Supplementary Methods

Study design and data sources

We conducted a two-sample Mendelian randomization (MR) analysis to investigate potential causal associations between genetically predicted plasma levels of 14 inflammation-related proteins and post-stroke outcome. These proteins were selected as they were significantly (false discovery rate [FDR] <0.05) or suggestively (P<0.05) associated with 3-month functional outcome after ischemic stroke in multivariable models adjusting for age, sex, day of blood draw, and stroke severity (i.e., National Institutes of Health Stroke Scale [NIHSS] score) in our previous observational study.[1]

Protein quantitative trait loci (pQTL) were derived from the UK Biobank Pharma Proteomics Project (UKB-PPP) as exposure data. The UKB-PPP characterized plasma proteomic profiles of randomly selected UK Biobank participants at a baseline visit, using the Olink Explore 3072 Proximity Extension Assay.[2] We collected summary statistics from the discovery cohort comprising 34,557 individuals of European ancestry. Single nucleotide polymorphisms (SNPs) in UKB-PPP were chosen as genetic instruments for plasma levels of the 14 proteins if they were located within ±200 kb of the encoding gene (i.e., *cis*-pQTLs) as they are more likely to have protein-specific effects than trans-pQTL.[3] Chosen SNPs also had to have a significance level of P< 0.001 in the UKB-PPP as well as a minor allele frequency >0.01 in the 1000 Genomes phase 3 project, European population. A summary of the genomic regions of the 14 eligible proteins and the resulting genetic instruments is provided in Supplementary Tables 1 and 2.

Summary statistics for post-stroke functional outcome were obtained from the Genetics of Ischemic Stroke Functional Outcome (GISCOME) network Genome-Wide Association Study (GWAS) meta-analysis, which included 6,165 ischemic stroke cases from 12 studies in Europe, Australia, and United States (all of European ancestry).[4] Functional outcome was assessed using the modified Rankin Scale (mRS) approximately 90 days after the index ischemic stroke (60–190 days permitted). For this study, we used summary statistics from the primary analysis in the GISCOME GWAS, i.e., mRS dichotomized into favorable (mRS score 0–2, n=3,741) and poor (mRS score 3–6, n=2,280) outcome with a total of 6,021 cases.

Effect sizes for exposure data were harmonized with the outcome data and SNPs not available in the GISCOME dataset were excluded. Independent genetic instruments were selected using a clumping threshold r^2<0.01 using LDlink[5] based on 1000 Genomes Project population data[6] and were further filtered using Annovar annotation to exclude missense and splice site mutations, reducing the likelihood of false associations due to assay artifacts.[7] The strength of each genetic instrument was estimated by calculating the F-statistic.[8] Detailed information is available in Supplementary Table 3.

Collider bias adjustment

Genetic association analyses of post-stroke functional outcome inherently have a case-only design, as outcome phenotypes can only be observed in individuals who have experienced a stroke. This design introduces potential selection bias, specifically collider bias, in subsequent analyses.[9] To address this bias, we employed the Slope-Hunter method, rather than the Dudbridge method, because the latter assumes no correlation between genetic effects on incidence and progression/outcome. Slope-Hunter uses a cluster-based approach to categorize independent genetic variants into two groups: those affecting disease incidence only and those affecting both disease incidence and, in this case, functional outcome. The method estimates a correction factor through regression analysis of adverse outcomes on stroke risk associations for a subset of variants (termed "hunted variants"), which is then applied to the complete variant set under assumptions of linearity and no interactions between variant-stroke risk and variant-outcome associations.[9]

For the bias adjustment, we utilized the Slope-Hunter R package[9] with genome-wide summary statistics for the index event of stroke from the GIGASTROKE consortium.[10] To minimize population stratification effects, we used European ancestry GIGASTROKE GWAS results, encompassing 59,911 ischemic stroke cases and 665,176 controls. Effect sizes from this data were harmonized with GISCOME data in two analyses: one adjusted for stroke severity (NIHSS) and one unadjusted for NIHSS.

The Slope-Hunter analysis identified different sets of SNPs associated with stroke incidence in each analysis, for NIHSS adjusted GISCOME data 910 SNPs were identified, yielding a correction factor of 2.83 (95% confidence interval [CI]: 1.68 to 3.99), while for NIHSS unadjusted GISCOME data 8 SNPs were identified, yielding a correction factor of 1.21 (95% CI: 0.10 to 2.32). These correction factors were subsequently applied to all variants in their respective analyses.

Genetic instruments

A total of 7,461 SNPs were initially selected for the 14 proteins in the UKB-PPP, with the number of SNPs per protein ranging from 4 to 1,729 as detailed in Supplementary Table 2. After harmonization with the GISCOME dataset and linkage disequilibrium (LD) clumping, 181 SNPs remained as genetic instruments for the 14 proteins (Supplementary Table 2). When correcting for collider bias with Slope-Hunter, 50 SNPs were absent from

GIGASTROKE. This left 131 SNPs representing 13 out of the 14 proteins, and notably, IL-6 was excluded at this stage as it did not have any available SNPs (Figure 1 and Supplementary Table 2). All remaining SNPs were robust, with F-statistics exceeding 10 (Supplementary Table 3). However, three proteins (STAMBP, 4E-BP1, and OSM) were excluded because they had fewer than 3 SNPs and an r^2<0.005, limiting their causal inference reliability. After these exclusions, 10 proteins were eligible for downstream MR analysis.

