The Role of Adiponectin in Coronary Heart Disease Risk: A Mendelian Randomization Study

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ABSTRACT

Rationale: Hypoadiponectinemia correlates with several coronary heart disease (CHD) risk factors. However, it is unknown whether adiponectin is causally implicated in CHD etiology.

Objective: We aimed to investigate the causal effect of adiponectin on CHD risk.

Methods and Results: We undertook a Mendelian randomization study using data from genome-wide association studies (GWAS) consortia. We used the ADIPOGen consortium to identify genetic variants that could be used as instrumental variables for the effect of adiponectin. Data on the association of these genetic variants with CHD risk were obtained from CARDIoGRAM (22,233 CHD cases and 64,762 controls of European ancestry) and from CARDIoGRAMplusC4D Metabochip (63,746 cases and 130,681 controls; ~ 91% of European ancestry) consortia. Data on the association of genetic variants with adiponectin levels and with CHD were combined to estimate the influence of blood adiponectin on CHD risk.

In the conservative approach (restricted to using variants within the adiponectin gene as instrumental variables), each 1 unit increase in log blood adiponectin concentration was associated with an odds ratio for CHD of 0.83 (95%CI: 0.68; 1.01) in CARDIoGRAM and 0.97 (95%CI: 0.84, 1.12) in CARDIoGRAMplusC4D Metabochip. Findings from the liberal approach (including variants in any locus across the genome) indicated a protective effect of adiponectin that was attenuated to the null following adjustment for known CHD predictors

Conclusions: Overall, our findings do not support a causal role of adiponectin levels in CHD etiology.

Keywords:
Adiponectin, coronary artery disease, cardiovascular disease, mendelian randomization analysis, genetic epidemiology.

Nonstandard Abbreviations and Acronyms:

C4	conservative instrumental variable analysis approach
CARDIoGRAM	Coronary ARtery DIsease Genome-wide Replication And Meta-analysis
CARDIoGRAMplusC4D Metabochip	CARDIoGRAMplusC4D Metabochip and GWAS meta-analysis
CEU	Utah residents with Northern and Western European ancestry
GIANT	Genetic Investigation of ANthropometric Traits
GLGC	Global Lipids Genetics Consortium
GWAS	genome-wide association studies
IVW method	inverse-variance weighted method
L17	liberal instrumental variable analysis approach
LD	linkage disequilibrium
MAGIC	Meta-Analyses of Glucose and Insulin-Related Traits Consortium
SNP	single nucleotide polymorphisms.

INTRODUCTION

Adiponectin, a 30 kDa protein produced mainly by mature adipocytes, has been implicated in a wide spectrum of biological pathways related to peripheral insulin sensitivity [1], inflammatory response [1, 2] and atherogenesis [2]. In contrast to most adipokines, adiponectin secretion is downregulated in obese individuals [3]. Observational epidemiological studies support that hypoadiponectinemia is associated with cardiovascular risk factors [4, 5] (e.g. insulin resistance and dyslipidaemia), and type 2 diabetes risk [6]; inconsistent findings have been observed regarding coronary heart disease (CHD) [7-10] and stroke risk [9, 11].

Mendelian randomization studies make use of genetic variants as instrumental variables to investigate the effect of environmental exposures and biomarkers on outcomes. Since alleles are randomly allocated during gametogenesis and genotype is a fixed exposure, Mendelian randomization studies are not as vulnerable to confounding and reverse causality, and can substantially improve causal inference from observational data [12]. Mendelian randomization is regarded as nature's analogue of randomized controlled trials and has successfully been used in cardiovascular research to investigate potential etiological mechanisms [13], validate and prioritize novel drug targets [14] and increase understanding of current therapies [15].

There is evidence of a shared allelic architecture of circulating adiponectin with CHD risk and carotid intima-media thickness [16, 17]; however, it remains unanswered if these findings implicate a causal effect of adiponectin on CHD risk or merely shared pleiotropic factors. Our aim was to investigate the causal effect of adiponectin on CHD risk using Mendelian randomization.

METHODS

Study design.

We performed a two-sample Mendelian randomization analysis using summary data from genome-wide association studies (GWAS) consortia. Single nucleotide polymorphisms (SNPs), previously reported to be associated with blood adiponectin levels, were used as instrumental variables for testing the causal effect of adiponectin on CHD risk. Data on the association of SNPs with (a) adiponectin levels (first samples) and (b) CHD risk (second samples) were combined to estimate the influence of blood adiponectin on CHD risk. In order to investigate the presence of potential bias (horizontal pleiotropy) or mediation of the effect of adiponectin on CHD via other CHD risk factors (vertical pleiotropy) (Online figure I), we also analyzed data on the association of the selected adiponectin related SNPs with a range of CHD risk factors: glycated hemoglobin (Hb_{A1c}), fasting insulin, high-density lipoprotein-cholesterol (HDL-c), low-density lipoprotein-cholesterol (LDL-c), triacylglycerols (TAG), body mass index (BMI), and BMI-adjusted waist circumference (WC).

