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# Sodium-glucose cotransporter protein 2 inhibition, plasma proteins, and ischemic stroke: A mediation Mendelian randomization and colocalization study

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ABSTRACT

*Purpose:* To determine the effect of the sodium-glucose cotransporter protein 2 (SGLT2) inhibition on ischemic stroke (IS) and investigate the circulating proteins that mediate the effects of SGLT2 inhibition on IS.
*Methods:* The effects of SGLT2 inhibition on IS were evaluated using two-sample Mendelian randomization (MR) analyses. The 4,907 circulating proteins from the plasma proteome were assessed to identify potential mediators. Sensitivity, colocalization, and external validation analyses were conducted to validate critical findings. MR analyses were also used to evaluate the associations of SGLT2 inhibition with magnetic resonance imaging (MRI)-based biomarkers and functional prognoses post-IS.
*Results:* SGLT2 inhibition was significantly associated with decreased risks of IS (odds ratio (OR): 0.39, 95 % confidence interval (CI): 0.25–0.61, $p = 3.53 \times 10^{-5}$) and cardioembolic stroke (OR: 0.16, 95 % CI: 0.07–0.37, $p = 1.82 \times 10^{-5}$); the effect of SGLT2 inhibition on IS was indirectly mediated through pathways involving tryptophanyl-transfer RNA synthetase (WARS) (β:0.08, 95 % CI:0.15 – -0.01, $p = 0.034$) and matrix metalloproteinase 12 (MMP12) (β:0.06, 95 % CI:0.12 – -0.01, $p = 0.016$), with mediation proportions of 8.2 % and 6.8 %, respectively. The external validation confirmed the WARS mediating effect. In addition, the sensitivity and colocalization analyses and MR analyses of MRI biomarker-based and functional prognostic outcomes supported these results.
*Conclusion:* In this study, we demonstrated from a genetic perspective that SGLT2 inhibitors prevent the development of IS and improve functional prognostic outcomes and brain microstructural integrity. WARS and MMP12 may act as potential mediators, presenting a novel approach for IS intervention.

## Introduction

Sodium-glucose cotransporter protein 2 inhibitors (SGLT2is), commonly called gliflozins, represent a novel class of oral hypoglycemic medications for managing type 2 diabetes mellitus (T2DM).[1] These agents maintain glucose homeostasis by targeting SGLT2 proteins, thereby inhibiting glucose reabsorption in the renal proximal tubules and enhancing glucose excretion in urine.[2] Ischemic stroke (IS), which is characterized by neurological deficits induced by disrupted cerebral circulation and subsequent tissue death in blood-deprived regions, poses a significant public health concern globally[3,4] T2DM represents a well-established risk factor for IS, and glucose-lowering medications have been demonstrated to be an effective intervention for IS.[5,6]

Notably, multiple large-scale randomized controlled trials have supported the efficacy of SGLT2is in reducing the risk of cardiovascular diseases, including nonfatal stroke[7] A meta-analysis also demonstrated the potential of SGLT2is in preventing stroke.[8] Takashima et al. demonstrated that pretreatment with low-dose luseogliflozin in a non-diabetic murine model enhanced ischemic tolerance in pericytes and attenuated ischemic brain injury without affecting blood glucose levels. This effect is associated with the activation of adenosine 5′ monophosphate-activated protein kinase and mitochondrial

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biogenesis.[9] Mechanistically, SGLT2is exert protective effects on cerebrovascular health by modulating various risk factors. They enhance vascular endothelial function by reducing blood glucose levels, regulating blood pressure, and attenuating oxidative stress.[10,11] Concurrently, they contribute to the mitigation of atherosclerosis by modulating inflammatory processes and improving insulin resistance.[12,13] Notably, numerous clinical studies have highlighted the significance of SGLT2 inhibition in mitigating IS risk; however, some discrepancies persist in the literature.[14,15] Therefore, further investigation of the role of SGLT2is in IS prevention is required to elucidate the specific molecular mechanisms involved, particularly in non-diabetic cohorts.

Due to the lower expression of SGLT2 in the central nervous system (CNS), it is postulated that SGLT2is may rely on specific mediators to improve IS, although they also directly impact IS-related risk factors.[16] Plasma proteins are integral to cellular and biological processes and serve as end-products of gene expression, making them crucial targets for investigating IS mechanisms.[17] These proteins are pivotal in regulating biological processes and act as major regulators of molecular pathways.[18] Notably, several proteomic studies have shown a relationship between plasma proteins and IS.[19–21] A recent study identified 48 plasma proteins that were significantly and independently associated with IS during the acute phase.[22] Proteomic analyses have indicated that SGLT2is can modify plasma protein profiles.[23] Therefore, employing plasma proteins as intermediate phenotypes may offer insights into the potential mechanisms through which SGLT2is mitigate IS.

Mendelian randomization (MR) is an emerging epidemiological technique that leverages genetic variation as an instrumental variable (IV) to facilitate causal inferences.[24] Grounded in Mendel's law of independent assortment, MR is less susceptible to the effects of confounding variables and reverse causation, thereby overcoming the limitations of observational studies.[25,26] Therefore, in this study, we aimed to utilize expression quantitative trait loci (eQTL) data of SGLT2-inhibited target genes as exposure variables to assess the impact of SGLT2 inhibition on IS through a two-sample MR (TSMR) design and identify the potential mediators of this relationship using integrated proteomics data and a two-step MR approach. We also conducted sensitivity, Bayesian colocalization, and external validation analyses to enhance the robustness of the association results. Finally, we assessed the impact of SGLT2 inhibition on IS-related magnetic resonance imaging (MRI)-based biomarkers and functional prognostic outcomes.

