorrhage, we extracted data from publicly available summary statistics of a GWAS meta-analysis on 1,545 cases and 1,481 controls of European ancestry.[19]

We computed *F*-statistics to quantify the strength of the selected instruments[20] and performed power calculations.[21] The *F*-statistic for the 489 instrument SNPs ranged from 17 to 789 (**Supplemental Table 3**), well above the threshold of $F > 10$ typically recommended for MR analyses.[22] Based on the sample size of MEGASTROKE, there was >80% power to detect significant associations with any stroke and any ischemic stroke for 18 of 23 cytokines at an effect size (OR [odds ratio]) of 1.10. Power was lower for the remaining 5 cytokines and for sub-analyses for ischemic stroke subtypes and intracerebral hemorrhage (**Supplemental Table 3**).

For validation of significant associations in MEGASTROKE, we used the UK Biobank dataset as detailed in the **Supplemental Methods**. We included cases of prevalent and incident stroke. Cases with an unconfirmed self-reported diagnosis of stroke were excluded from the analysis. The final sample size consisted of 369,419 individuals, including 4,985

cases with any stroke and 3,628 cases with any ischemic stroke. No data were available on ischemic stroke subtypes.

Cytokines that were significantly associated with stroke were subsequently explored for an association with etiologically related vascular outcomes. Publicly available summary statistics were extracted from the CARDIoGRAMplusC4D Consortium for CAD and MI (60,801 CAD and 43,676 MI cases; 123,504 controls),[23] and the AFGen Consortium for AF (17,931 cases; 115,142 controls).[24]

Statistical analysis

After extraction of data and harmonization of the effect alleles across GWASs, we computed individual MR estimates and standard errors from the SNP-cytokine and SNP-outcome effects using the Wald estimator and the Delta method that weight all estimates based on the effect size of the SNP-cytokine association.[25] The MR effect of each cytokine on stroke was estimated after pooling individual SNP MR estimates using fixed-effects inverse-variance weighted (IVW) meta-analysis.[25] Statistical significance for the MR associations with stroke was set at a *p*-value corrected for multiple comparisons (based on number of cytokines) using the Bonferroni method. We further report on results corrected for both the number of cytokines and the number of examined phenotypes. A $p < 0.05$ but above the Bonferroni-corrected threshold was considered as suggestive for association. The IVW MR approach assumes that instruments affect the outcome only through the exposure under consideration, and not by some alternative pathway.[25] Any violation of this assumption would represent horizontal pleiotropy of the instrument and could introduce bias to the MR estimate. In the absence of any such horizontal pleiotropy, there would not be any expected heterogeneity in the MR estimates obtained from different instruments. As such, heterogeneity markers (I^2

>25% or Cochran *Q*-derived p <0.05) from the IVW MR were used as indicators of possible horizontal pleiotropy.[26]

For cytokines showing either significant or suggestive associations or significant heterogeneity in the primary IVW MR analysis, we conducted additional sensitivity analyses that vary in their underlying assumptions regarding the presence of pleiotropic genetic variants that may be associated with the outcome independently of the exposure. Particularly, we used MR-Egger regression, which requires that the strengths of the instruments are independent of their direct effect on the outcome,[27] and the weighted median method, which requires that at least half of the information for the MR analysis comes from valid instruments.[28] We used the intercept obtained from the MR-Egger regression as a measure of directional pleiotropy (p <0.05 was considered significant),[27] and also tested for outlier SNPs using MR-PRESSO.[29]

To generate MR estimates unaffected by the presence of pleiotropic pathways acting through cardiovascular risk factors, we performed regression-based multivariable MR with summary genetic association estimates[30] that adjusted for the genetic association of instruments with circulating lipid levels (LDL cholesterol, HDL cholesterol, triglycerides), type 2 diabetes (T2D), and blood pressure measurements (systolic and diastolic blood pressure, hypertension). Genetic association estimates for these phenotypes were extracted from the GLGC consortium,[31] the DIAGRAM consortium,[32] and the UK Biobank GWAS published by the Neale lab (https://sites.google.com/broadinstitute.org/ukbbgwasresults), respectively.

Instrument SNPs for cytokines showing significant associations with stroke were mapped to the nearest gene using the GRCh37/hg19 reference genome. We used the STRING database[33] to look for protein-protein interactions between gene products and the cytokines and identified interacting subnetworks. As a sensitivity analysis and to gain further insight into the

biological processes involved in the examined associations, we performed IVW MR analysis with SNPs restricted to the specific subnetworks.

The GWAS used to select cytokine instruments included no replication and its effect estimates were further adjusted for BMI, besides age and sex.[14] As a sensitivity analysis for any possible bias that may be introduced by this BMI adjustment or winner's curse,[34] we also calculated an unweighted allele score for any cytokines demonstrating a significant effect in our main IVW MR analysis.[35] Such an unweighted allele score may offer evidence of a causal effect of the exposure on the outcome without suffering from bias in the genetic association estimates for the exposure, although this is at the cost of not being able to estimate the magnitude of any such effect.[35] Statistical analysis was conducted in Stata 13.1 (StataCorp).

Meta-analysis of observational studies

For the cytokines that showed significant associations with stroke in MR, we performed a meta-analysis of observational studies. We searched Medline until December 10, 2017 (search strategy is available in the **Supplemental Methods**), for case-control studies comparing the circulating cytokine levels between stroke patients and controls, and cohort studies exploring the association of baseline levels with incident or recurrent stroke. We extracted relevant data and applied random-effects meta-analyses for Hazard ratios (cohort studies) or standardized mean differences (case-control studies). We evaluated heterogeneity with the I^2 and the Cochran Q.