Data sources.

Summary data on the association between SNPs and the phenotypes of interest were extracted from public databases of different consortia: ADIPOGen for adiponectin [18]; CARDIoGRAM (Coronary ARtery DIsease Genome-wide Replication And Meta-analysis) [19] and CARDIoGRAMplusC4D Metabochip (CARDIoGRAMplusC4D Metabochip and GWAS meta-analysis) [20] for CHD; MAGIC (Meta-Analyses of Glucose and Insulin-Related Traits Consortium) for Hb_{A1c} [21] and fasting insulin [22]; GLGC (Global Lipids Genetics Consortium) for HDL-c, LDL-c and TAG [23]; and GIANT (Genetic Investigation of ANthropometric Traits) for BMI [24] and WC [25]. Details about each data source are displayed in Online table I. CARDIoGRAMplusC4D Metabochip includes data from CARDIoGRAM GWAS.

Instrumental variables.
The SNPs for our main instrumental variables analyses (n = 17 SNPs) were selected from 145 SNPs strongly ($p < 5*10^{-8}$) associated with blood adiponectin levels in the European ancestry GWAS meta-analysis from the ADIPOGen consortium [18]. Independent SNPs were previously selected by Dastani et al (2013) [16] by linkage disequilibrium (LD) prunning of the genome-wide significant SNPs, retaining SNPs that explained most variance in adiponectin levels in each LD block (LD threshold: $R^2 < 0.05$ in HapMap CEU population (Utah residents with Northern and Western European ancestry)) (Table 1).

We used two sets of instruments (Figure 1):

1. A conservative instrumental variable analysis, in which only SNPs within the *ADIPOQ* locus (± 50 kb) were considered eligible (n = four SNPs) (C4). *ADIPOQ* is mainly expressed in adipose tissue and encodes adiponectin. We considered this approach unlikely to be biased by horizontal pleiotropy given the functional relationship of *ADIPOQ* to adiponectin levels.

2. A liberal analysis, in which independent SNPs from any locus that had reached a genome-wide significant association ($p < 5*10^{-8}$) with adiponectin levels in the ADIPOGen consortia GWAS (n = 17 SNPs) were included (L17), as previously reported by Dastani et al. [16]. These 17 SNPs included the four SNPs within the *ADIPOQ* locus.

Ten of the 17 selected SNPs could be found in CARDIoGRAMplusC4D Metabochip data, three of which were proxy SNPs ($R^2 > 0.95$ for CEU population). For the remaining seven SNPs, data from CARDIoGRAM GWAS was used. One of the 17 selected SNPs could not be found in GLGC data rs1108842, a proxy SNP (rs13083798), in perfect LD, was used instead ($R^2 = 1.0$ for CEU population).

Validation of instrumental variable assumptions.
Validity of Mendelian randomization analyses results can be compromised if the instrumental variable assumptions are violated. In Online table II, we described the three core assumptions of instrumental variable analysis and the strategies used to address these.

Estimation of causal effect.
For both liberal and conservative approaches, the beta coefficient (log odds ratio of CHD per one natural log greater adiponectin level) and its standard error were calculated using the inverse-variance weighted (IVW) method as described by Burgess et al. [26] (See Web-supplemental methods).

For the liberal approach, we also used the IVW method to estimate the combined effect of adiponectin levels on cardiovascular risk factors (Hb_{A1c}, fasting insulin levels, HDL-c, LDL-c, TAG, BMI, and WC). Where we found evidence of an effect of the SNPs on these risk factors, estimates of the association between adiponectin and CHD were adjusted for these risk factors to reduce the possibility that horizontal pleiotropy biased our findings [27] (See Web-supplemental methods).

Sensitivity analyses.
Assuming that all valid instrumental variables identify the same causal parameter, substantial heterogeneity would be suggestive of pleiotropic SNPs. We evaluated heterogeneity in our IVW estimates using standard tools from the meta-analysis literature: forest plot of per SNP ratio estimate, Cochran's Q test, and I^2 values.[28-30] In addition, in order to identify overly influential SNPs, additional meta-analyses were carried out by removing one SNP at a time and recalculating the overall instrumental variable estimates.