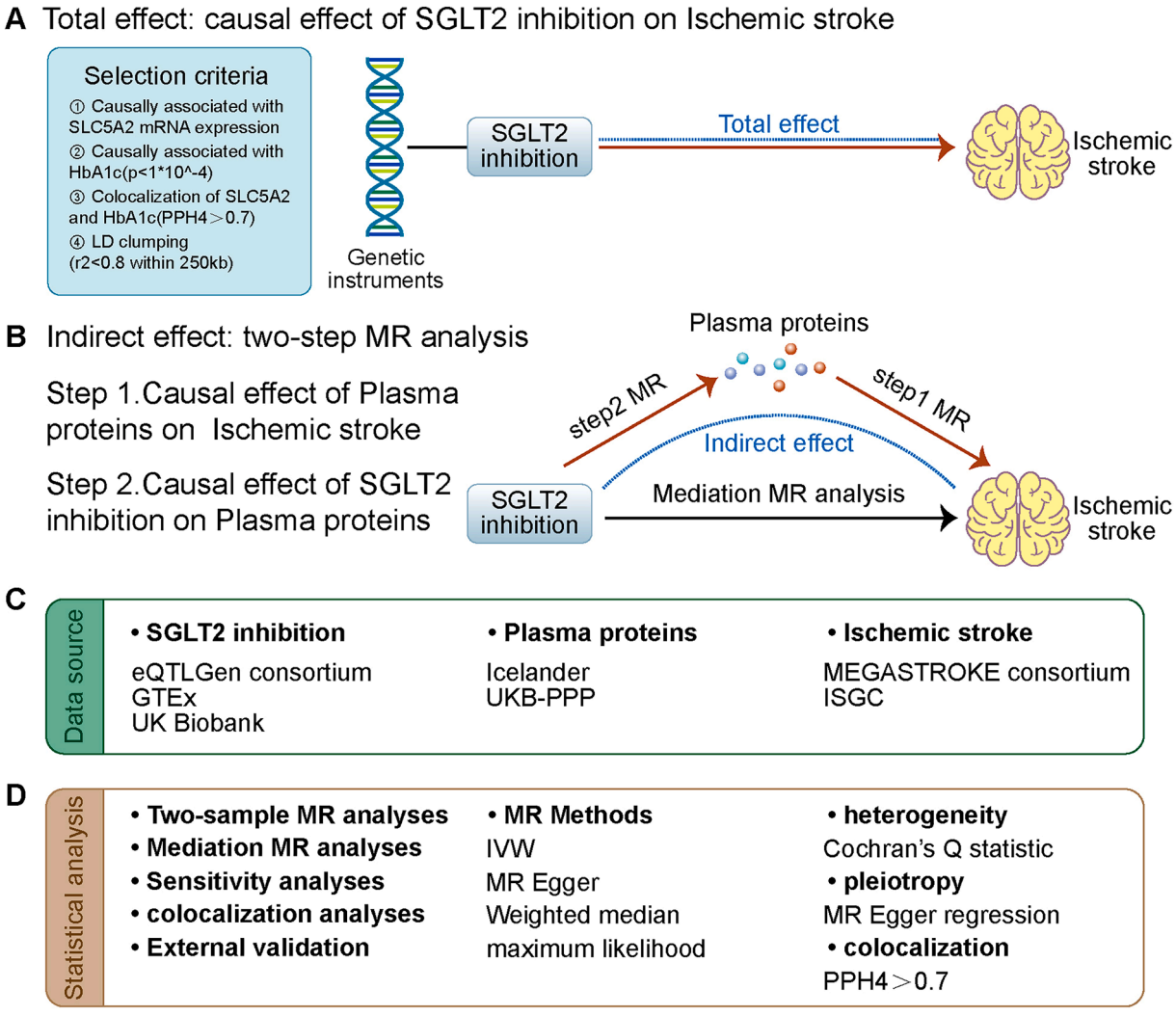


**Fig. 1.** Overview of study design. (A) Genetic variables associated with SGLT2 inhibition were identified, and the impact of SGLT2 inhibition on IS was investigated using two-sample MR analysis. (B) A mediated MR analysis was conducted using a two-step MR framework to investigate plasma proteins that may potentially mediate the pathway by which SGLT2 inhibition associates with IS, and to calculate indirect effects. (C) The data sources utilized for the exposure variable (SGLT2 inhibition), mediator variable (plasma proteins), and outcome variable (IS), including the discovery and validation sets for plasma proteins and IS. (D) The analytical procedures employed in the study included two-sample MR analysis (four methods), mediated MR analysis, sensitivity analysis (Cochran's Q test and MR Egger regression analysis), colocalization analysis, and external validation.

## Materials and methods

### Study design

Fig. 1 provides a summary of the study's design framework. Initial MR and subsequent two-step MR analyses were conducted to explore the potential relationship between SGLT2 inhibition and IS and determine whether plasma-circulating proteins could serve as potential therapeutic targets that mediate these effects. The MR analyses were predicated on the following three core assumptions[27]: (1) genetic variation is strongly associated with exposure; (2) genetic variation is independent of any confounding factors; (3) genetic variation can influence outcomes only through exposure. The research adhered to the Strengthening the Reporting of Observational Studies in Epidemiology using Mendelian Randomization (STROBE-MR) guidelines,[28] as detailed in the Additional File. Supplementary Table S1 provides a thorough summary of the fundamental attributes of the genome-wide association study (GWAS) data utilized in this investigation. In this study, we utilized previous GWAS summary data for analysis and did not involve the recruitment of new human participants or the use of animals. Ethical approval and consent to participate were obtained for all original studies from which the data were derived. Therefore, no additional ethical approval was required for our study.

### Genetic instruments for SGLT2 inhibition

Furthermore, to identify genetic variants representative of SGLT2 inhibition, this investigation was based on four rigorous steps outlined previously.[29] Genetic variants associated with messenger RNA (mRNA) expression levels of solute carrier family 5 member 2 (SLC5A2) (gene encoding for SGLT2) were identified using eQTLGen consortium[30] and Genotype Tissue Expression[31] data. Subsequent MR analyses were conducted to calculate associations between each genetic variant and glycated hemoglobin (HbA1c) levels; only those with significant associations ($p < 1 \times 10^{-4}$) were selected for analysis. The HbA1c data based on 344,182 unrelated participants without diabetes were obtained from the UK Biobank. Subsequently, genetic colocalization analyses were performed to ascertain the existence of a shared causal variant linking SLC5A2 and HbA1c expression. Posterior probability $> 0.7$ was considered evidence of colocalization. Finally, the standard clumping process was employed for linkage disequilibrium (LD) pruning, with $r^2 = 0.8$ and kb = 250 as the thresholds. Ultimately, 14 variants strongly associated with SGLT2 inhibition were retained for subsequent MR analysis (Supplementary Table S2), each of which exhibited an F-statistic $> 10$, thereby ensuring a high level of statistical power.