Access to publicly available data

The analyses for this study were based on publicly available summary statistics from GWAS Consortia. The web-links for downloading the data are provided in **Supplemental Table 1**

along with descriptive characteristics of the Consortia. The retrieved summary data for the current analysis and the code script are available upon reasonable request to the corresponding author. As all analyses have been based on publicly available summary statistics and not individual-level data, no ethical approval from an institutional review board was required.

RESULTS

Genetically determined circulating levels of cytokines and risk of stroke

The primary results of the MR analyses for the 23 cytokines are presented in **Figure 2**. Following Bonferroni correction for testing multiple cytokines (p <0.05/23=2.2x10^{-3}), the only cytokine showing statistically significant associations with stroke was the CC chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2). As depicted in **Figure 3A** and **Supplemental Figure 1**, genetically determined higher circulating MCP-1 levels (1-SD increase) were associated with 6% increased odds for both any stroke (OR: 1.06, 95%CI: 1.02-1.09, p=9x10^{-4}; 523,047 individuals; 66,856 cases) and any ischemic stroke (OR: 1.06, 95%CI: 1.02-1.10, p=1.8x10^{-3}; 511,551 individuals; 60,341 cases) in MR analyses. Corresponding analyses for ischemic stroke subtypes revealed significant associations for large artery stroke (OR: 1.19, 95%CI: 1.09-1.30, p=1.7x10^{-4}; 245,201 individuals; 6,688 cases) and cardioembolic stroke (OR: 1.14, 95%CI: 1.06-1.23, p=3.5x10^{-4}; 361,858 individuals; 9,006 cases), but not for small vessel stroke (OR: 1.03, 95%CI: 0.95-1.11, p=0.50; 298,777 individuals; 11,710 cases). We further found no significant association of genetically determined MCP-1 levels with intracerebral hemorrhage (OR: 1.24, 95%CI: 0.94-1.64, p=0.13), although this might be related to the lower sample size (3,026 individuals; 1,545 cases). Importantly, the results for large artery stroke and cardioembolic stroke remained significant when further correcting for both the number of examined cytokines and the number of examined phenotypes (p <0.05/138=3.6x10-4; **Figure 2**). Sub-analyses

restricted to lobar (OR: 1.25, 95%CI: 0.88-1.79, p=0.22; 2,145 individuals; 664 cases), and nonlobar intracerebral hemorrhage (OR: 1.03, 95%CI: 0.72-1.49, p=0.16; 2,362 individuals; 881 cases) also showed no significant associations with genetically determined MCP-1 levels. The individual SNPs associated with MCP-1 levels explained 14.7% of the variance of MCP-1 levels (**Supplemental Table 3**) and are presented in **Supplemental Table 4**.

There was no evidence for heterogeneity in any of the MCP-1 associations as measured by I^2 and Cochran Q (**Figure 3A**) and no outlier SNPs were detected with the MR-PRESSO method. Also, there was no indication for directional pleiotropy effects as assessed by the MR-Egger intercept (any stroke, p=0.41; any ischemic stroke, p=0.39; large artery stroke, p=0.98; cardioembolic stroke, p=0.67; small vessel stroke, p=0.70; intracerebral hemorrhage, p=0.94). The weighted median estimator and the MR-Egger regression analysis provided estimates of the same magnitude as the fixed-effects IVW meta-analysis for large artery stroke (OR: 1.22, 95%CI: 1.07-1.40, $p=2\text{x}10^{-3}$ and OR: 1.19, 95%CI: 0.93-1.53, p=0.13, respectively) and cardioembolic stroke (OR: 1.13, 95%CI: 1.01-1.27, p=0.04 and OR: 1.21, 95%CI: 0.96-1.53, p=0.09, respectively, **Figure 3B**); although with wider confidence intervals as would be expected given the lower statistical power of these approaches.[27, 28] Use of an unweighted allele score for the MCP-1 instrument SNPs also showed statistically significant associations with risk of large artery ($p=1.5\text{x}10^{-4}$) and cardioembolic stroke ($p=2.8\text{x}10^{-4}$). The significant effect of MCP-1 on outcomes was retained both when restricting the analysis to individuals of European ancestry (**Supplemental Figure 2**), and when applying the more conservative threshold of $p<5\text{x}10^{-8}$ for instrument selection (**Supplemental Figure 3**).

To explore whether the MR effect of genetically determined MCP-1 levels on stroke was attributable through pleiotropic pathways relating to cardiovascular risk factors, we conducted multivariable MR analysis adjusting for circulating lipid levels, T2D, and blood pressure. The

results remained stable regardless of the model (unadjusted, single or fully-adjusted model), thus supporting an independent effect of MCP-1 levels on stroke and stroke subtypes (**Table 1**).

None of the genetic instruments for MCP-1 was within or close to the *MCP1* gene. Looking at genes closest to the instruments for MCP-1 we noted that several of them encoded proteins that show a biological relationship with MCP-1, e.g. CCR2 the main receptor for MCP1 (**Supplemental Table 4**). To minimize the risk of using unspecific instruments that might exert pleiotropic effects we performed an additional sensitivity analysis focusing on instruments in the vicinity of these genes. Using the STRING database, we found the chemokine receptors CCR2, CCR1, CCR3, and CCR9, the chemokine binding protein CCBP2, and the receptor of the complement C5a (C5aR1) to integrate into a subnetwork of established interactions with MCP-1 (**Supplemental Figure 4A)**. Restricting the MR analysis to the respective SNPs, resulted in significant effect estimates for large artery (OR per 1-SD increase in MCP-1 levels: 1.25, 95%CI: 1.08-1.45, $p=2x10^{-3}$) and cardioembolic stroke (OR: 1.21, 95%CI: 1.07-1.37, $p=3x10^{-3}$), as well as intracerebral hemorrhage (OR: 2.19, 95%CI: 1.30-3.69, $p=3x10^{-3}$) (**Supplemental Figure 4B**).

