Causal associations of insulin resistance with coronary artery disease and ischemic stroke: a Mendelian randomization analysis

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ABSTRACT

Introduction The relationship between insulin resistance (IR) and cardiovascular diseases is unclear. We aimed to examine the causal associations of IR with cardiovascular diseases, including coronary artery disease, myocardial infarction, ischemic stroke and its subtypes, using Mendelian randomization.

Research design and methods Due to low sample size for gold standard measures and in order to well reflect the underlying phenotype of IR, we used 53 single nucleotide polymorphisms associated with IR phenotypes (ie, fasting insulin, high-density lipoprotein cholesterol and triglycerides) from recent genome-wide association studies (GWASs) as instrumental variables. Summary-level data from four GWASs of European individuals were used. Data on IR phenotypes were obtained from meta-analysis of GWASs of up to 188 577 individuals and data on the outcomes from GWASs of up to 446 696 individuals. Mendelian randomization (MR) estimates were calculated with inverse-variance weighted, simple and weighted-median approaches and MR-Egger regression was used to explore pleiotropy.

Results Genetically predicted 1-SD increase in IR phenotypes were associated with a substantial increase in risk of coronary artery disease (OR=1.79, 95% CI: 1.57 to 2.04, p<0.001), myocardial infarction (OR=1.78, 95% CI: 1.54 to 2.06, p<0.001), ischemic stroke (OR=1.21, 95% CI: 1.05 to 1.40, p=0.007) and the small-artery occlusion subtype of stroke (OR=1.80, 95% CI: 1.30 to 2.49, p<0.001), but not associated with the large-artery atherosclerosis and cardioembolism subtypes of stroke. There was no evidence of pleiotropy. Results were broadly consistent in sensitivity analyses using simple and weighted-median approaches accounting for potential genetic pleiotropy.

Conclusions This study provides evidence to support that IR was causally associated with risk of coronary artery disease, myocardial infarction, ischemic stroke and the small-artery occlusion subtype of stroke.

Significance of this study

What is already known about this subject?
► Previous observational studies showed that insulin resistance was associated with an increased risk of cardiovascular diseases.
► Whether this reflects a causal association remains to be established.

What are the new findings?
► Genetically predicted insulin resistance phenotypes was associated with an increased risk of coronary artery diseases, myocardial infarction, ischemic stroke and the small-artery occlusion subtype of stroke.

How might these results change the focus of research or clinical practice?
► Mechanism underlying the association of insulin resistance with cardiovascular diseases requires further investigation.
► Further validations are needed in studies with large sample sizes for the risk of stroke subtypes.

lipoprotein cholesterol (HDL-C) levels and high triglycerides (TGs) levels are three hallmarks of common IR.[1 2] Due to absence of well-powered genome-wide association studies (GWASs) for gold standard measures of IR derived from euglycemic clamp and in order to well reflect the underlying phenotypes of IR, Lotta *et al*[2] identified 53 genetic variants for IR based on these three phenotypes, and Wang *et al*[3] further generated a composite genetic instrument for IR phenotypes by meta-analyzing these genetic variants.

IR is considered as a key risk factor of adverse metabolic and cardiovascular disease.[4 5] Previous observational studies showed that IR was positively associated with an increased risk of coronary artery diseases (CAD)[6–8] and ischemic stroke[9 10] in the general population. However, positive association was not observed in other studies.[11] Whether this reflects a

INTRODUCTION
Insulin resistance (IR) is the clinical state of a reduced sensitivity to insulin with an impaired ability of insulin to maintain normal glucose metabolism. IR is a complex trait, whereas high fasting insulin levels, low high-density

causal association remains to be established since observational epidemiological studies suffer from potential biases and reverse causation which limits their ability to robustly identify causal associations.[12] Whereas, recent clinical trials also demonstrated that insulin sensitizing agents that ameliorated IR prevented vascular events.[13 14] Previous Mendelian randomization (MR) analyses also showed causal associations of IR-related traits (diabetes and obesity) with CAD and cerebrovascular disease.[15–17] Other studies showed genetic evidence of association of insulin or IR with CAD.[17–19] To confirm and strengthen the emerging association of IR with cardiovascular outcomes, we sought to explore the effects of a recently described multitrait genetic instrument of IR on CAD, myocardial infarction (MI) and ischemic stroke.

MR, using genetic variants as instrumental variables, is a method that can control potential confounding factors that may bias observational studies.[12] Genetic variants are randomly allocated at meiosis and independent of other factors. Therefore, MR analysis with genetic variants as instrumental variables can prevent confounding and reverse causation, thus make stronger causal inferences between an exposure and risk of diseases. In the present study, we aimed to use MR analysis to determine whether IR is causally associated with cardiovascular diseases, including CAD, MI, ischemic stroke and its subtypes.

RESEARCH DESIGN AND METHODS
Study design
MR analysis was designed to evaluate the causal associations between IR and risk of cardiovascular diseases (figure 1). Genetic variants associated with IR phenotypes were selected as instrumental variable for the MR analysis. We used published summary-level data from four GWASs of European individuals.[20–24] Data on the exposure (IR phenotypes) were derived from meta-analysis of GWASs of up to 188 577 individuals[3 20–22] and data on the outcome (CAD and ischemic stroke) were obtained from GWASs of up to 446 696 individuals.[23 24] Characteristics of these GWASs are presented in table 1 and online supplementary methods 1. Analyses of all phenotypes were based on subjects of European ancestry only.