Even after adjusting for cardiovascular risk factors associated with our instrument, the liberal approach estimates could still be biased by unknown horizontal pleiotropic pathways that link the adiponectin genetic instrumental variable to CHD independently of path through adiponectin. To explore the presence of this possible bias, the MR-Egger regression method was used. [31]. See Web-supplemental methods for a description of this method.

We also undertook a positive control analysis that consisted of a Mendelian randomization analysis in which LDL-c was the biomarker of interest and CHD risk was the outcome (using the IVW and MR-Egger method), due to its established causal role in CHD development (see Supplemental methods).

RESULTS

Association of the genetic instrument with adiponectin and CHD risk.

Figure 2 shows the associations of SNPs, used as instrumental variables in the conservative (n = 4 SNPs within *ADIPOQ* gene) and liberal analyses (n = 17 SNPs across the genome), with adiponectin levels and CHD risk. For the conservative approach, each adiponectin increasing allele was associated with 2.3% reduction in CHD risk (95% CI: -4.1; -0.4%) in CARDIoGRAM data and 0.6% reduction in CHD risk (95% CI: -1.9; 1.0%) in CARDIoGRAMplusC4D Metabochip. For the liberal approach, each adiponectin increasing allele was associated with 2.3% reduction in CHD risk (95% CI: -3.2; -1.5%) in CARDIoGRAM data and 1.7% reduction in CHD risk (95% CI: -2.3; -1.1%) in CARDIoGRAMplusC4D Metabochip. Of the 17 SNPs, there was some evidence of heterogeneity ($p < 0.05$) between studies that contributed to each consortium for three SNPs: two SNPs in CARDIoGRAM (rs1108842 and rs6488898) and one SNP in CARDIoGRAMplusC4D Metabochip (rs3774261).

Association of the genetic instruments with CHD risk factors.

Over 50% of individual SNPs were associated with one or more CHD risk factor (Hb_{A1c}, fasting insulin levels, HDL-c, LDL-c, TAG, BMI, and WC), none of these SNPs were located within *ADIPOQ* gene (± 50 kb) (Table 2). In general, adiponectin increasing variants were not associated with CHD risk factors in the conservative approach but were related to lower fasting insulin, higher HDL-c, lower TAG, lower WC, and higher BMI in the liberal approach (Figure 3).

Effect of blood adiponectin concentration on CHD risk.

Figure 4 shows the results of all Mendelian randomization analyses assessing the association of genetically-predicted adiponectin with CHD risk. Using the conservative approach (including only the four SNPs within *ADIPOQ* gene), each unit increase in log adiponectin concentration was associated with an odds ratio for CHD of 0.83 (95% CI: 0.68; 1.01) in CARDIoGRAM and 0.97 (95% CI: 0.84; 1.12) in CARDIoGRAMplusC4D Metabochip dataset. Using the liberal approach (including 17 SNPs), the OR for the effect of each unit increase in log adiponectin concentration on CHD was 0.76 (95%CI: 0.65; 0.89) in CARDIoGRAM and 0.83 (95% CI: 0.74; 0.93) in CARDIoGRAMplusC4D Metabochip. When we adjusted these liberal approach results for the CHD risk factors associated with the genetic instrument (fasting insulin, HDL-c, TAG, WC, and BMI) the OR was 0.88 (95%CI: 0.75; 1.03) in CARDIoGRAM and 1.00 (95% CI: 0.90; 1.12) in CARDIoGRAMplusC4D Metabochip.

Sensitivity analyses.

There was substantial heterogeneity in IVW estimates among the 17 SNPs from the liberal approach in both CARDIoGRAM (I^2 = 65.2; P = $1*10^{-4}$) and CARDIoGRAMplusC4D Metabochip (I^2 = 72.4; P = $2*10^{-6}$) data (Online figure II). The effect of removing one SNP at a time on the overall estimate showed that no single SNP could explain the observed protective effect in the liberal analysis. Though, the inclusion of the SNPs rs17366568 and rs8047711 slightly underestimated findings from the IVW method in CARDIoGRAM dataset (Online figure III).

By using the MR-Egger method with our liberal instrument, we observed further evidence of directional pleiotropy, i.e. the instrument was associated with a decreased log odds of CHD independently of its effect on adiponectin in CARDIoGRAM (log OR: -0.03; 95%CI: -0.05; -0.02 for the intercept) and in CARDIoGRAMplusC4D Metabochip (log OR: -0.03; 95%CI: -0.05; -0.02 for the intercept) (Online figure IV). According to Mendelian randomization estimates using the MR-Egger method, each unit increase in log adiponectin concentration was associated with an odds ratio for CHD of 1.25 (95% CI: 0.96; 1.63) in CARDIoGRAM and 1.30 (95% CI: 1.06; 1.58) in CARDIoGRAMplusC4D Metabochip dataset (Figure 4). In the influence meta-analysis, in which we removed one of the 17 SNPs at a time from the pooled estimates, all of the results for the remaining 16 SNPs were in the same (positive) direction but the magnitude of this varied somewhat (Online figure III).