Several other cytokines not reaching the Bonferroni-corrected threshold showed suggestive ($p<0.05$) associations with risk of stroke in MR analyses: genetic predisposition to higher levels of eotaxin, IP-10, MIG, PDGF-bb, and VEGF were associated with an increased risk of stroke whereas predisposition to higher levels of SCF and SCGF-b were associated with lower risk of stroke (**Figure 2**).

Genetically determined circulating levels of MCP-1 and risk of stroke in UK Biobank

We next explored the MR effect of genetically determined MCP-1 levels on risk of any stroke and risk of any ischemic stroke in the independent UK Biobank sample and meta-analyzed the

MEGASTROKE and UK Biobank data (**Figure 4A and Supplemental Figure 5**). Effect estimates in UK Biobank were similar to MEGASTROKE for any stroke (OR per 1-SD increase: 1.08, 95%CI: 0.99-1.17, p=0.09; 369,419 individuals, 4,985 cases) and any ischemic stroke (OR: 1.07, 95%CI: 0.97-1.18, p=0.17; 369,419, 3,628 cases), but did not reach statistical significance. Genetically elevated circulating MCP-1 levels were significantly associated with both any stroke (OR: 1.06, 95%CI: 1.03-1.09, $p=2x10^{-4}$) and any ischemic stroke (OR: 1.06, 95%CI: 1.03-1.10, $p=7x10^{-4}$) in the meta-analysis of MEGASTROKE and UK Biobank

Circulating levels of MCP-1 and risk of stroke: meta-analysis of observational studies

Next, we compared the MR estimates with those derived from a meta-analysis of observational studies. Our search yielded 17 case-control studies of ischemic stroke patients and controls, two cohort studies on patients with a history of stroke or cardiovascular disease exploring the risk of recurrent ischemic stroke, and one case-cohort study of incident ischemic stroke in a community population (**Supplemental Table 5** and **Supplemental Figure 6**). Patients with any ischemic stroke were found to have significantly higher MCP-1 levels than controls in the case-control studies (Hedges' g: 0.66, 95%CI: 0.18-1.15 [corresponding to a medium to strong effect size[36]]; 1137 cases, 717 controls; heterogeneity: I^2=89%, p<0.001; **Figure 4B** and **Supplemental Figure 7A**). Studies on recurrent stroke (2,642 individuals, 605 events) yielded a HR of 1.11 (95%CI: 0.92-1.33) for 1 SD increase in MCP-1 levels (heterogeneity: I^2=32%, p=0.23; **Figure 4B** and **Supplemental Figure 7B**), whereas the single study examining incident ischemic stroke (95 cases, 190 controls) reported a HR of 0.99 (95%CI: 0.68-1.45).

Genetically determined circulating levels of MCP-1 and etiologically related vascular outcomes

Figure 5 depicts the MR effect of genetically determined MCP-1 levels on the risk of CAD, MI and AF. Genetic predisposition to higher MCP-1 levels was associated with CAD (OR per 1-SD increase: 1.04, 95%CI: 1.00-1.08, p=0.04; 184,305 individuals, 60,801 cases) and MI (OR: 1.05, 95%CI: 1.01-1.09, p=0.02; 167,180 individuals, 43,676 cases). Given the association of MCP-1 with cardioembolic stroke, we further explored the relationship between genetically determined MCP-1 levels and risk of AF in MR analysis, but found no association (OR: 0.96, 95%CI: 0.91-1.01, p=0.09).

DISCUSSION

Exploring 41 cytokines in a two-sample MR approach involving the largest GWAS datasets available, we found that genetic predisposition to higher levels of MCP-1/CCL2 is associated with increased risk of any stroke, any ischemic stroke, large artery stroke, and cardioembolic stroke. The results were stable in alternative MR methods and sensitivity analyses and remained significant after adjustment for cardiovascular risk factors. Moreover, effect sizes for any stroke and any ischemic stroke were similar in the UK Biobank. We further found associations between genetic predisposition to higher MCP-1 levels and increased risk of CAD and MI as etiologically related outcomes. Collectively, our findings suggest that lifelong elevated circulating MCP-1 levels increase risk of stroke.

The directionality of the MR effect of increased levels of MCP-1 on risk of large artery stroke is consistent with experimental data showing a key role for this chemokine in atherogenesis and atheroprogression. Acting mainly through its receptor CCR2, MCP-1 is the prototypical CC family chemokine that is upregulated by chronic inflammatory conditions and attracts monocytes to the subendothelial space of the atherogenic arterial wall.[37] Mice lacking MCP-

1[38] or CCR2[39] are less susceptible to atherosclerosis and anti-MCP-1 gene therapy,[40] MCP-1 competitors,[41] and CCR2 antagonists[42] reduce plaque size and inhibit plaque progression and destabilization in experimental atherosclerosis. Conversely, overexpression of MCP-1 leads to inflammation, accumulation of lipids, and smooth muscle cell proliferation in atherosclerotic plaques.[43]

We further found an MR association between genetic predisposition to higher MCP-1 levels and risk of cardioembolic stroke, although the mechanisms underlying this association remain elusive. MCP-1 has been reported to promote myocardial fibrosis,[44] an established risk factor for AF.[45] However, we found no association between the genetic instruments for MCP-1 and AF risk. Other investigators have found an association between circulating MCP-1 levels and the presence of atrial thrombi in patients with AF.[46] Hence, it might be that MCP-1 increases the risk of cardioembolic stroke by promoting thrombus formation in patients with established AF. Alternative explanations for the association between circulating MCP-1 levels and cardioembolic stroke might include less frequent causes of cardioembolism such as valvular disease and misclassification of patients with multiple competing stroke etiologies including atherosclerosis.