Generation of genetic instrumental variables
Due to absence of well-powered GWASs for gold standard measures of IR derived from euglycemic clamp and in order to well reflect the underlying phenotype of IR, we used 53 single nucleotide polymorphisms (SNPs) implicated in IR phenotypes identified through meta-analysis of GWASs by Lotta et al.[2] Using an integrative genomic approach, Lotta et al identified 53 SNPs that were associated with three components of IR phenotypes (ie, high fasting insulin, low HDL-C and high TGs) at p<0.005 for each trait in up to 188 577 individuals from genome-wide results.[2] These 53 SNPs were the lead insulin-associated SNP at each 1 Mb region. Genetic risk score based on these 53 SNPs have been validated to be associated with gold standard measures of IR in independent samples from the Fenland study and four other cohorts. Having a greater number of 53-SNP score was substantially associated with lower insulin sensitivity as measured by euglycemic clamp or insulin suppression test in 2764 individuals ($p=4.3\times10^{-6}$) and lower insulin sensitivity index by oral glucose tolerance test in 4769 individuals ($p=7.3\times10^{-10}$).[2] The triad of these phenotypes has been proposed as a metric to characterize the genetic architecture of IR.[1 2] Summary statistics for association of each SNP with fasting insulin adjusted for body mass index
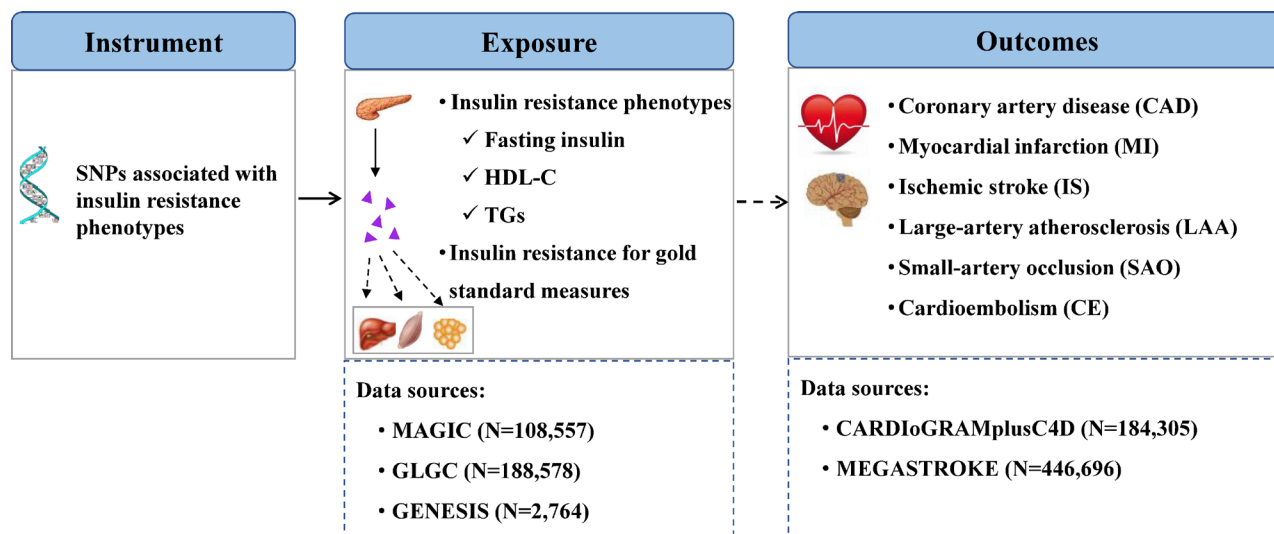

Figure 1 Conceptual framework for the Mendelian randomization analysis of insulin resistance and risk of coronary artery disease and stroke. CARDIoGRAMplusC4D, Coronary ARtery DIsease Genome-wide Replication And Meta-Analysis Plus Coronary Artery Disease Genetics; GENESIS, GENEticS of Insulin Sensitivity; GLGC, Global Lipids Genetics Consortium; HDL-C, high-density lipoprotein cholesterol; MAGIC, Meta-Analyses of Glucose and Insulin-related traits Consortium; MEGASTROKE, Multiancestry Genome-wide Association Study of Stroke; SNP, single nucleotide polymorphisms; TGs, triglycerides.