To investigate any differences between CARDIoGRAM and CARDIoGRAMplusC4D Metabochip, we compared Mendelian randomization results of the effect of LDL-c on CHD risk (positive control analysis). The OR for CHD for each standardized unit increase in LDL-c was 1.70 (95% CI: 1.54; 1.88) in CARDIoGRAM and 1.57 (95% CI: 1.47; 1.67) in CARDIoGRAMplusC4D Metabochip. After accounting for unknown confounding (MR-Egger method), estimates were: 1.96 (95% CI: 1.59; 2.33) for CARDIoGRAM and 1.92 (95% CI: 1.65; 2.17) for CARDIoGRAMplusC4D Metabochip.

DISCUSSION

Taken together, our results are not supportive of a protective causal effect of adiponectin on CHD risk. First, we found no consistent evidence that genetic predisposition to elevated blood adiponectin levels is associated to reduced risk of CHD in the analysis restricted to *ADIPOQ* SNPs (conservative approach). Secondly, in the more liberal analysis, using variants associated with adiponectin across the genome, there was evidence of a protective effect, but this was due to horizontal pleiotropy. This conclusion regarding horizontal pleiotropy resulting in a biased apparent protective effect with our liberal approach is supported by both multivariable Mendelian randomization and MR-Egger. Some of the variants strongly associated with circulating adiponectin, in our liberal analysis, are related to loci of potential importance for LDL-c signalling in endothelial cells (*CDH13*) and for vascular biology (e.g. *TRIB1* and *VEGFA*), which might explain their pleiotropic effects regarding CHD aetiology [18]. Lastly, our results are strengthened by the consistent strong positive associations of LDL-c with CHD when we use the same methods used for adiponectin to test this known causal effect.

Few previous studies have conducted Mendelian randomization analysis to investigate the effect of adiponectin on metabolic diseases. Two smaller studies found evidence that genetically raised adiponectin levels were positively associated with insulin sensitivity [32, 33]. However, a larger study did not provide evidence of a causal role of adiponectin in insulin resistance or type 2 diabetes [34], but found that genetically raised insulin levels are associated with lower adiponectin levels, suggesting that the association was possibly because higher insulin levels caused lower adiponectin, rather than the other way round.

We have undertaken the first large Mendelian randomization study of the causal effect of adiponectin on cardiovascular disease risk using GWAS consortia data from CARDIoGRAM (22,233 CHD cases and 64,762 controls) and CARDIoGRAMplusC4D Metabochip (60,801 cases and 123, 504 controls) with detailed phenotyping of coronary artery disease, myocardial infarction, or both. We applied a rigorous analyses plan to assess the validity and consistency of our findings. This included **(I)** adopting a systematic prespecified approach to selecting SNPs for our instrumental variables; **(II)** exploring different scenarios from the plausibly valid (but less well powered) conservative MR approach (restricted to SNPs within adiponectin locus) to the well-powered (but vulnerable to horizontal pleiotropy) liberal MR approach (using SNPs across the genome); **(III)** extensively investigating the presence of bias due to horizontal pleiotropy by using data from other CHD-related phenotypes (e.g. glycaemic, lipid and anthropometric traits) and methods to account for it (adjusted IVW method and MR-Egger method); **(IV)** testing our hypotheses in two datasets (CARDIoGRAM and CARDIoGRAMplusC4D Metabochip); **(V)** using a very large sample size which provides us with 100% power to detect an odds ratio of 0.80 and 81% to detect and odds ratio of 0.90 with a 0.05% type 1 error rate (Online table III) **(VI)** checking the consistency of our findings by performing influence metanalysis and a positive control analysis and (**VII**) using two-sample Mendelian randomization to avoid statistical overfitting in comparison to Mendelian randomization where all analyses are conducted in the same participants[35] (in a one-sample setting, results could be biased in the presence of weak instruments due to genetic variants correlating with confounders by chance).