### Proteomic data source

In the discovery phase (primary analysis), we obtained summary data on 4,907 plasma proteins from a large-scale protein quantitative trait loci (pQTL) study involving 35,559 Icelanders. Plasma proteins were measured using the SomaScan platform and were adjusted for covariates, including sex and trait.[32] Independent and significant IVs were extracted according to the following criteria[33,34]: (1) cis-pQTL located within ±1 Mb of the genetic distance of the gene encoding the protein; (2) a genome-wide level of significant association with plasma protein expression ($p < 5 \times 10^{-8}$); (3) no significant LD between the pQTL which based on 1000 Genomes European panel to be estimated ($r^2 < 0.001$, genetic distance $> 10$ Mb). In the replication phase, pQTL from the UK Biobank Pharma Proteomics Project (UKB-PPP) was used for validation and subjected to proteomic analysis using the Olink platform based on plasma samples from 54,219 participants; in these analyses, the summary data for 1,463 plasma proteins were collated.[35] In that phase, the IVs were selected using the same criteria as those in the discovery phase.

### Outcome data sources

During the discovery phase, IS summary data were acquired from the MEGASTROKE Consortium meta-analysis of GWAS data[36] derived from 29 cohorts; to mitigate heterogeneity resulting from diverse ethnic backgrounds, only the summary data for those of European descent were selected for the MR analysis, encompassing 34,217 cases and 406,111 controls. These cases were further stratified into distinct subtypes based on clinical and imaging criteria, including 4,373 cases of large artery stroke (LAS), 5,386 cases of small vessel stroke (SVS), and 7,193 cases of cardioembolic stroke (CES). In the replication phase, we obtained pooled IS data from the International Stroke Genetics Consortium (ISGC), which comprised 10,307 cases and 19,326 controls.[37] IS functional outcomes at 3 months post-onset were evaluated using the modified Rankin Scale (mRS), with scores ranging from 0 (asymptomatic) to 6 (death)[38]; the genetic summary data for the scores at this time point were obtained from another study.[39] Therefore, to gain a comprehensive understanding of the IS phenotype, the white matter hyperintensity (WMH) volume was analyzed, which is an MRI-based biomarker of cerebral small-vessel disease and refers to the increased luminance on T2-weighted gradient-recalled echo sequences underlying subcortical lacunar IS pathology.[40] The WMH dataset (n = 32,114) was sourced from a recent GWAS on brain imaging phenotypes conducted by the UK Biobank.[41]

### MR analysis

A TSMR approach was used to investigate the relationship between SGLT2 inhibition and IS. For exposures with a single IV, the Wald ratio method was utilized for analysis. However, in cases involving at least two IVs, four MR methods were used, including the inverse variance-weighted (IVW), weighted median, maximum likelihood, and MR-Egger methods. The IVW method, which calculates and combines Wald ratios for each single nucleotide polymorphism (SNP) through meta-analysis, is the most efficient method with the greatest statistical efficacy. However, this method is based on the assumption that all genetic variants are valid IVs, and the results obtained may be biased if the IVs show horizontal pleiotropy.[42] Therefore, the other three methods were used to complement and increase the robustness of the results. Furthermore, given the repeated calculations, the false discovery rate (FDR) method was applied after per-analysis of the plasma proteins with a single outcome to adjust the statistical significance thresholds to account for multiple comparisons in the primary analytical methods (IVW and Wald ratio methods) to mitigate the likelihood of type I errors.[26] Positive FDR (PFDR) values $< 0.05$ were considered statistically significant, and results were considered to be robust only if the directions of the associations derived from the four MR methods were consistent.

In the sensitivity analysis phase, horizontal pleiotropy and heterogeneity were assessed. Horizontal pleiotropy was evaluated using the intercept p-value from the MR-Egger regression, with $p < 0.05$ indicating the presence of IV-associated horizontal pleiotropy.[43] Heterogeneity among IVs was estimated using Cochran's Q test, with $p < 0.05$ indicating significant heterogeneity. In cases of heterogeneity, a random-effects IVW model was employed; however, a fixed-effects IVW model was used in the absence of heterogeneity.[44] The MR analyses were performed using R software (version 4.3.2) and the "TwoSampleMR" package (version 0.5.8).[45]

### Mediation analysis

Mediation analyses employing a two-step MR design were conducted to elucidate the potential mediating role of plasma proteins in the relationship between SGLT2 inhibition and IS. The initial TSMR identified plasma proteins exhibiting a significant effect on IS (PFDR $< 0.05$); the impact of SGLT2 inhibition on these plasma proteins was also subsequently assessed using TSMR. The overall impact of SGLT2 inhibition

on IS could be attributed to direct or indirect effects; the "product of coefficients" method was employed to quantify the latter.[46] The proportion of the total effect mediated by plasma proteins was determined by dividing the indirect effect (β1 × β2) by the total effect (β3), where β1, β2, and β3 denote the impact of SGLT2 inhibition on plasma proteins, the effect of plasma proteins on IS, and the effect of SGLT2 inhibition on IS, respectively. Additionally, the standard error (SE) and confidence intervals (CIs) were calculated using the "delta method".[47]

### *Colocalization analysis*

The two-step MR analysis identified matrix metalloproteinase 12 (MMP12) and tryptophanyl transfer-RNA synthetase (WARS) as mediators of the effects of SGLT2 inhibition on IS; therefore, colocalization analyses were conducted using the "coloc" package (version 5.2.3) to detect shared causal variation between IS and pQTL of those proteins.[48] A posterior probability of H4 (PPH4) (indicating shared causal variation between two traits) > 0.7 was considered supporting evidence for colocalization, strengthening the inference.[49,50]

### *External validation*

Furthermore, to ensure the robustness of the associations between the protective effects of SGLT2 inhibition and IS development, the findings were verified through external validation using GWAS data from the ISGC, with statistical significance set at $p < 0.05$. Furthermore, we employed proteomic data from the UKB-PPP to validate the mediating role of plasma proteins.