In contrast, our analysis provides no evidence for an association of genetically determined MCP-1 levels with small vessel stroke even though the sample size was larger than for other stroke subtypes. The lack of a signal with deep intracerebral hemorrhage, which like small vessel stroke is attributed to small vessel disease,[19] is in line with this result. In fact, we found none of the cytokines to be associated with small vessel stroke (all $p>0.05$, **Figure 2**). Overall, these observations agree with the notion that inflammatory processes are less important in small vessel disease[47] than in large artery atherosclerosis although this has so far not been systematically examined.

Our meta-analysis of case-control studies revealed higher circulating MCP-1 levels in patients with ischemic stroke compared to healthy controls. However, our systematic search identified only one prospective cohort study on incident events.[48] Also, ischemic stroke subtypes were not considered in any of these studies, precluding meaningful comparisons with our MR results. Interestingly, observational cohort studies on CAD found higher MCP-1 levels to be associated with increased risk of incident[49] and recurrent[50] events consistent with the observed association with atherosclerotic stroke. Serial measurements of MCP-1 in large population-based cohorts with data on ischemic stroke subtypes would offer further insights into the relationship between MCP-1 and risk of stroke.

Targeting specific inflammatory cytokines might reduce vascular risk. The recent multicenter CANTOS trial showed that canakinumab, a monoclonal antibody against IL-1β, decreases the rate of recurrent cardiovascular events, including nonfatal myocardial infarction, nonfatal stroke and cardiovascular mortality, among patients with MI and elevated circulating CRP levels.[9] Unfortunately, the original cytokine GWAS did not identify any genetic instruments for IL-1β circulating levels[14] thus precluding a comparison of the MR results with the results of the CANTOS trial.[9] The MCP-1/CCR2 pathway was targeted in a small phase II clinical trial in patients with risk factors for atherosclerosis and elevated circulating CRP levels. MLN1202, a humanized monoclonal antibody against CCR2 reduced CRP levels after 4 and 12 weeks.[51] However, effects on clinical endpoints were not assessed[51] and would need to be determined in a larger trial.

This study has several methodological strengths. We used the most recent and comprehensive dataset for cytokine levels and the largest available GWAS dataset for stroke and stroke subtypes. Results were confirmed through sensitivity analyses for pleiotropy including alternative MR methods, in sub-analyses on a biologically plausible protein-protein interaction network, and in analyses on etiologically related outcomes (CAD and MI).

Our study also has limitations. First, none of the SNPs used as instruments for MCP-1 were located in the vicinity of the *MCP1* gene thus precluding analyses restricted to SNPs within this locus. Consequently, while we found no statistical evidence for pleiotropy, we cannot preclude unspecific effects of the MCP-1 instruments. Second, our instrument selection was based on a single discovery GWAS that adjusted for BMI. While this might have introduced bias into the MR effect estimates, the consistency of the association for MCP-1 when using an unweighted allele score argues against this possibility. Third, we could not obtain reliable genetic instruments for 18 cytokines and several analyses for ischemic stroke subtypes were underpowered. Thus, we might have missed associations for several cytokines that have previously been implicated in vascular disease such as IL-1β, TNF-α and IL-6. Targeted studies incorporating further GWAS data on individual cytokines might reveal additional associations not captured by our approach. Fourth, genetic instruments were selected using an FDR-based approach, which might have weakened the instruments. However, the *F*-statistics were high and the results were in line with those derived when selecting instruments based on the genome-wide threshold ($p < 5 \times 10^{-8}$). Finally, the UK Biobank analysis was rather underpowered and did not include stroke subtypes. Yet, the consistency of both the direction and magnitude of the effects for any stroke and any ischemic stroke supports our results.

In conclusion, this study demonstrates that lifelong elevated circulating MCP-1 levels are associated with increased risk of stroke and particularly with the large artery and the cardioembolic subtypes. Interventions aimed at targeting MCP-1 or its downstream effectors seem a promising strategy for lowering stroke risk.