Some limitations of this study should be considered. First, we were not able to test for effect modification by sex, age or previous disease due to the use of summary data only. In observational studies, the association between adiponectin levels and CHD outcomes is modified by factors such as the type of event (incident vs. prevalent) [10] and age of the participant [36]. Surprisingly, we did find a positive association between circulating adiponectin and CHD risk in the MR-Egger analysis with CARDIoGRAMplusC4D Metabochip dataset, which is likely to be reflecting a false positive finding since it was generally inconsistent with results from the conservative approach. We aimed to estimate the causal effect of total adiponectin concentrations, but high molecular weight adiponectin is thought to be the biologically active fraction and we are not able to specifically assess its effect. Whilst we have explored possible violation of

the assumptions of Mendelian randomization (Online table II) we cannot rule out bias due to possible compensatory mechanisms, known as canalization (e.g. counter-regulation of adiponectin receptors expression due to variations in blood adiponectin concentration). That said we are not aware of any evidence that this might be the case.

The two-sample Mendelian randomization assumes that both samples come from comparable populations. For our discovery analyses this was the case, whereas in CARDIoGRAMplusC4D Metabochip, though the majority of the participants were of European ancestry (the same as in ADIPOGen), 9% were from other ethnic backgrounds. However, we think it is unlikely that this will have resulted in a major source of bias. First, double genomic control for ethnicity was undertaken in CARDIoGRAMplusC4D Metabochip to control for confounding by population stratification. Second, we found very little evidence of heterogeneity in the association of SNPs with CHD in the two consortia, which suggests (strong) effect modification by genomic ancestry is unlikely. Lastly, in a positive control study we showed that two-sample Mendelian randomization produced similar evidence for the expected positive causal effect of LDL-c on CHD.

Adiponectin concentration in the blood ranges from one to 30 ng/mL in healthy adults, which is approximately 10^3 to 10^6 folds higher than the concentration of many hormones and cytokines [37]. Blood adiponectin concentration is a modifiable risk factor that can be efficiently targeted by lifestyle modifications, mainly weight loss and dietary changes [38]. Our results reinforce that Mendelian randomization studies can be helpful in prioritizing potential drug or lifestyle targets, which could substantially reduce the high costs associated with the development and evaluation of large numbers of compounds or lifestyle changes that fail along the development process.

Overall, our findings are not supportive of a protective role of adiponectin in CHD and indicate that the association of genetically increased adiponectin levels and lower risk of CHD is mainly driven by horizontal pleiotropy.

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None.

AUTHOR CONTRIBUTIONS
Study design: MCB, DAL, CO, BLH, AJDB
Analysis plan: MCB, DAL, JW, AJDB
Data acquisition (from public data basis): MCB, CO
Analyses: MCB
Writing first draft of paper: MCB
Critical comments and contributions to final writing of paper: DAL, CO, JW, BLH, AJDB

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FIGURE LEGENDS

Figure 1. Analysis plan. Summary data from SNP-phenotypes was extracted from GWAS consortia datasets (ADIPOGen, CARDIoGRAM, C4D, MAGIC, GLGC, and GIANT). The effect of adiponectin on CHD was estimated using a conservative Mendelian randomization approach (instrumental variable: SNPs within *ADIPOQ* locus (± 50 kb)) and a liberal approach (instrumental variable: SNPs in any locus). For the conservative approach, IVW method was used. For the liberal approach, IVW method was used in both crude and adjusted analysis for known pleiotropic factors and MR-Egger regression in the analysis accounting for hidden pleiotropy (sensitivity analysis). Hb_{A1c}: glycated hemoglobin; BMI: body mass index; CARDIoGRAM: Coronary Artery Disease Genomewide Replication and Meta-analysis; CARDIoGRAMplusC4D Metabochip: CARDIoGRAMplusC4D Metabochip meta-analysis; CHD: coronary heart disease; GIANT: Genetic Investigation of Anthropometric Traits; GLGC: Global Lipids Genetics Consortium; HDL: high density lipoprotein; IV: instrumental variable; IVW method: inverse-variance weighted method; LDL: low density lipoprotein; MAGIC: Meta-Analyses of Glucose and Insulin-related traits Consortium; MR: Mendelian randomization; SNPs: single nucleotide polymorphisms; TAG: triacylglycerol; WC: waist circumference.

Figure 2. Forest plots of mean difference in log adiponectin levels and odds ratio of coronary heart disease per allele of SNPs according to the conservative (A) and liberal (B) approaches. A: Conservative approach including 4 SNPs within *ADIPOQ* gene associated with adiponectin at genome-wide significant levels ($p < 5*10^{-8}$) (C4); B: liberal approach including 17 SNPs across the genome associated with adiponectin at genome-wide significant levels ($p < 5*10^{-8}$) (L17). CHD: coronary heart disease; Chr: chromosome; OR: odds ratio; SNP: single nucleotide polymorphism . Results for log adiponectin included 29,347 individuals from ADIPOGen consortium and for CHD risk included 86,995 individuals (22,233 CHD cases) from CARDIoGRAM and 194,427 individuals (63,746 CHD cases) from CARDIoGRAMplusC4D Metabochip consortium.