### *Extended analysis of IS-related traits*

In addition, to comprehensively elucidate the impact of SGLT2 inhibition on IS, we broadened the spectrum of IS-related phenotypes under investigation. TSMR was used to evaluate the effect of SGLT2 inhibition on the MRI WMH volume and mRS scores at 3 months post-IS onset. Therefore, assessing the impact of SGLT2 inhibition on cerebral white matter protection and post-stroke rehabilitation could extend the therapeutic application of SGLT2is across various IS stages.

## Results

### *Effect of SGLT2 inhibition on IS*

Fourteen independent SNPs were selected as IVs to investigate the effect of SGLT2 inhibition on IS, as previously described (Guo et al., 2024) (Supplementary Table S2). The F-statistic for each IV was > 10, indicating that any potential bias arising from weak IV strength could be disregarded.

During the discovery phase, the primary findings of the IVW approach indicated that SGLT2 inhibition significantly reduced the risk of IS overall and its three subtypes. Specifically, the genetic prediction of SGLT2 inhibition was associated with decreased risks of IS (IVW OR: 0.39, 95 % CI: 0.25 – 0.61, $p = 3.53 \times 10^{-5}$), LAS (IVW OR: 0.13, 95 % CI: 0.04 – 0.48, $p = 3.19 \times 10^{-3}$), CES (IVW OR: 0.16, 95 % CI: 0.07 – 0.37, $p$

| Exposure | Outcome | MR Method | OR (95%CI) | P−value |
|---|---|---|---|---|
| SGLT2 inhibition | IS | Inverse variance weighted | 0.39 (0.25−0.61) | 3.53e−05 |
| SGLT2 inhibition | IS | Weighted median | 0.41 (0.23−0.73) | 2.28e−03 |
| SGLT2 inhibition | IS | Maximum likelihood | 0.41 (0.26−0.64) | 8.82e−05 |
| SGLT2 inhibition | IS | MR Egger | 0.83 (0.12−5.81) | 0.853942 |
| SGLT2 inhibition | LAS | Inverse variance weighted | 0.13 (0.04−0.48) | 2.19e−03 |
| SGLT2 inhibition | LAS | Weighted median | 0.13 (0.03−0.58) | 7.6e−03 |
| SGLT2 inhibition | LAS | Maximum likelihood | 0.14 (0.04−0.42) | 5.38e−04 |
| SGLT2 inhibition | LAS | MR Egger | 4.76 (0.02−1319.09) | 0.596 |
| SGLT2 inhibition | CES | Inverse variance weighted | 0.16 (0.07−0.37) | 1.82e−05 |
| SGLT2 inhibition | CES | Weighted median | 0.11 (0.04−0.34) | 1.24e−04 |
| SGLT2 inhibition | CES | Maximum likelihood | 0.16 (0.07−0.40) | 5.37e−05 |
| SGLT2 inhibition | CES | MR Egger | 0.11 (0−4.97) | 0.275 |
| SGLT2 inhibition | SVS | Inverse variance weighted | 0.19 (0.07−0.52) | 1.38e−03 |
| SGLT2 inhibition | SVS | Weighted median | 0.16 (0.04−0.62) | 8.42e−03 |
| SGLT2 inhibition | SVS | Maximum likelihood | 0.20 (0.07−0.56) | 2.24e−03 |
| SGLT2 inhibition | SVS | MR Egger | 1.39 (0.02−121.92) | 0.887 |

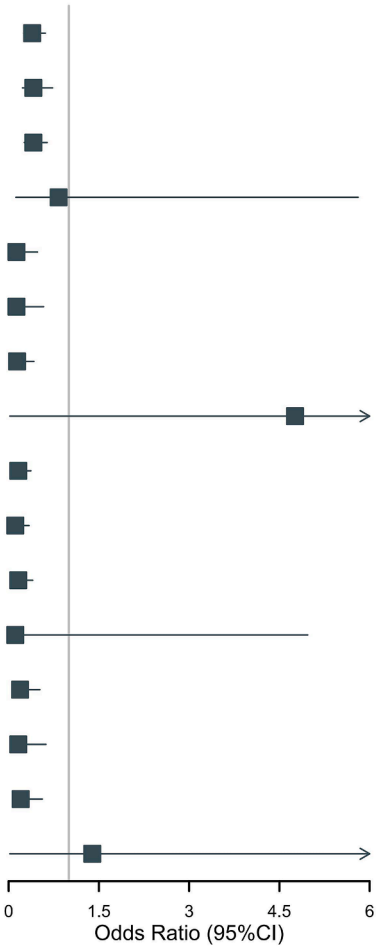


**Fig. 2.** This forest plot depicts the results based on 4 MR methods during the discovery phase, demonstrating the effect of SGLT2 inhibition on IS and its subtypes.

$= 1.82 \times 10^{-5}$), and SVS (IVW OR: 0.19, 95 % CI: 0.07 – 0.52, $p = 1.38 \times 10^{-3}$) for every standard deviation decrease in HbA1c levels induced by SGLT2 inhibition (Fig. 2). These associations remained significant after correcting for multiple comparisons using the FDR. Furthermore, the IVW, weighted median, and maximum likelihood methods consistently yielded significant relationships and consistent estimates of the direction of the relationships between SGLT2 inhibition and the occurrence of IS or its three subtypes, confirming that the findings were robust. However, the direction of the association of SGLT2 inhibition with LAS or SVS derived from the MR-Egger method was inconsistent with the results of the other three methods and must be interpreted cautiously. Therefore, the most rigorous approach was adopted, and the association was ignored. Furthermore, Cochran's Q test for heterogeneity between the IVW and MR-Egger methods did not reveal any significant heterogeneity ($p > 0.05$) (Supplementary Table S3). The test for horizontal pleiotropy based on the intercept term of the MR-Egger regression analysis indicated the findings were unaffected by horizontal pleiotropy ($p > 0.05$) (Supplementary Table S3).