Table 1 Characteristics of the GWASs used in this study

Phenotype	Consortium	N	Ethnicity	Genotype data	PMID
Exposure (insulin resistance phenotypes)*					
Fasting insulin adjusted for BMI	MAGIC	Up to 108 557 individuals	European	GWAS array and metabochip array	22885924 to 22581228
HDL-C and triglycerides	GLGC	Up to 188 577 individuals	European	GWAS array and metabochip array	24097068 29046328
Insulin sensitivity for gold standard measures	GENESIS	2764 individuals	European	GWAS array	25798622
Outcomes					
Coronary artery disease	CARDIoGRAMplusC4D	Up to 184 305 individuals (60 801 cases and 123 504 controls)	European	GWAS array	26343387
Myocardial infarction	CARDIoGRAMplusC4D	Up to 171 876 individuals (43 677 cases and 128 199 controls)	European	GWAS array	26343387
Ischemic stroke	MEGASTROKE	Up to 446 696 individuals (40 585 cases and 406 111 controls)	European	GWAS array	29531354
Large-artery atherosclerosis	MEGASTROKE	Up to 440 328 individuals (34 217 cases and 406 111 controls)	European	GWAS array	29531354
Small-artery occlusion	MEGASTROKE	Up to 411 497 individuals (5386 cases and 406 111 controls)	European	GWAS array	29531354
Cardioembolism	MEGASTROKE	Up to 413 304 individuals (7193 cases and 406 111 controls)	European	GWAS array	29531354

*Lotta et al[2] identified 53 genetic variants for insulin resistance phenotypes by combining published GWAS results for fasting insulin adjusted for BMI, HDL-C and triglycerides, and Wang et al[3] generated a composite genetic instrument for insulin resistance phenotypes by meta-analysis of these genetic variants.
BMI, body mass index; CARDIoGRAMplusC4D, Coronary ARtery DIsease Genome-wide Replication And Meta-Analysis Plus Coronary Artery Disease Genetics; GENESIS, GENEtiCS of Insulin Sensitivity; GLGC, Global Lipids Genetics Consortium; GWAS, genome-wide association study; HDL-C, high-density lipoprotein cholesterol; MAGIC, Meta-Analyses of Glucose and Insulin-related traits Consortium; MEGASTROKE, Multiancestry Genome-wide Association Study of Stroke; PMID, PubMed unique identifier.

(BMI) were acquired from the Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC)[20 21] and that with HDL-C or TGs levels from the Global Lipids Genetics Consortium (GLGC).[22] A subset 25 of these 53 loci had previously been associated with HDL-C or TGs levels at genome-wide significance, whereas 28 had not.[2] All the SNPs were in different genomic regions and in linkage equilibrium (online supplementary table 1). Potential pleiotropic effects (whether the genetic variants affect the outcome independently of the exposure of interest) of these SNPs were assessed through the MR-Egger regression method. The slope of the MR-Egger regression represents pleiotropy-corrected causal estimates and the intercept represents the average pleiotropic effects across all SNPs.

As Lotta et al[2] did not provide beta-coefficient and SE for the association of these individual SNPs with the IR phenotype, we used the composite genetic instrument for IR phenotypes generated based on these 53 SNPs estimates by Wang et al.[3] An estimate of each of the 53 SNP associations with the composite IR phenotypes were generated through meta-analysis of the absolute values of the standardized beta-coefficient for each SNP association with the individual components of IR phenotypes (ie, high fasting insulin adjusted for BMI, low HDL-C and high TGs) using a fixed-effect inverse-variance weighted (IVW) method.[3] We used this meta-analyzed value as the SNP-exposure (IR phenotypes) estimate (online supplementary table 1); 1-SD genetically higher IR phenotypes was associated with 55% higher fasting insulin adjusted for BMI, 0.46 mmol/L lower HDL-C and 0.89 mmol/L higher TGs.[3]

As Wang et al[3] reported, most of the SNPs had a similar contribution of the three traits to the composite IR phenotypes with the exception of the SNP rs1011685 (near *LPL*), which had a much weaker effect on insulin adjusted for BMI. The heterogeneity of association between the composite IR phenotypes and the three traits was substantially reduced after exclusion of rs1011685 (for insulin: Q=235.29, p<0.001, I^2=78% to Q=49.71, p=0.52, I^2=0%; for HDL-C: Q=73.76, p=0.03, I^2=30% to Q=57.25, p=0.25, I^2=11%; for TGs: Q=139.17, p<0.001, I^2=63% to

Q=57.19, p=0.26, I^2=11%). Therefore, sensitivity analyses were conducted after exclusion of rs1011685 from the instrument.[3]

Outcomes

Summary statistics for the association of each SNP with CAD and MI were acquired from the previously published Coronary ARtery DIsease Genome-wide Replication And Meta-Analysis Plus Coronary Artery Disease Genetics (CARDIoGRAMplusC4D) 1000 Genomes-based GWAS,[23] and that with ischemic stroke as a whole and the three main subtypes (large-artery atherosclerosis (LAA), small-artery occlusion (SAO), cardioembolism (CE)) from the previously published GWAS of Multiancestry Genome-wide Association Study of Stroke (MEGASTROKE) consortium,[24] respectively (table 1 and online supplementary method 1). The associations of the 53 individual SNPs for the IR phenotypes with CAD and MI, and ischemic stroke and its subtypes are presented in online supplementary tables 2 and 3, respectively.