Figure 3. Standardized mean difference (and 95%CI) in cardiovascular risk biomarkers per 1 unit increase in genetically instrumented log adiponectin levels. A: Conservative approach including 4 SNPs within *ADIPOQ* gene associated with adiponectin at genome-wide significant levels ($p < 5*10^{-8}$) (C4); B: liberal approach including 17 SNPs across the genome associated with adiponectin at genome-wide significant levels ($p < 5*10^{-8}$) (L17). BMI: body mass index; CI: confidence interval; Hb_{A1c}: glycated hemoglobin; HDL-c: high-density lipoprotein-cholesterol; LDL-c: low-density lipoprotein-cholesterol; SNP: single nucleotide polymorphism; TAG: triacylglicerols; WC: waist circumference.

Figure 4. Mendelian randomization estimates of odds ratio (and 95%CI) of coronary heart disease risk per 1 unit increase in genetically instrumented log adiponectin levels. CHD: coronary heart disease; OR: odds ratio; IVW: inverse variance weighted method; MR-Egger: Mendelian randomization-Egger method; SNP: single nucleotide polymorphism.

Novelty and Significance

What Is Known?

- Adiponectin is a protein produced mainly by mature adipose cells.
- Higher circulating adiponectin levels are associated with lower cardiometabolic risk.
- Genetic variants are associated with both circulating adiponectin and coronary heart disease risk

What New Information Does This Article Contribute?

- Our findings do not support association of circulating adiponectin levels with the risk of coronary heart disease (CHD).
- Genetic variants that are associated with both circulating adiponectin levels and CHD have pleiotropic effects and do not reflect a direct role of circulating adiponectin in CHD development.

Higher circulating adiponectin levels are associated with better cardiometabolic profile; however, it is unknown whether this association is causal or merely correlative due to confounding factors. We used genetic variants associated with circulating adiponectin levels to test whether adiponectin is causally involved in CHD development, a technique known as Mendelian randomization. Overall, our findings do not support a causal effect of adiponectin on CHD risk, indicating that primary perturbation of circulating adiponectin is unlikely to be a major cause of CHD. Interventions targeting total circulating adiponectin might not be appropriate therapeutic strategies for primary CHD prevention.

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Table 1. Characteristics of SNPs selected for each analytical approach