MR analyses

Slope-Hunter adjusted values were applied to the GISCOME data. The random effects inverse-variance weighted (IVW) method[11] was used as the primary MR method. In line with the primary analysis in the GISCOME GWAS, in our primary MR analyses, we adjusted for age, sex, ancestry, and stroke severity (NIHSS score). Also, in line with the GISCOME GWAS, we performed secondary analyses without adjustment for stroke severity for comparison.[4]

To address multiple testing, the FDR correction was applied. Many of these proteins are in the same pathway and are highly correlated.[12] It is noted that adjustment for multiple comparisons by number of investigated proteins (n=10 for the main Slope-Hunter adjusted MR analyses) does not equate to 10 independent comparisons and is arguably too stringent. Therefore, we also consider associations with *P*-value <0.05 as suggestive.

Sensitivity analyses

A series of analyses were performed to check for potential disruptions of MR assumptions and to verify the robustness of the findings. The MR-Egger regression intercept was used to test for directional pleiotropy[13] and additional robustness checks included the weighted median[14] and the MR-Robust Adjusted Profile Score (MR-RAPS)[15] method to account for weak or invalid genetic instruments. To assess the findings and the potential impact of collider bias, a sensitivity analysis was conducted on suggestive or significant proteins by repeating the MR analysis without adjusting for collider bias using Slope-Hunter (detailed in Supplementary Table 8 for the primary analysis and Supplementary Table 13 for the secondary analysis). MR-Pleiotropy RESidual Sum and Outlier (MR-PRESSO)[16] was employed to detect and correct for outliers that could bias results. These approaches offer robust protection against assumption violations. All analyses were performed in R (version 4.4.1; R Foundation for Statistical Computing, Vienna, Austria) with the TwoSampleMR (version 0.6.8), MendelianRandomisation (version 0.10.0), MR-PRESSO (version 1.0), and MR-RAPS (version 0.2) packages.

Data availability

Publicly available data were used for all analyses. Data not provided in the article because of space limitations may be shared at the request of any qualified investigator for purposes of replicating procedures and results.

Standard protocol approvals, registrations, and patient consents

This study was conducted in adherence to the guidelines for Strengthening the Reporting of Observational Studies in Epidemiology-MR. All data sources were derived from publicly available, European ancestry summary statistics, with ethical approval and participant consent obtained in each respective study.

Supplementary Results

Assessment of MR assumptions and sensitivity analyses

The three key MR assumptions were evaluated as follows: the relevance assumption was supported by strong F-statistics for all genetic instruments (range 11–554, median 15); the independence assumption was strengthened by the use of *cis*-pQTLs and strict LD clumping (r^2<0.01); and for the exclusion restriction assumption, the MR-Egger intercept tests showed no evidence of directional pleiotropy (i.e., was non-significant). To further check for potential disruptions of MR assumptions, we conducted a variety of sensitivity analyses. For the significant and/or suggestive associations (i.e., CASP-8, HGF, S100A12, and MCP-3), directionally concordant results were observed in the weighted median analysis, MR-Egger, and MR-RAPS analyses. The MR-PRESSO estimates aligned with the IVW results for all proteins and the global test did not identify any significant outliers. Taken together, these tests support the validity of our genetic instruments (summarized in Supplementary Tables 5-7 for the primary analysis and Supplementary Tables 10-12 for the secondary analysis).

Methodological differences between the present MR-design and the design by Xu et al.

It is noteworthy that our MR finding of a protective effect for HGF differs from that reported in a recent MR study that suggests a detrimental effect.[17] Several key methodological differences between the two studies may account for this discrepancy, as summarized in Supplementary Table 14. Firstly, we adjusted for collider bias using Slope-Hunter. Secondly, we selected genetic instruments from a recent large GWAS of plasma proteins, the UKB-PPP, while Xu et al.[17] selected their SNPs from a meta-analysis of GWASs on plasma proteins measured by the Olink

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CVD-I panel performed within the SCALLOP consortium.[18] Thirdly, the thresholds for selecting SNPs ($P<0.001$ in the present study vs. $P<5\times10^{-8}$) and for LD clumping ($r^2<0.01$ vs. $r^2<0.3$) differed. It is also of importance to note that we only used *cis*-variants, while Xu et al.[17] used both *cis*- and *trans*-variants. More specifically, the genetic instrument used by Xu et al.[17] comprised 3 *cis*-SNPs and 10 *trans*-SNPs (all of which were located on another chromosome), and these 13 SNPs had no overlap with our 10 SNPs. Finally, and most importantly, we utilized GWAS data from the primary GISCOME analysis as the outcome, i.e., mRS score 0–2 vs. 3–6 (n=6,021) with adjustment for stroke severity, while Xu et al.[17] used data from a secondary analysis of outcome on a subgroup of cases that could be dichotomized as mRS score 0–1 vs. 2–6 (n=4,363).

Supplementary References

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