Statistical analysis

The SNP-IR phenotypes and SNP-outcome associations were used to compute estimates of IR phenotypes-outcome associations using MR analyses. We used a conventional IVW MR analysis in which the SNP-outcome estimate is regressed on the SNP-IR phenotypes estimate, weighted by the inverse-variance of SNP-outcome estimate and with the y-axis intercept is fixed to zero.[25] The IVW estimate is an efficient analysis method when all genetic variants are valid instruments. In sensitivity analyses, we also conducted MR-Egger, simple median, weighted-median methods of MR analyses, which are more robust to the inclusion of pleiotropic instruments. The MR-Egger method can identify and control for bias due to directional pleiotropy (ie, whether causal estimates from weaker variants tend to be skewed in one direction) and provide an effect estimate which is not subject to some violations of the standard instrumental variable assumptions.[26] The slope of the MR-Egger regression can provide pleiotropy-corrected causal estimates. An I_{GX}^2 statistic was also calculated to test the presence of measurement error in MR-Egger results; I_{GX}^2 statistic >0.90 was considered no obvious violation of 'No Measurement Error' assumption.[27] The weighted-median method can provide a consistent estimate of the causal effect even when up to 50% of the information contributing to the analysis comes from genetic variants that are invalid instruments.[28] These approaches may assess the robustness of estimates to potential violations of the instrumental variable assumptions.

In addition to the 53-SNPs instruments, we also conducted sensitivity analyses based on: 1) 52-SNPs instruments with the exclusion of rs1011685 (near *LPL*), which as described above, did not show consistent associations across individual phenotypes of IR; 2) 28-SNPs instruments reported in Lotta *et al*[2] that were not in loci previously associated with HDL-C or TGs at genome-wide

significance; 3) 44-SNPs instruments after exclusion of 9 SNPs individually associated with BMI at p<0.001 using Genetic Investigation of ANthropometric Traits summary statistics from 53-SNPs instrument identified by Lotta *et al*[2 3 29]; 4) 12-SNPs instruments reported by the MAGIC investigators that were associated with fasting insulin (BMI adjusted) at genome-wide significance (online supplementary table 4)[20]; 5) 5-SNPs instruments for gold standard measures of IR, such as euglycemic clamp or insulin suppression test, identified by GENEticS of Insulin Sensitivity consortium through GWAS in 2764 European individuals (online supplementary table 4).[30]

Genetic effect estimates of the exposure-outcome associations are presented as OR with their 95% CI of outcome (CAD, MI, ischemic stroke and its subtypes) per 1-SD genetically higher IR phenotypes. To gain insight into the association of the composite genetic IR phenotypes with its individual components and the outcomes, we quantified the association of a 1-SD higher genetically elevated IR phenotypes on the individual components of IR phenotypes (fasting insulin adjusted for BMI, HDL-C and TGs) and the outcomes (CAD, MI, ischemic stroke and its subtypes). To ensure the validity of our conclusions, we took a conservative approach and applied a Bonferroni-corrected significance threshold calculated as 0.05 divided by 6 (ie, 0.0083; 0.05/6 for six outcomes). We considered a statistical test with an observed two-sided p value <0.05 as nominally significant evidence for a potential, but yet to be confirmed, causal association; and an observed two-sided p value <0.0083 as statistically significant evidence for a causal association.[31] All analyses were conducted with R V.3.5.1 (R Development Core Team).

RESULTS

Causal association of IR with CAD

The IVW method showed that 1-SD increase in IR phenotypes was causally associated with a substantial increase in risk of CAD (OR=1.79, 95% CI: 1.57 to 2.04, p<0.001) and MI (OR=1.78, 95% CI: 1.54 to 2.06, p<0.001) at the Bonferroni-adjusted level of significance (p<0.0083) using the 53-SNPs instrument (figure 2). MR-Egger regression showed no evidence of directional pleiotropy for the association of IR phenotypes with CAD (intercept=0.002, p=0.67) and MI (intercept=0.000, p=0.98) (table 2). Similar magnitudes of association and no evidence of directional pleiotropy were observed using the 52-SNPs and 28-SNPs instruments. There was a low risk of bias with MR-Egger because of measurement error using 53-SNPs instrument (I_{GX}^2 statistic=94.6% for CAD and 94.6% for MI) but not using 28-SNPs and 52-SNPs instrument (I_{GX}^2 statistic=9.6% and 78.9% for CAD, 8.3% and 79.5% for MI). Associations between each variant with IR phenotypes and risk of CAD and MI are displayed in figure 3 and online supplementary figure 1.