### *Mediation analyses of potential plasma proteins*

#### *Effect of plasma proteins on IS*

MEGASTROKE Consortium GWAS and proteomics data from Icelandic sources were used as the discovery cohort for the MR analysis, which revealed statistically significant relationships between genetically predicted levels of three proteins and IS after controlling for the FDR (Fig. 3A and Supplementary Table S4). Specifically, elevated plasma levels of aldehyde dehydrogenase 2 (ALDH2) (Wald ratio OR: 1.78, 95 % CI: 1.36 – 2.32, $p = 2.22 \times 10^{-5}$) and WARS (IVW OR: 1.19, 95 % CI: 1.09 – 1.30, $p = 7.33 \times 10^{-5}$) significantly increased IS risk, whereas elevated MMP12 levels (IVW OR: 0.88, 95 % CI: 0.83 – 0.93, $p = 5.93 \times 10^{-6}$) decreased IS risk. However, no significant horizontal pleiotropy or heterogeneity was detected in the preliminary analyses (Supplementary Table S4).

#### *Effect of SGLT2 inhibition on plasma proteins*

We estimated the effects of SGLT2 inhibition on ADLH2, WARS, and MMP12 to assess their potential as mediators of IS risk. The primary results demonstrated a significant correlation between SGLT2 inhibition and WARS (IVW OR: 0.65, 95 % CI: 0.46 – 0.91, $p = 0.012$) and MMP12 (IVW OR: 1.63, 95 % CI: 1.16 – 2.28, $p = 4.67 \times 10^{-3}$) expression, implying that SGLT2 inhibition affects the abundance of plasma proteins (Fig. 3B). A consistent direction of effect was observed for the other MR methods (Supplementary Table S5). The sensitivity analyses confirmed that the associations were unaffected by horizontal pleiotropy or heterogeneity (Supplementary Table S5).

#### *Mediation effects of plasma proteins*

A two-step MR method and a "product of coefficients" approach were used to assess the role of plasma WARS and MMP12 levels in mediating the association between SGLT2 inhibition and IS. WARS indirectly mediated the overall effect of SGLT2 inhibition on IS (β:0.08, 95 % CI:0.15 – -0.01, $p = 0.034$) and MMP12 (β:0.06, 95 % CI:0.12 – -0.01, $p = 0.016$), with mediation proportions of 8.2 % (95 % CI: 0.2–16.6 %) and 6.8 % (95 % CI: 0.4–13.3 %), respectively (Table S1).

#### *Colocalization analysis*

Bayesian colocalization analysis was conducted on the potential mediators identified in the two-step MR analyses to assess the probability of the existence of shared genetic variants associated with IS among the pQTL of WARS and MMP12. Plasma levels of WARS (PPH4 = 0.764) and MMP12 (PPH4 = 0.760) exhibited high posterior probabilities of an association with IS, implying potential shared genetic

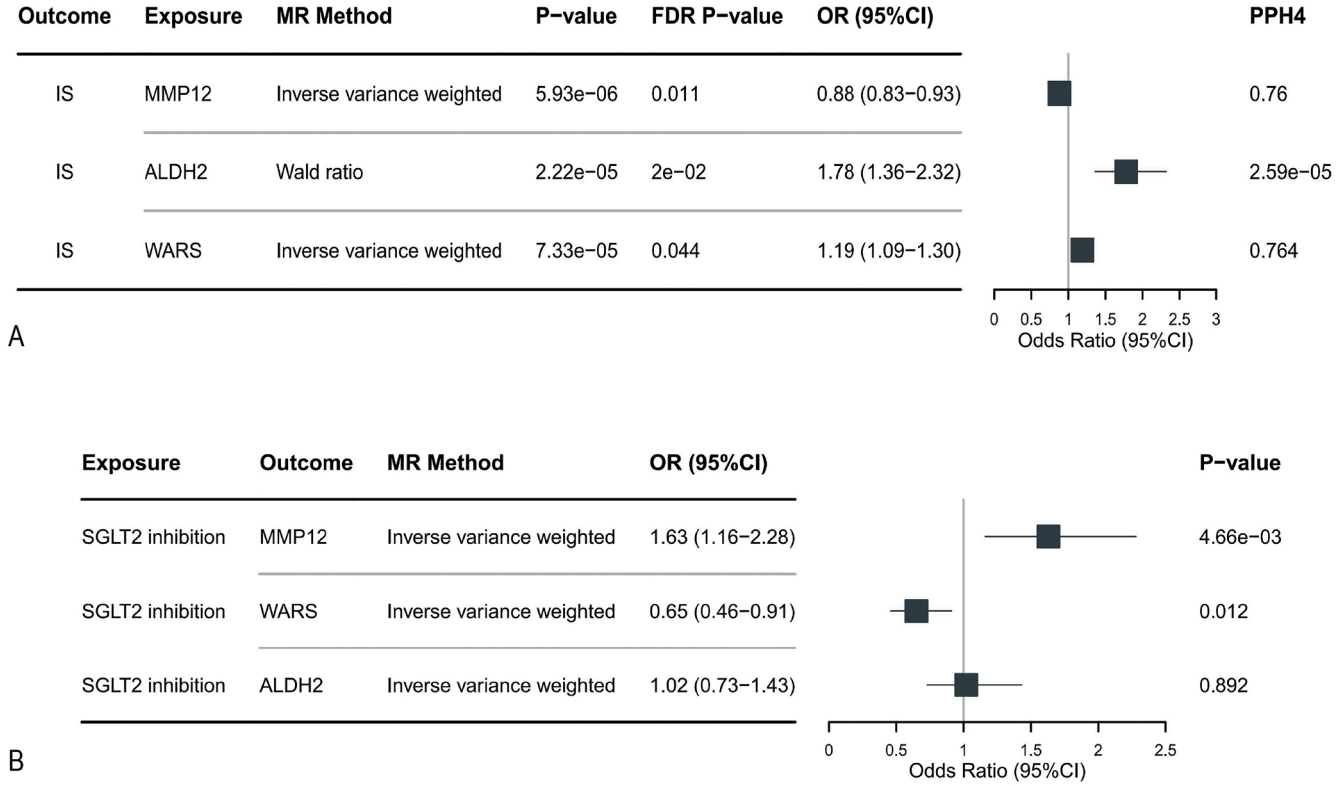


**Fig. 3.** In the discovery phase, a mediated MR analysis based on a two-step MR framework was used to identify plasma proteins that may potentially mediate the pathway by which SGLT2 inhibition associates with IS. (A) Forest plot demonstrating the three plasma proteins that exhibited a statistically significant effect on IS following correction for FDR technique (FDR < 0.05). (B) Forest plot demonstrating the impact of SGLT2 inhibition on three plasma proteins identified as statistically significant.

influences (Fig. 4). This evidence is crucial for reinforcing causal relationships.