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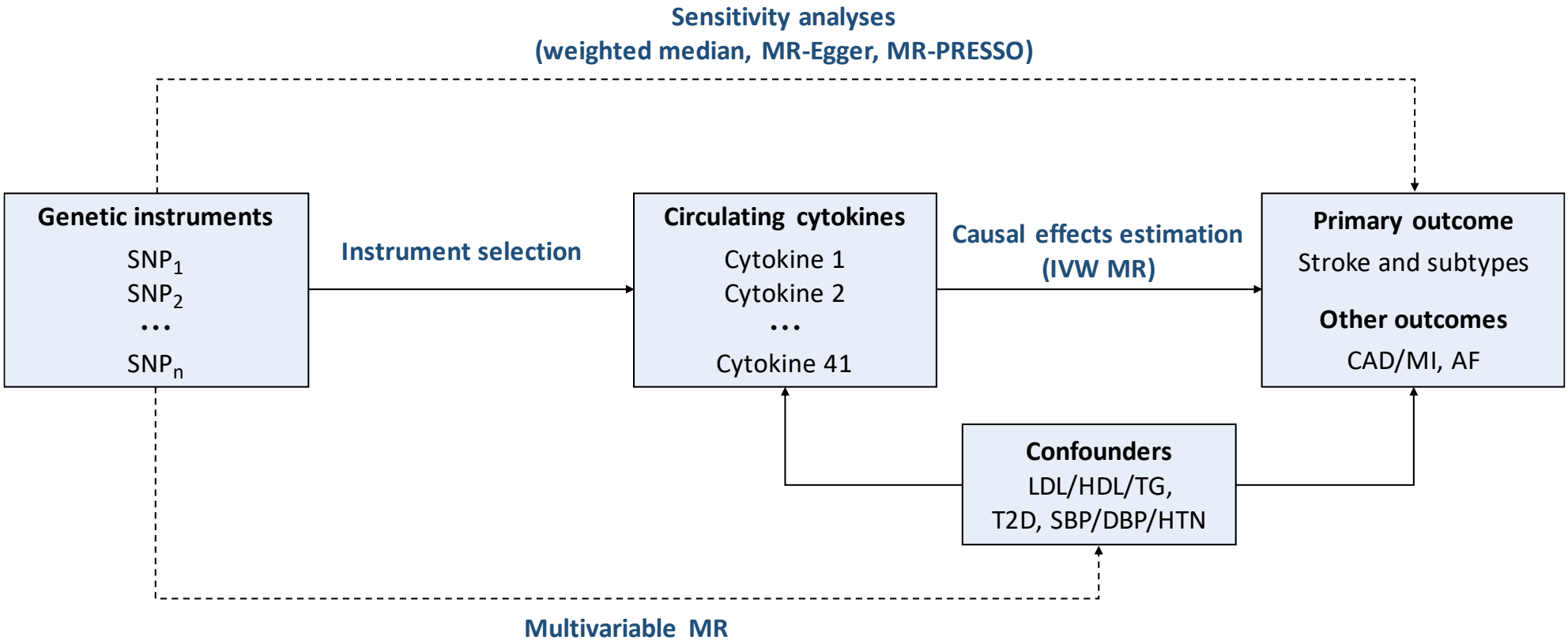

Figure 1. Schematic representation of the study design. Methods used to test for causal effects and for violations of the Mendelian randomization assumptions (dashed lines).

AF, atrial fibrillation; CAD, coronary artery disease; DBP, diastolic blood pressure; HDL, high-density lipoprotein cholesterol; HTN, hypertension; IVW, inverse-variance weighted; LDL, low-density lipoprotein cholesterol; MI, myocardial infarction; MR: Mendelian randomization; MR-

PRESSO: Mendelian Randomization Pleiotropy RESidual Sum and Outlier; SBP, systolic blood pressure; SNP, Single-nucleotide polymorphism; T2D. type 2 diabetes mellitus; TG, triglycerides.