In sensitivity analyses using the simple median and weighted-median method of MR analyses, similar
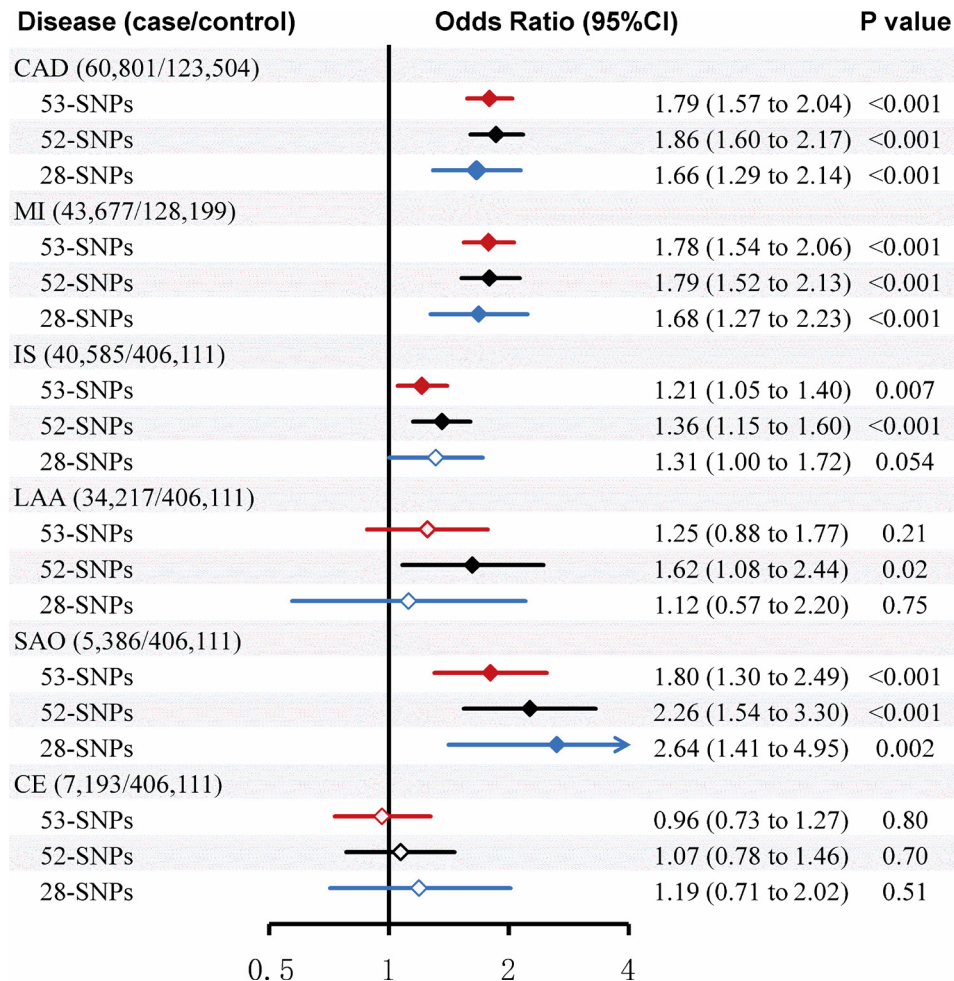
Figure 2 Causal effect estimates of genetically predicted insulin resistance phenotypes on coronary artery disease and ischemic stroke. Estimates are derived from inverse-variance weighted method of Mendelian randomization analysis and represented OR (95% CI) per 1-SD insulin resistance phenotypes. Open and closed symbols indicate p≥0.05 and p<0.05, respectively. CAD, coronary artery disease; CE, cardioembolism; IS, ischemic stroke; LAA, large-artery atherosclerosis; MI, myocardial infarction; SAO, small-artery occlusion; SNP, single nucleotide polymorphism.

association were observed using 53-SNPs, 52-SNPs and 28-SNPs instruments (table 2). However, nominal associations using the 52-SNPs instruments (p=0.01, p=0.045), but no significant association using 28-SNPs instrument (p=0.92, p=0.74), were observed both for the risk of CAD and MI using MR-Egger regression method. Further sensitivity analysis using 44-SNPs instruments that were not associated with BMI at p<0.001 and 12-SNPs instruments that were associated with fasting insulin (BMI adjusted) at genome-wide significance showed significant association of IR phenotypes with the risk of CAD and MI (all p<0.001; online supplementary figure 2). However, association was not observed for CAD or MI using 5-SNPs instruments for gold standard measures of IR (p=0.17; p=0.12).

Causal association of IR with ischemic stroke

The IVW method showed that 1-SD increase in IR phenotypes was causally associated with a substantial increase in risk of ischemic stroke (OR=1.21, 95% CI: 1.05 to 1.40, p=0.007) and the SAO subtype of stroke (OR=1.80,

95% CI: 1.30 to 2.49, p<0.001) at the Bonferroni-adjusted level of significance (p<0.0083), but no significant association for the LAA (OR=1.25, 95% CI: 0.88 to 1.77, p=0.21) and CE (OR=0.96, 95% CI: 0.73 to 1.27, p=0.80) subtypes of stroke using the 53-SNPs instrument (figure 2). MR-Egger regression showed no evidence of directional pleiotropy for the associations of IR phenotypes with ischemic stroke (intercept=0.007, p=0.053), LAA (intercept=0.006, p=0.48) and CE subtypes (intercept=0.008, p=0.24), but marginal significant for the SAO subtype (intercept=0.017, p=0.046) (table 2). Similar magnitudes of association and no evidence of directional pleiotropy were observed using the 52-SNPs and 28-SNPs instruments. Additionally, nominal association were observed between 1-SD increase in IR phenotypes and risk of the LAA subtype (OR=1.62, 95% CI: 1.08 to 2.44, p=0.02) using 52-SNPs instrument. There was a low risk of bias with MR-Egger because of measurement error using 53-SNPs instrument (I_{GX}^2 statistic=94.7%, 94.8%, 94.7% and 94.7% for IS, LAA, SAO and CE, respectively)