*External validation*

In the external validation phase, we employed other datasets as replication cohorts for the exposure and outcome variables. Replication of the primary outcome using the IVW method confirmed the association of SGLT2 inhibition with IS and the mediating role of plasma proteins observed in the discovery phase. Fig. 5 demonstrates the successful validation of the protective effects of SGLT2 inhibition on IS and its subtypes based on the ISGC data. Notably, based on the UKB-PPP dataset, WARS was also identified as a mediator in the reduced IS risk associated with SGLT2 inhibition, with a mediating proportion of 11.2 % (95 % CI: 1.5–21.0 %) (Table 2).

*Extended analysis of IS-related traits*

Changes in the WMH volume and mRS scores 3 months post-IS were evaluated through TSMR to comprehensively assess the effects of SGLT2 inhibition on the pathological imaging characteristics and functional outcomes of IS. A significant reduction in the WMH volume (IVW OR: 0.40, 95 % CI: 0.23 – 0.68, $p = 6.62 \times 10^{-4}$) and improvement in functional outcomes (IVW OR: 0.17, 95 % CI: 0.03 – 0.82, $p = 0.03$) followed the genetically predicted SGLT2 inhibition (Supplementary Table S6). Furthermore, the direction of the associations was consistent, regardless of the MR method used, and no evidence of heterogeneity or horizontal pleiotropy was identified in the sensitivity analyses (Supplementary Table S6).

## Discussion

This study employed TSMR and mediation analyses to elucidate a relationship between SGLT2 inhibition, circulating plasma protein levels, and IS. SGLT2 inhibition generally prevented IS and CES in particular, and the mediating influence of MMP12 and WARS in this relationship was confirmed. Furthermore, the colocalization analysis revealed that cis-pQTL for MMP12 and WARS exhibited genetic overlap with IS, and the mediating effect of WARS was successfully replicated in the external validation phase. Finally, additional MR analyses confirmed that SGLT2 inhibition reduced the WMH volume on MRI and improved the functional prognosis of IS.

Notably, many clinical studies and meta-analyses have substantiated the efficacy of SGLT2is in preventing and treating IS; however, most have focused on patients with T2DM, thereby limiting the generalizability of the findings and the mechanistic understanding of the effects of SGLT2is in non-T2DM populations. The present study employed MR techniques and proteomics to investigate the genetic underpinnings and potential pathways through which SGLT2is exert their effects on IS.

SGLT2is have a pronounced hypoglycemic effect due to their ability to regulate glucose levels, and SGLT2is reduce IS risk more significantly in patients with HbA1c levels of ≥8 %.[8] In addition, SGLT2is can slow atherosclerosis progression and stabilize plaques by regulating dyslipidemia, improving insulin resistance, reducing oxidative stress, inhibiting vascular inflammation, minimizing endothelial cell apoptosis, and preventing foam cell formation.[12,51,52] Atherosclerosis of the intracranial arteries represents the most significant factor affecting IS pathogenesis, and acute thrombosis at unstable plaque sites can result in acute vascular occlusion. The diverse pharmacological properties of SGLT2is offer a crucial theoretical foundation for preventing and managing IS in non-T2DM patients with dyslipidemia, hypertension, and other cerebrovascular disease risk factors. In our study, SGLT2 inhibition significantly reduced the WMH volume, which is a potential MRI biomarker whose changes can be observed before IS onset and are closely associated with cerebral small vessel disease. This effect may be associated with their ability to protect cellular and myelin ultrastructure in neurovascular units.[53] Furthermore, SGLT2is have been demonstrated to facilitate functional recovery in diabetic mice post-stroke.[54], consistent with our findings that SGLT2is exert cerebroprotective effects, some of which may be impacted by WARS activity.

WARS is an essential conserved enzyme that catalyzes the ligation of tryptophan to homologous transfer RNAs during the translation of mRNAs into proteins. Its catalytic function is vital for ensuring cellular viability.[55] In addition to its known classical activity in the nucleus, recent research has highlighted the significant role of WARS in various extracellular processes, including innate immunity, cell death, and atherogenesis.[56–58] Its role in promoting arterial atherosclerosis could explain our findings that WARS increased IS risk. Specifically, atherosclerotic foam cell formation is regulated by the interferon-gamma (IFN-γ)/mini-WARS signaling axis.[58] IFN-γ, highly expressed in