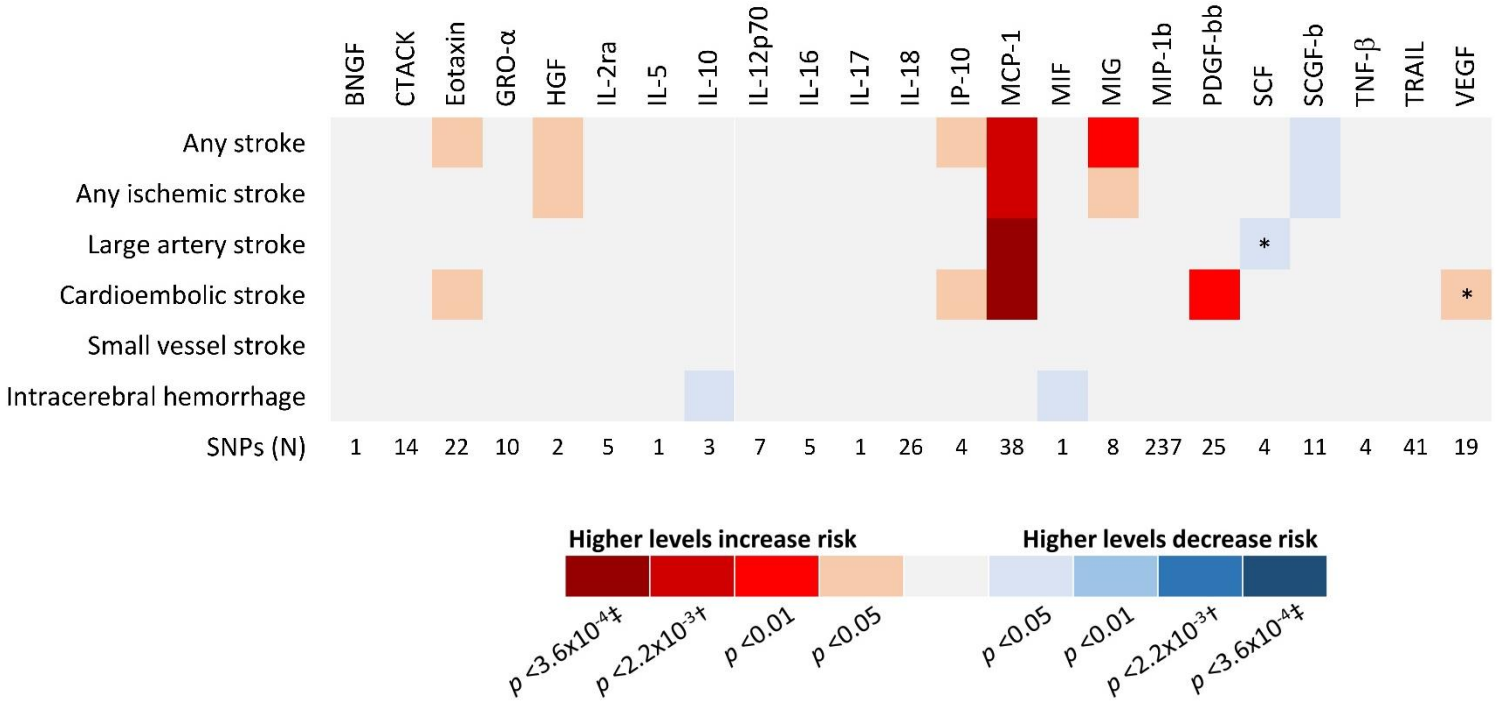

Figure 2. Mendelian randomization associations of circulating cytokine and growth factor levels with stroke and stroke subtypes. Shown are the results derived from the fixed-effects inverse-variance weighted (IVW) meta-analysis.

* Significant heterogeneity (I2>25% or Cochran Q-derived p <0.05)

† Bonferroni-corrected threshold for number of tested cytokines

‡ Bonferroni-corrected threshold for number of cytokines and number of phenotypes

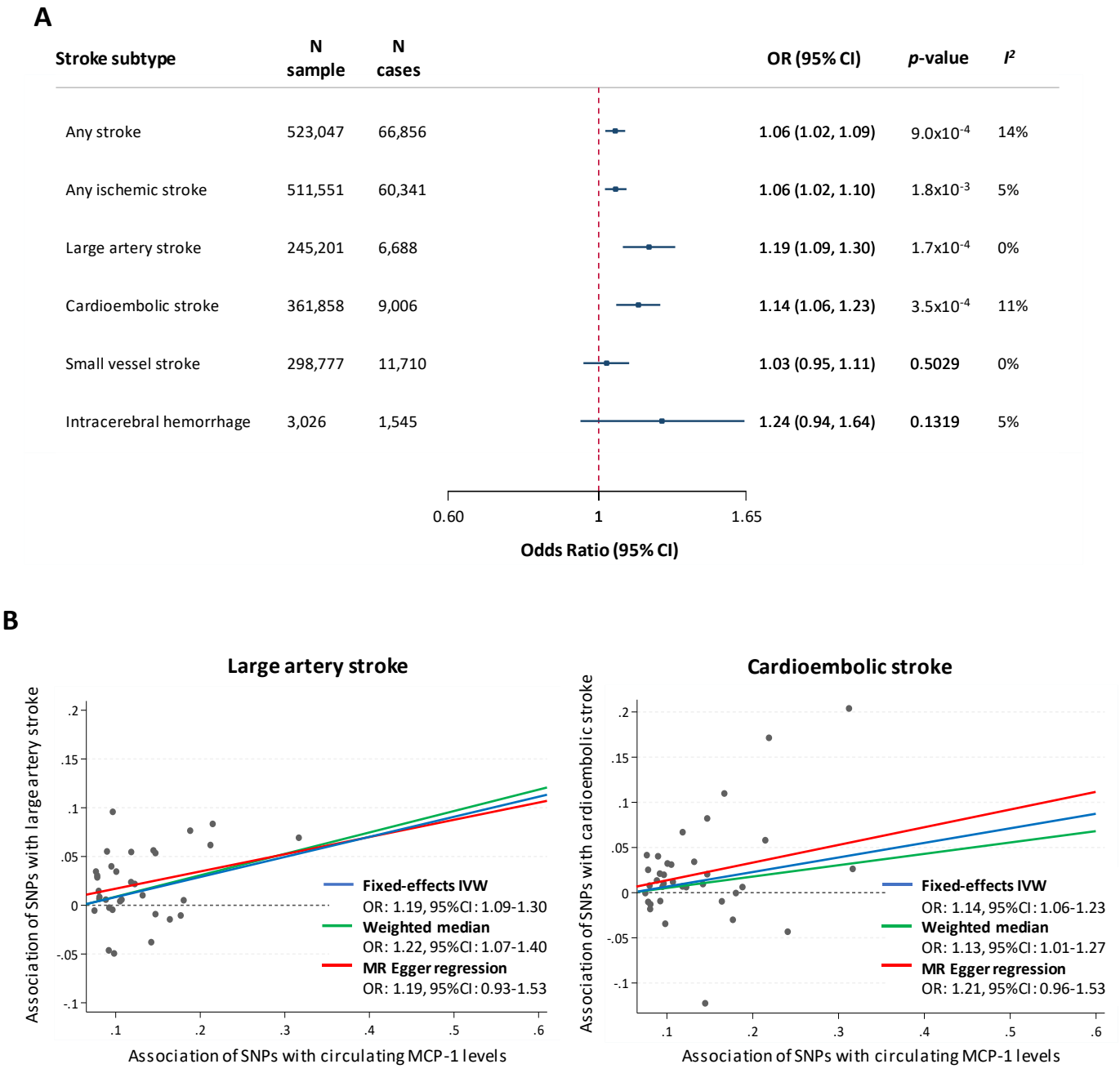

Figure 3. Mendelian randomization analysis for circulating MCP-1 levels and risk of stroke.

(A) MR-derived effects of circulating MCP-1 levels (1-SD increase) on risk of any stroke and stroke subtypes. (B) Effects of circulating MCP-1 levels on risk of large artery (left) and cardioembolic (right) stroke based on different MR methods. I^2 refers to heterogeneity in the Mendelian randomization analysis (inverse-variance weighted method). CI, confidence intervals; IVW, inverse-variance weighted; OR, Odds Ratio; SNP, single nucleotide polymorphism.