Table 2 MR statistical sensitivity analyses

Outcome (case/control)	MR-Egger					Simple median		Weighted-median	
	OR (95% CI)	P value	Intercept (95% CI)	P value for intercept	I^2_{GX}	OR (95% CI)	P value	OR (95% CI)	P value
CAD (60 801/123 504)									
53-SNPs	1.68 (1.22 to 2.32)	0.002	0.002 (−0.006 to 0.009)	0.67	94.6%	2.02 (1.60 to 2.54)	<0.001	1.73 (1.39 to 2.16)	<0.001
52-SNPs	2.12 (1.18 to 3.82)	0.01	−0.003 (−0.014 to 0.009)	0.65	78.9%	2.03 (1.59 to 2.57)	<0.001	2.09 (1.66 to 2.64)	<0.001
28-SNPs	0.94 (0.30 to 2.99)	0.92	0.009 (−0.009 to 0.028)	0.32	9.6%	1.97 (1.37 to 2.83)	<0.001	1.86 (1.30 to 2.67)	0.001
MI (43 677/128 199)									
53-SNPs	1.78 (1.27 to 2.49)	0.001	0.000 (−0.007 to 0.008)	0.98	94.6%	1.74 (1.36 to 2.23)	<0.001	1.76 (1.35 to 2.30)	<0.001
52-SNPs	1.87 (1.01 to 3.44)	0.045	−0.001(−0.013 to 0.011)	0.89	79.5%	1.76 (1.35 to 2.29)	<0.001	1.97 (1.52 to 2.56)	<0.001
28-SNPs	0.80 (0.20 to 3.13)	0.74	0.012 (−0.010 to 0.034)	0.27	8.3%	2.02 (1.34 to 3.06)	0.001	1.99 (1.32 to 3.02)	0.001
IS (40 585/406 111)									
53-SNPs	0.92 (0.66 to 1.27)	0.61	0.007 (0.000 to 0.053)	0.053	94.7%	1.39 (1.09 to 1.78)	0.009	1.15 (0.92 to 1.44)	0.21
52-SNPs	1.32 (0.71 to 2.44)	0.38	0.001 (−0.011 to 0.012)	0.92	78.8%	1.39 (1.09 to 1.78)	0.009	1.39 (1.09 to 1.77)	0.008
28-SNPs	2.07 (0.41 to 10.42)	0.38	−0.007 (−0.033 to 0.018)	0.57	0.0%	1.55 (1.02 to 2.36)	0.04	1.63 (1.08 to 2.48)	0.02
LAA (34 217/406 111)									
53-SNPs	0.97 (0.44 to 2.16)	0.94	0.006 (−0.011 to 0.024)	0.48	94.8%	1.33 (0.72 to 2.44)	0.36	1.00 (0.59 to 1.72)	0.99
52-SNPs	5.69 (1.30 to 24.78)	0.02	−0.025 (−0.053 to 0.003)	0.08	79.1%	1.33 (0.73 to 2.44)	0.36	1.86 (1.01 to 3.40)	0.046
28-SNPs	6.03 (0.17 to 216.59)	0.33	−0.027 (−0.084 to 0.029)	0.34	0.0%	1.03 (0.38 to 2.78)	0.95	0.90 (0.33 to 2.44)	0.84
SAO (5386/406 111)									
53-SNPs	0.94 (0.45 to 1.96)	0.87	0.017 (0.000 to 0.033)	0.046	94.7%	2.28 (1.28 to 4.08)	0.005	1.09 (0.66 to 1.81)	0.74
52-SNPs	1.49 (0.35 to 6.27)	0.59	0.008 (−0.019 to 0.036)	0.55	78.8%	2.56 (1.44 to 4.53)	0.001	2.28 (1.28 to 4.04)	0.005
28-SNPs	14.43 (0.58 to 359.96)	0.10	−0.028 (−0.078 to 0.023)	0.29	0.0%	2.28 (0.90 to 5.75)	0.08	2.24 (0.89 to 5.64)	0.09
CE (7193/406 111)									
53-SNPs	0.71 (0.39 to 1.28)	0.25	0.008 (−0.005 to 0.021)	0.24	94.7%	0.96 (0.61 to 1.51)	0.86	0.72 (0.46 to 1.14)	0.16
52-SNPs	0.82 (0.26 to 2.60)	0.74	0.005 (−0.017 to 0.027)	0.65	79.2%	0.97 (0.61 to 1.55)	0.90	1.12 (0.70 to 1.78)	0.64
28-SNPs	0.95 (0.05 to 19.85)	0.97	0.004 (−0.044 to 0.052)	0.88	0.0%	0.97 (0.45 to 2.11)	0.94	0.98 (0.45 to 2.13)	0.96

CAD, coronary artery disease; CE, cardioembolism; IS, ischemic stroke; LAA, large-artery atherosclerosis; MI, myocardial infarction; MR, Mendelian randomization; SAO, small-artery occlusion; SNP, single nucleotide polymorphism.

but not using 28-SNPs and 52-SNPs instrument (I^2_{GX} statistic=0.0% using 28-SNPs instrument; 78.8%, 79.1%, 78.8% and 79.2% for IS, LAA, SAO and CE, respectively using 52-SNPs instrument). Associations between each variant with IR phenotypes and risk of ischemic stroke, the LAA and SAO subtypes of stroke are displayed in figure 3 and online supplementary figure 1.