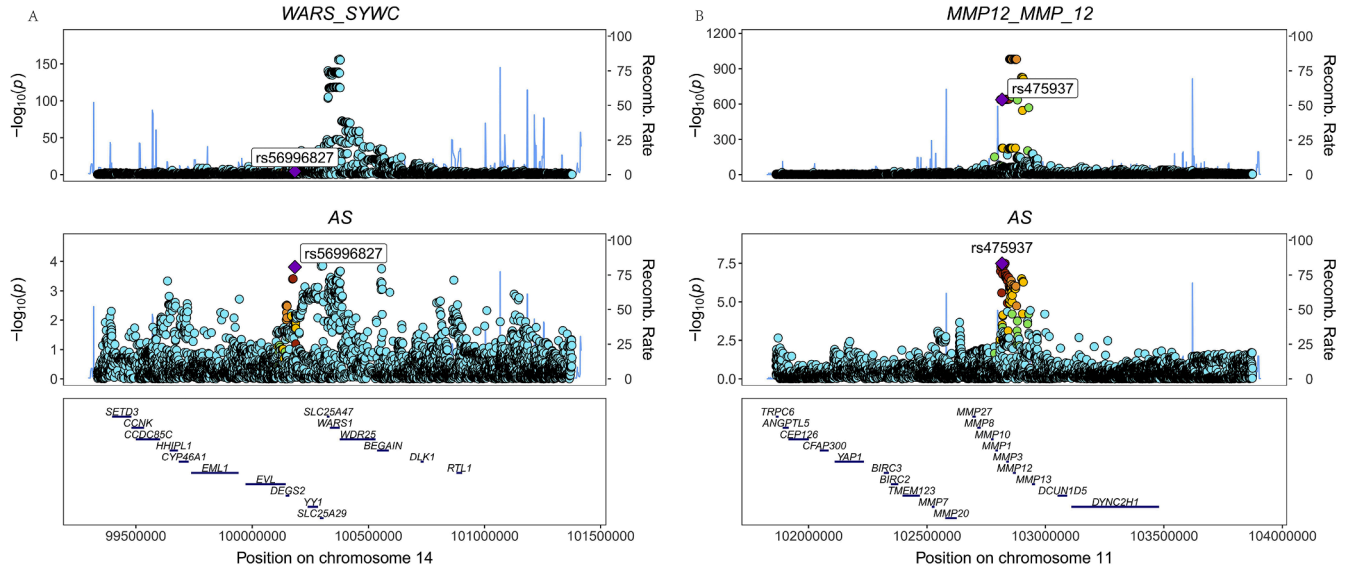


**Fig. 4.** Regional association and matrix plots of colocalization analysis results. (A) and (B) represent the colocalization of pQTL for proteins WARS and MMP12 with GWAS for IS, respectively; the upper level represents the distribution of P-values for loci 1000 KB above and below the pQTL; the middle level represents the distribution of P-values for IS corresponding to IVs of WARS and MMP12. Each point represents an SNP. horizontal coordinates indicate the physical location on the chromosome, and the vertical axis indicates the -log P-value of the pQTL or the GWAS of the IS.

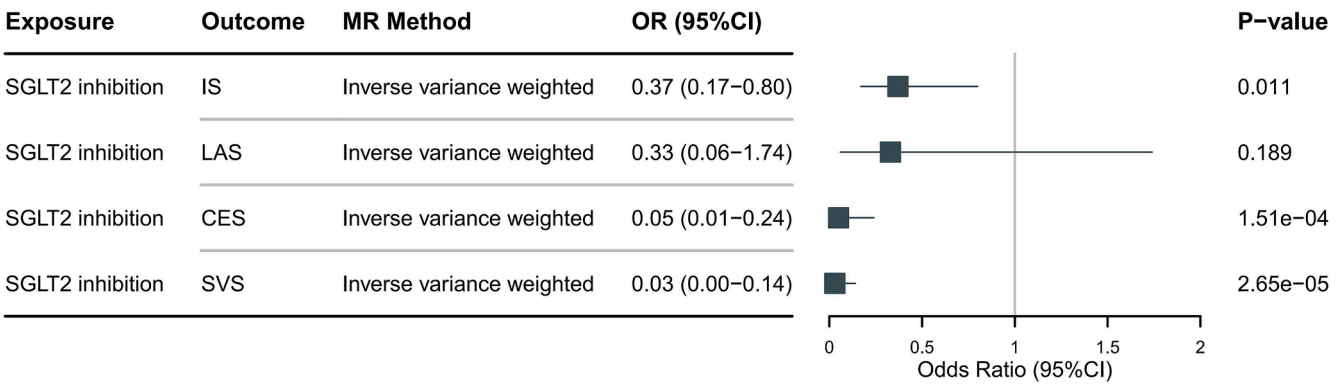


**Fig. 5.** Forest plot showing the effect of SGLT2 inhibition on IS during the external validation phase. GWAS summary data for IS from the ISGC consortium.

**Table 1**
The mediation effect of SGLT2 inhibition on IS via plasma proteins (WARS and MMP12) in the discovery phase.

| Mediator | Total effect β (95 % CI) | Direct effect A β (95 % CI) | Direct effect B β (95 % CI) | Mediation effect β (95 % CI) | p | Mediated proportion (%) (95 % CI) |
|---|---|---|---|---|---|---|
| WARS | -0.94 (-1.39, -0.49) | -0.43 (-0.78, -0.09) | 0.17 (0.09, 0.26) | -0.08 (-0.15, -0.01) | 0.034 | 8.2 (0.2, 16.6) |
| MMP12 | -0.94 (-1.39, -0.49) | 0.49 (0.15, 0.82) | -0.13 (-0.19, -0.07) | -0.06 (-0.12, -0.01) | 0.016 | 6.8 (0.4, 13.3) |

The term "total effect" denotes the impact of SGLT2 inhibition on IS. "Direct effect A" signifies the influence of SGLT2 inhibition on plasma proteins (WARS and MMP12). "Direct effect B" represents the effect of plasma proteins on IS. The term "mediation effect" denotes the impact of SGLT2 inhibition on IS through plasma proteins.

**Table 2**
External validation was performed using the protein dataset from the UKB-PPP study to replicate the involvement of plasma proteins in mediating the effects of SGLT2 inhibition on IS.

| Mediator | Total effect β (95 % CI) | Direct effect A β (95 % CI) | Direct effect B β (95 % CI) | Mediation effect β (95 % CI) | p | Mediated proportion (%) (95 % CI) |
|---|---|---|---|---|---|---|
| WARS | -0.94 (-1.39, -0.49) | -0.57 (-0.89, -0.25) | 0.18 (0.10, 0.27) | -0.10 (-0.18, -0.03) | 0.007 | 11.2 (1.5, 21.0) |

The method of interpretation for Table 2 is consistent with that of Table 1.

atherosclerotic lesions, can induce mini-WARS transcription, indirectly initiating a phenotypic switch in vascular smooth muscle cells (VSMCs)[59] from a quiescent contractile to an activated synthetic phenotype. In the synthetic state, VSMCs phagocytose oxidized low-density lipoproteins (oxLDLs) to form foam cells, which gradually accumulate and eventually cause atherosclerosis.[60] One study administered D-tryptophan to inhibit the IFN-γ/mini-WARS pathway and observed that VSMCs could preserve their contractile phenotype when exposed to IFN-γ *in vitro*,[61] underscoring the significance of mini-WARS in atherosclerosis development.

Moreover, WARS expression becomes upregulated during inflammation, and it is involved in the innate immune response. Upon microbial infection, WARS is promptly secreted from monocytes into the extracellular mesenchyme without requiring de novo synthesis.[62] Concurrently, antigen presentation by dendritic cells to T cells stimulates the production of IFN-γ, which subsequently enhances the expression of WARS.[57] This protein directly interacts with Toll-like receptor 2/4 on macrophages, thereby activating innate immune responses, including the production of chemokines and the enhancement of neutrophil phagocytosis.[63] Furthermore, WARS can activate triggering receptors expressed on myeloid cells-1 and induce downstream mediators, myeloid differentiation primary response 88, and TIR-domain-containing adaptor inducing interferon-β, functioning synergistically.[64] Activated phagocytes internalize oxLDLs to form foam cells, which subsequently serve as a source of inflammation. Furthermore, SGLT2is demonstrate significant potential in IS treatment by reducing the abundance of WARS.