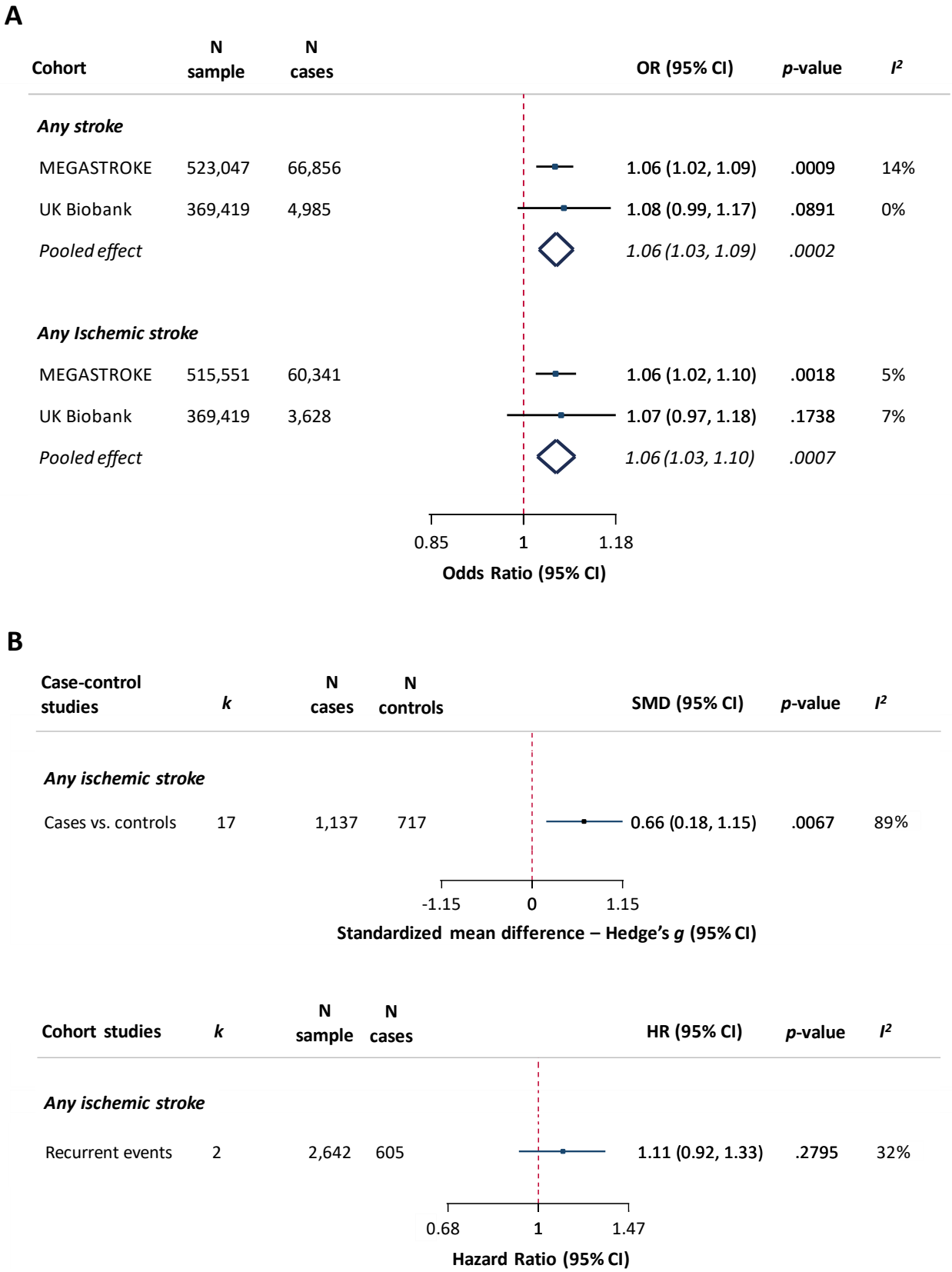

Figure 4. Effects of circulating MCP-1 levels on risk of stroke in Mendelian randomization and in observational studies. (A) MR-derived effects of circulating MCP-1 levels (1-SD increase) on risk of

any stroke and any ischemic stroke in MEGASTROKE, in UK Biobank, and a meta-analysis of both samples. (B) Meta-analysis-derived effects of circulating MCP-1 levels (1-SD increase) on risk of ischemic stroke in case-control and cohort studies. *k* refers to number of included studies. I^2 *i*n Figure 4A refers to heterogeneity in the Mendelian randomization analysis (inverse-variance weighted method) and in Figure 4B in the random-effects meta-analyses of observational studies.

CI, confidence interval; HR, hazard ratio; OR, odds ratio; SMD, standardized mean difference; SNP, single nucleotide polymorphism.