In sensitivity analyses, significant association was observed for risk of ischemic stroke using weighted-median method with 52-SNPs instrument (p=0.008). Nominal associations were observed for the risk of ischemic stroke using the simple median with 53-SNPs (p=0.009), 52-SNPs (p=0.009) and 28-SNPs (p=0.04) instruments and weighted-median method with 28-SNPs
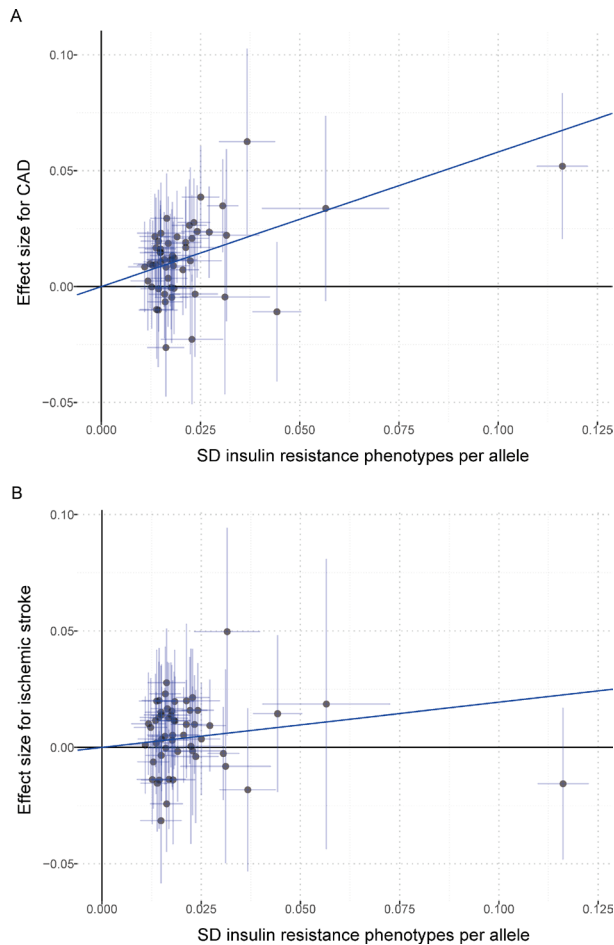
Figure 3 Associations of IR phenotypes variants with risk of CAD (A) and ischemic stroke (B). The blue line indicates the estimate of effect using inverse-variance weighted method. Circles indicate marginal genetic associations with IR phenotypes and risk of outcome for each variant. Error bars indicate 95% CIs. CAD, coronary artery disease; IR, insulin resistance.

(p=0.02) instruments, and for the risk of the LAA subtype using MR-Egger regression and weighted median methods with the 52-SNPs instrument (p=0.02; p=0.046) (table 2). Significant associations were observed for the risk of the SAO subtype using the simple median method with the 53-SNPs and 52-SNPs instruments (p=0.005; p=0.001) and the weighted-median method with the 52-SNPs instrument (p=0.005). Further sensitivity analysis using 44-SNPs instruments that were not associated with BMI at p<0.001 showed significant association of IR phenotypes with ischemic stroke (p=0.008) and nominal association with SAO (p=0.009) (online supplementary figure 2). Using 12-SNPs instruments that were associated with fasting insulin (BMI adjusted) at genome-wide significance and 5-SNPs instruments for gold standard measures of IR, nominal associations were observed for SAO (p=0.02 and p=0.01). No significant association was observed in analyses using other methods or instruments.

DISCUSSION

Using MR analysis, our study provides genetic evidence in support that higher level of IR may lead to increased risk of cardiovascular diseases. In this study, genetically predicted higher level of IR phenotypes was associated with an increased risk of CAD, MI, ischemic stroke and the SAO subtype of stroke. Higher level of IR phenotypes was potentially, yet to be confirmed, causally associated with an increased risk of the LAA subtype of stroke. However, no significant association was observed between IR phenotypes and risk of the CE subtype of stroke.

The findings were consistent with previous observational studies showing a positive association of IR with risk of CAD.[6 7] Our results also were consistent with previous MR analysis based on the Finnish dataset that revealed causal effects between glycemic traits (insulin and glucose) and coronary heart disease.[19] However, results from the Northern Manhattan Study showed that IR was associated with risk of combined outcomes (ischemic stroke, MI and vascular death) after controlling for demographic factors but was attenuated and no longer significant after controlling for metabolic syndrome status or after adjustment for vascular risk factors.[9] Women's Health Initiative Biomarkers studies also implicated that IR measures were no longer associated with cardiovascular risk after adjustment for HDL-C in post-menopausal women without diabetes mellitus.[8] The reason for the discrepancy between our study and these studies is unclear. The potential explanation might be that the above-mentioned observational studies were overadjusted since metabolic syndrome and high HDL-C were considered as pathophysiological consequences or traits of IR.[5] MR associations were attenuated or abolished after using the 28-SNPs instruments which excluded SNPs that had been associated with HDL-C or TGs (a similar adjustment for HDL-C and TGs), indicating that the major contribution of the IR composite phenotype to the CAD/MI outcome is via its effect on lipids. This may also due to the quality of this analysis given the low I^2_{GX} statistic values. Attenuated associations were not observed in MR analyses using 44-SNPs instrument which excluded SNPs that were associated with BMI, indicating that the association of IR and cardiovascular events were not mainly mediated by BMI. These results suggest that a composite assessment of IR phenotypes that includes HDL-C and TGs is a better proxy of IR and predictor of cardiovascular outcomes.