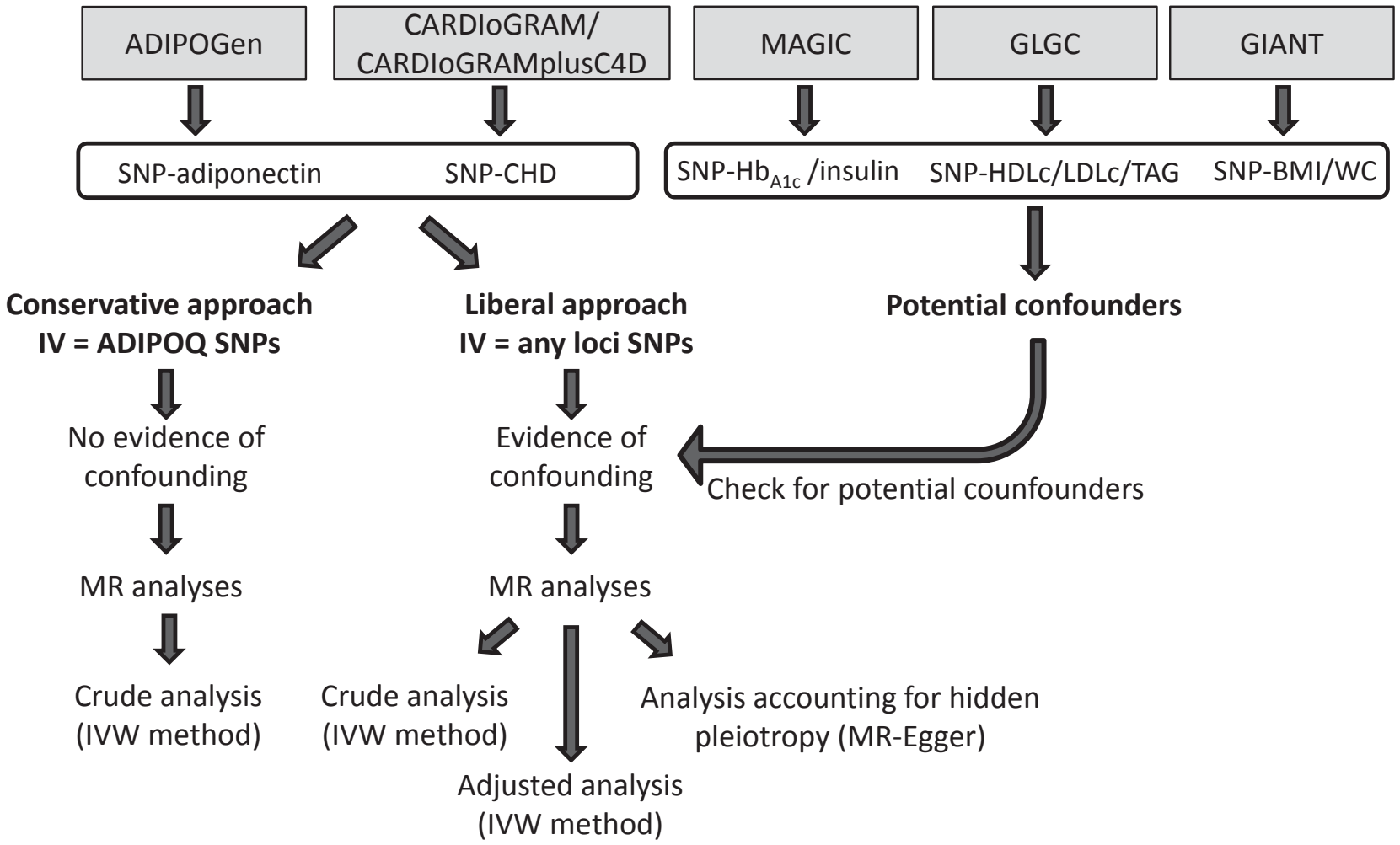
SNP	Chr	Position*	Closest gene	EA	NEA	EAF†	C4	L17
rs1415293	1	219730006	ZC3H11B	T	A	0.25		√
rs1108842	3	52720080	GNL3	C	A	0.49		√
rs6810075	3	186548565	ADIPOQ	T	C	0.61	√	√
rs16861209	3	186563114	ADIPOQ	A	C	0.08	√	√
rs17366568	3	186570453	ADIPOQ-AS1, ADIPOQ	G	A	0.93	√	√
rs3774261	3	186571559	ADIPOQ-AS1, ADIPOQ	A	G	0.50	√	√
rs998584	6	43757896	VEGFA	C	A	0.54		√
rs2980880	8	126480972	TRIB1	A	G	0.71		√
rs7955516	12	20498036	PDE3A	C	A	0.28		√
rs601339	12	123174743	HCAR2	G	A	0.25		√
rs6488898	12	124203832	ATP6V0A2	A	G	0.98		√
rs7978610	12	124468572	ZNF664, FAM101A	C	G	0.27		√
rs2925979	16	81534790	CMIP	C	T	0.71		√
rs7200895	16	82644606	CDH13	T	C	0.69		√
rs8047711	16	82667671	CDH13	G	A	0.92		√
rs12929479	16	82997853	CDH13	G	A	0.42		√
rs731839	19	33899065	PEPD	A	G	0.54		√