Another significant protein is MMP12, a metalloelastase produced by macrophages that is pivotal in the degradation of extracellular matrix components and biomolecules.[65,66] The involvement of MMP12 in IS has been demonstrated in numerous studies.[67,68] It is highly expressed in carotid atherosclerotic plaques and has been shown to reduce plaque stability by enhancing elastin degradation and macrophage invasion.[69] Mechanistically, MMP12 activates the downstream matrix metalloproteinases MMP2 and MMP9, which in turn disrupt the tight junctions of the blood-brain barrier (BBB), promote neuroinflammation and neuronal apoptosis, and ultimately lead to ischemic brain injury.[70,71] An observational study demonstrated a significant correlation between MMP12 levels and the severity of IS, with elevated MMP12 levels indicating a poorer clinical prognosis.[68] The knockout of MMP12 resulted in a notable reduction in infarct size and facilitated motor and cognitive recovery in an IS mouse model.[70] Notably, our MR study revealed a decrease in the risk of IS associated with MMP12. This finding was consistently replicated in the external validation phase; however, the result should be interpreted cautiously as it may represent a false positive. Notably, several other MR studies have also reported the protective role of MMP12 in atherosclerosis and IS, further bolstering our results and suggesting that they are not merely coincidental.[20,72,73] There are two possible reasons for the discrepancy between observational and genetic studies. First, MR studies assess the lifetime impact of specific plasma proteins on disease, which may not accurately capture short-term effects at particular time points. Second, the IVs used in MR studies are derived from GWAS, allowing for the minimization of confounders and reverse causation through a genetic perspective. However, other studies measure plasma protein expression solely through proteomic techniques, and various post-translational modifications influence the protein translation process.

This is the first study to comprehensively investigate the relationship between SGLT2is, plasma protein levels, and IS. We combined genomics and proteomics to provide genetic evidence for the mechanism of action of SGLT2is using genetic variants as IVs. This mechanism was shown to be mediated by the modulation of plasma levels of WARS and MMP12. These two proteins may influence biological processes such as BBB permeability, inflammatory responses, and atherosclerosis, which are crucial in IS pathogenesis. Considering the important role that plasma proteins play in regulating biological processes as major modulators of

molecular pathways, our study provides a new direction to unravel the mechanism of SGLT2is in IS protection, especially providing a theoretical basis for their application in non-diabetic patients. We also found that SGLT2is have a beneficial effect on WMH and functional outcomes after IS, further supporting their potential application in all stages of IS prevention and treatment. Future studies should further validate the efficacy of SGLT2is in patients with different types of IS, and animal studies should be conducted to verify the plasma protein-mediated effects. In patients with T2DM who also have comorbid atrial fibrillation, SGLT2is may serve as an effective pharmacological intervention for the prevention of CES. However, additional clinical studies are warranted to substantiate these findings.

However, this study has some limitations. First, the selection of IVs of SGLT2 inhibition resulted in a reduction in the targeting based on HbA1c levels and *SLC5A2* gene expression, which may not fully reflect the inhibitory effect of SGLT2is. Second, the inhibitory effects are associated with several factors, including the dose of SGLT2is used, the frequency of administration, and pharmacokinetic factors affecting drug metabolism; the inability to account for these factors could have influenced the results. Third, the IV screening criteria for plasma proteins were too strict. However, they reduced the bias associated with weak IVs; they resulted in some proteins having no qualified SNPs, limiting the number of candidates. Fourth, our study's exclusive focus on cis-pQTLs, without accounting for trans-pQTLs, overlooks the intricate mechanisms involved in protein expression regulation, potentially constraining the interpretative scope of our findings. Fifth, the study undertook a series of sensitivity analyses, colocalization analyses, and external validation to substantiate our findings; however, the potential impact of horizontal pleiotropy on the results could not be entirely excluded. Moreover, the mediating role of MMP12 proteins was not consistently replicated in the UKBPPP plasma proteomic dataset. This inconsistency may stem from measurement differences, as protein assessments across different studies utilized diverse populations, biological samples, and measurement platforms. However, our study is exploratory and aimed at discovering more potential mediating proteins, but the findings are not conclusive. Therefore, to resolve this discrepancy in future research, it will be essential to integrate cellular experiments, animal model studies, and clinical sample analyses to enhance the robustness and reliability of the results. Finally, the population included only individuals of European descent; while this reduced the degree of heterogeneity, it also limited the generalizability of the results to other ethnic populations. Furthermore, the genetic architecture and environmental determinants influencing cerebrovascular disease may differ across ethnic groups. Therefore, additional MR analyses or experimental studies focusing on diverse ethnic populations are necessary to enhance the generalizability of the findings.

## Conclusion

This study provides genetic evidence supporting a link between SGLT2 inhibition, circulating plasma protein levels, and IS. More specifically, SGLT2 inhibition decreases the likelihood of IS, with WARS and MMP12 acting as potential mediators in this relationship. These findings contribute novel insights into the preventive and therapeutic benefits of SGLT2is for IS and offer valuable information for future clinical and experimental investigations.

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## Availability of code

The source code is available for free download from https://github.com/baohenacl155/Mendelian-Randomization-Codes-for-a-publication-in-JSCVD

## CRediT authorship contribution statement

**Zhiqing Chen:** Writing – original draft, Validation, Supervision, Software, Conceptualization. **Hongmei Meng:** Funding acquisition, Formal analysis. **Yujin Guo:** Formal analysis, Data curation. **Huaiyu Sun:** Visualization, Formal analysis. **Wuqiong Zhang:** Data curation. **Yu Guo:** Data curation. **Shuai Hou:** Writing – review & editing, Supervision, Funding acquisition.

## Declaration of competing interest

This study was conducted without any potential conflicts of interest arising from business or financial affiliations.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jstrokecerebrovasdis.2024.108136.

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