Our study also observed that genetically predicted IR phenotypes was positively associated with an increased risk of ischemic stroke, substantially the SAO subtype and potentially the LAA subtype of stroke. These results were consistent with previous observational studies.[9 10 32] Both observational results from the Northern Manhattan Study[9] and the Cardiovascular Health Study[10] showed that IR were associated with increased risk of incident ischemic stroke in non-diabetic populations. Results from the REasons for Geographic And Racial Differences in Stroke Study indicated a marginal positive association of

IR with risk of ischemic stroke in white population but no association in blacks.[32] However, the association was not validated in the Rotterdam Study.[11] In contrast, studies of the association between IR and risk of etiological subtypes of ischemic stroke are limited. IR was showed to be associated with intracranial and carotid atherosclerosis but can be largely explained by the clustered expression of components of the metabolic syndrome.[33 34] A cross-sectional study in Korea showed that IR was an independent risk factor of silent lacunar infarction presence and its severity.[35] The present study adds the evidence of causal impact of genetic-predicted IR phenotypes and risk of ischemic stroke and its subtypes using MR analysis, a method that may control unmeasured confounding factors and its potential to ascertain causal relationship.[36] For ischemic stroke and SAO subtype, the weighted-median method with 53-SNPs instruments completely attenuated the significance, but the 52-SNPs instruments which removed the outlying SNP rs1011685 recovered this. This may be because the results of weighted-median method with 53-SNPs instruments were much driven by the SNP rs1011685, which had a negative association with the outcomes but large weight in weighted-median method.

IR results from defective intracellular signaling that affects glucose transport. The pathophysiological consequences of IR include hypertension, dyslipidemia, abnormal fibrinolysis, hyperglycemia, hyperinsulinemia, systemic inflammation, altered vascular endothelial function and atherogenesis.[5] Recent MR analysis based on GWASs showed that IR causally affects all branched-chain amino acids (isoleucine, leucine, valine) and inflammation, whose metabolism lie on a causal pathway from IR to type 2 diabetes.[3] These metabolic and cellular changes may then promote atherosclerosis and subsequent clinical events, including CAD and ischemic stroke.[5] Using genetic data via an MR approach, we assessed the causal relationship between IR phenotypes and risk of cardiovascular diseases. The results showed that genetic predisposition to IR phenotypes were related to higher risk of CAD, MI, ischemic stroke and the SAO subtype of stroke, and potentially the LAA subtype of stroke.

Strengths of our study is the design of MR analysis based on large-scale GWASs using multiple IR phenotypes-related SNPs, which enable us to perform a comprehensive evaluation of IR and increase the precision of the estimates. The design of MR analysis can prevent reverse causation and potential confounders, such as dietary and lifestyle preference, thus ascertain causal inferences.[12] Our analysis distinguishes itself from previous MR study[19] by performing a comprehensive evaluation of causal associations of IR with risk of CAD and ischemic stroke as well as its subtypes. Comprehensive evaluation of subsequent clinical events with CAD and stroke may help better understanding of the clinical consequences of IR.

Our study had several limitations. First, our analyses were conducted using European datasets and generalization of the findings to population of non-European ancestry was limited. However, recent studies are providing evidence of shared genetic architecture for metabolic diseases between Europeans and non-Europeans.[37] The uniformity of the included subjects ensures minimal risk of confounding by population admixture. Second, the identification of IR phenotype was through proxy IR based on a meta-GWAS of three traits (higher fasting insulin levels adjusted for BMI, lower HDL-C and higher TGs levels). As we known, the 'gold standard' for quantifying IR is the euglycemic hyperinsulinemic glucose clamp technique. Due to lack of data regarding large-scale GWAS on gold standard measures of insulin sensitivity and in order to well reflect the underlying phenotype of IR, we used this proxy measure of IR with SNP-phenotype associations at $p<0.005$ for each of the three traits. The selection condition with $p<0.005$ creates a concordance of all three by selection rather than biology and the proxy measure of IR might just represent a very specific weighted sum of the three traits. This may cause misclassification bias. However, the identified loci were strongly associated with risk of diabetes and gold standard measures of insulin sensitivity in the validation population in the original paper by Lotta *et al*.[2] Third, there was a risk of bias because of measurement error using 28-SNPs and 52-SNPs instrument and the results may be biased by potential pleiotropy (SNPs may tag heterogeneous pathways) since we used MR design.[38] Caution is needed to explain the results as no significant association was observed either for the risk of CAD or MI using 28-SNPs instrument and MR-Egger regression method, which may provide pleiotropy-corrected causal estimates.[26] However, pleiotropic effects were not observed in MR-Egger regression analyses and sensitivity analyses with exclusion of non-specific SNPs showed mostly similar results. Finally, sample size of GWAS for stroke subtypes was limited and the causal inferences of IR phenotypes and stroke subtypes need further validation based on GWASs with larger sample sizes.

CONCLUSIONS

Our MR analysis provide new evidences of causal associations between IR and risk of cardiovascular diseases, especially for the risk of CAD, MI, ischemic stroke and the SAO subtype of stroke. However, further validations are needed in other studies with large sample sizes for the risk of stroke subtypes.

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