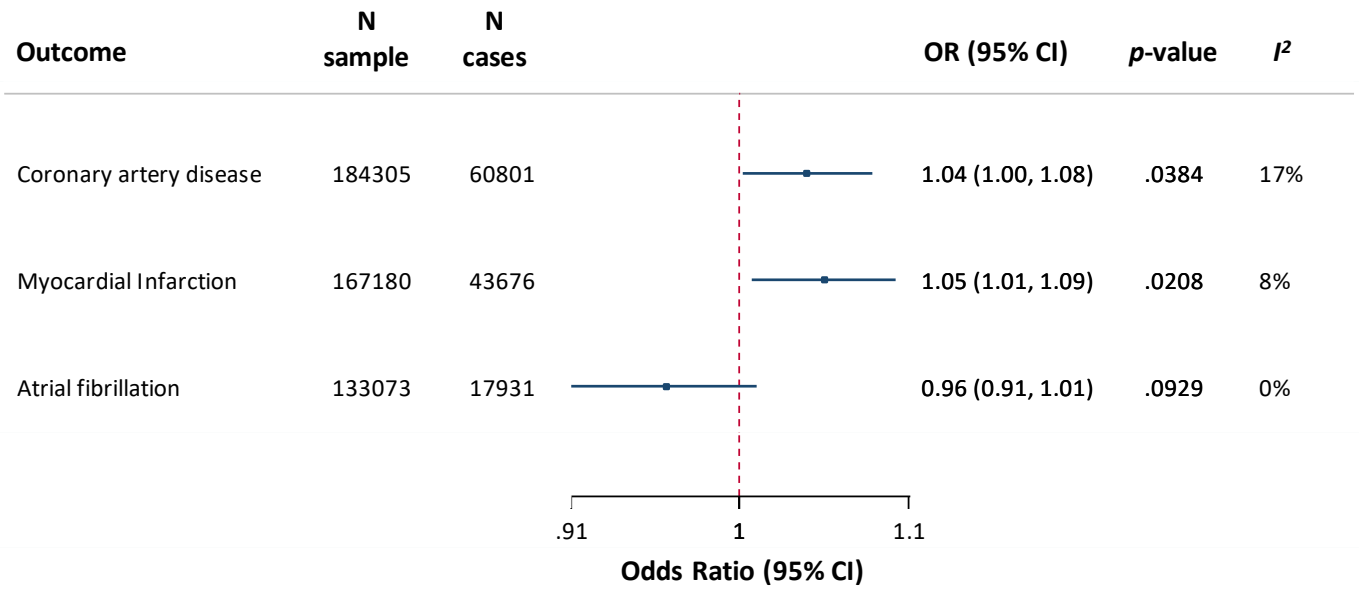

Figure 5. Mendelian randomization analysis for circulating MCP-1 levels and etiologically related vascular outcomes. MR-derived effects of genetically determined circulating MCP-1 levels (1-SD increase) on risk of coronary artery disease, myocardial infarction, and atrial fibrillation. I^2 refers to heterogeneity in the Mendelian randomization analysis (inverse-variance weighted method).

Table 1. Multivariable Mendelian randomization effects of circulating MCP-1 levels on the risk for stroke and its subtypes adjusting for cardiovascular risk factors.

	Any stroke	**Any ischemic stroke**	**Large artery stroke**	**Cardioembolic stroke**	**Small vessel stroke**	**Intracerebral hemorrhage**
N sample	523,047	511,551	245,201	361,858	298,777	3,026
N cases	66,856	60,341	6,688	9,006	11,710	1,545
Unadjusted model	1.06 (1.02-1.09)	1.06 (1.02-1.10)	1.19 (1.09-1.30)	1.14 (1.06-1.23)	1.03 (0.95-1.11)	1.24 (0.94-1.64)
Adjusted for T2D	1.07 (1.03-1.11)	1.07 (1.03-1.11)	1.22 (1.12-1.33)	1.17 (1.08-1.27)	1.03 (0.97-1.10)	1.06 (0.94-1.20)
Adjusted for LDL	1.06 (1.02-1.10)	1.06 (1.02-1.11)	1.20 (1.10-1.31)	1.16 (1.06-1.24)	1.03 (0.98-1.09)	1.26 (0.93-1.71)
Adjusted for HDL	1.07 (1.03-1.11)	1.07 (1.02-1.11)	1.21 (1.11-1.33)	1.15 (1.06-1.25)	1.04 (0.97-1.10)	1.27 (0.94-1.72)
Adjusted for TG	1.06 (1.02-1.10)	1.06 (1.02-1.10)	1.19 (1.09-1.30)	1.16 (1.06-1.26)	1.03 (0.97-1.10)	1.28 (0.94-1.73)
Adjusted for SBP	1.08 (1.04-1.12)	1.09 (1.05-1.14)	1.23 (1.12-1.35)	1.20 (1.10-1.32)	1.03 (0.96-1.11)	1.81 (1.13-1.90)
Adjusted for DBP	1.08 (1.04-1.13)	1.09 (1.05-1.14)	1.22 (1.11-1.34)	1.20 (1.10-1.32)	1.04 (0.96-1.11)	1.53 (0.89-2.65)
Adjusted for HTN	1.07 (1.03-1.11)	1.07 (1.03-1.11)	1.19 (1.09-1.29)	1.18 (1.08-1.29)	1.03 (0.95-1.11)	1.03 (0.93-1.14)
Fully-adjusted model (T2D, LDL*, SBP †)	1.08 (1.03-1.12)	1.09 (1.04-1.13)	1.23 (1.11-1.35)	1.20 (1.10-1.32)	1.04 (0.97-1.12)	1.06 (0.92-1.21)

The results are presented as Odds Ratios (95% Confidence Intervals) for the effect of 1 standard deviation increase in MCP-1 levels.

* restricted to LDL to avoid collinearity with HDL and TG levels. † restricted to SBP to avoid collinearity with DBP and HTN.

DBP, diastolic blood pressure; HDL, high-density lipoprotein cholesterol; HTN, hypertension; LDL, low-density lipoprotein cholesterol; SBP: systolic blood pressure; T2D, type 2 diabetes mellitus; TG, triglycerides.