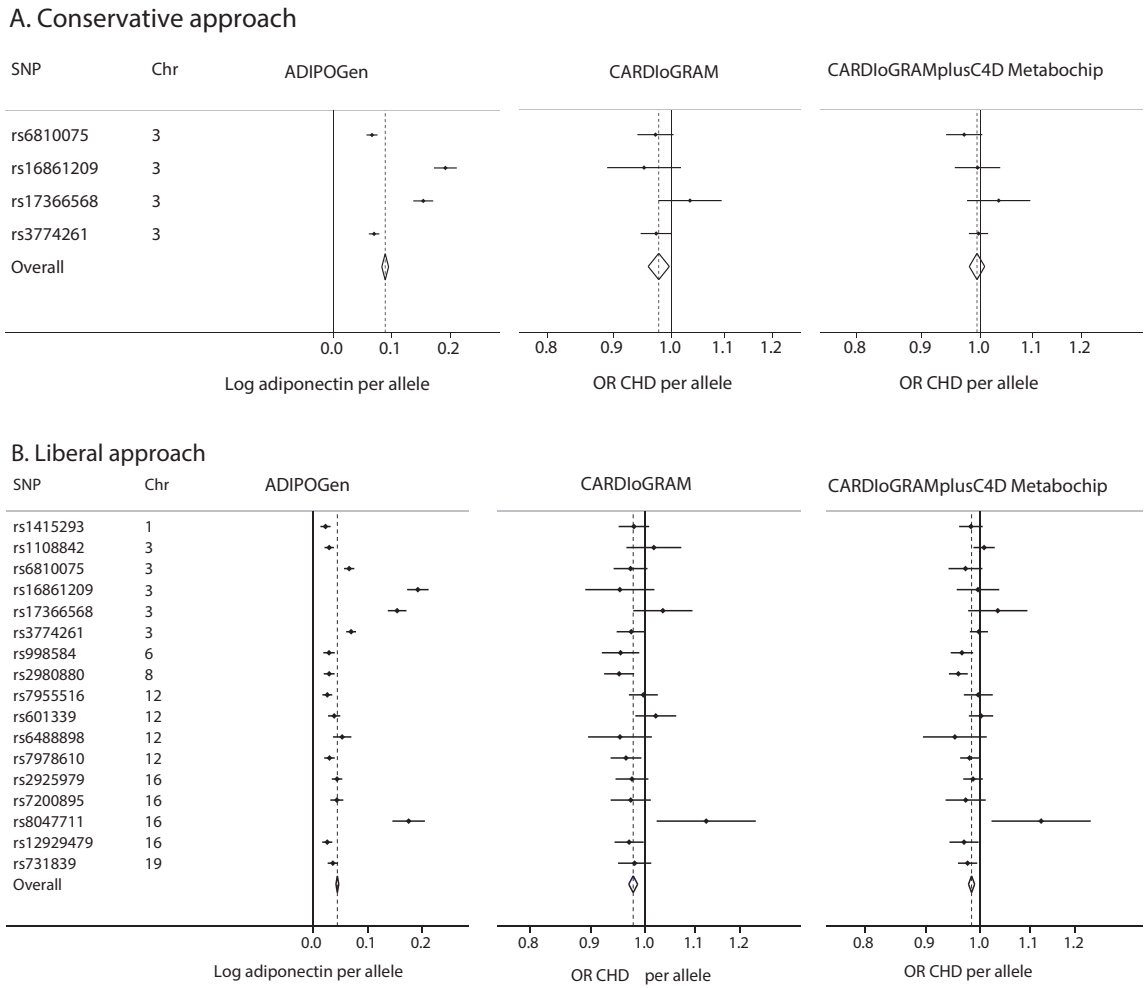
Chr: chromosome; EA: effect allele; NEA: non effect allele; EAF: effect allele frequency: C4: the four SNPs used in the conservative analyses; L17: 17 SNPs used in the liberal analyses (SNPs selected on the basis of reaching genome-wide significant levels in association with adiponectin - $p < 5*10^{-8}$). *Genome Reference Consortium Human Build 37; †1000 Genomes.

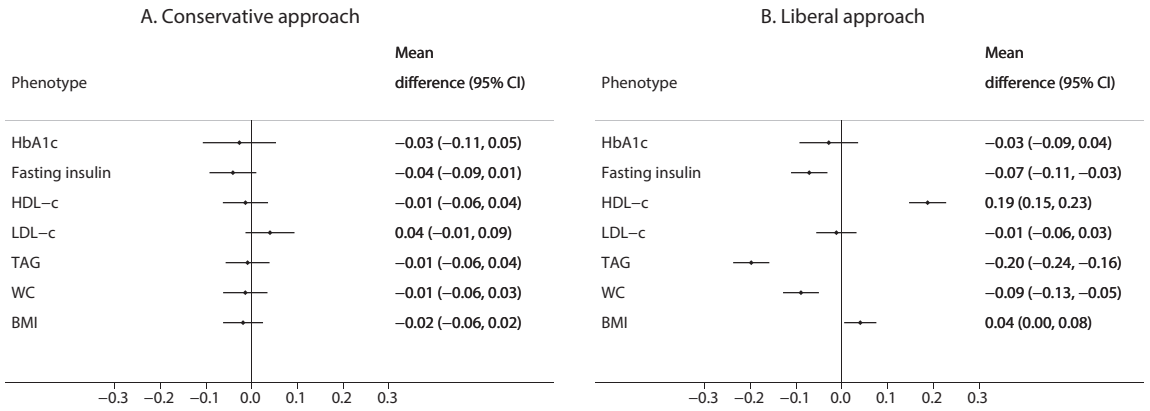
Table 2. Standardized mean difference (and p-values) of cardiovascular risk factors per allele of SNPs used in Mendelian randomization analyses

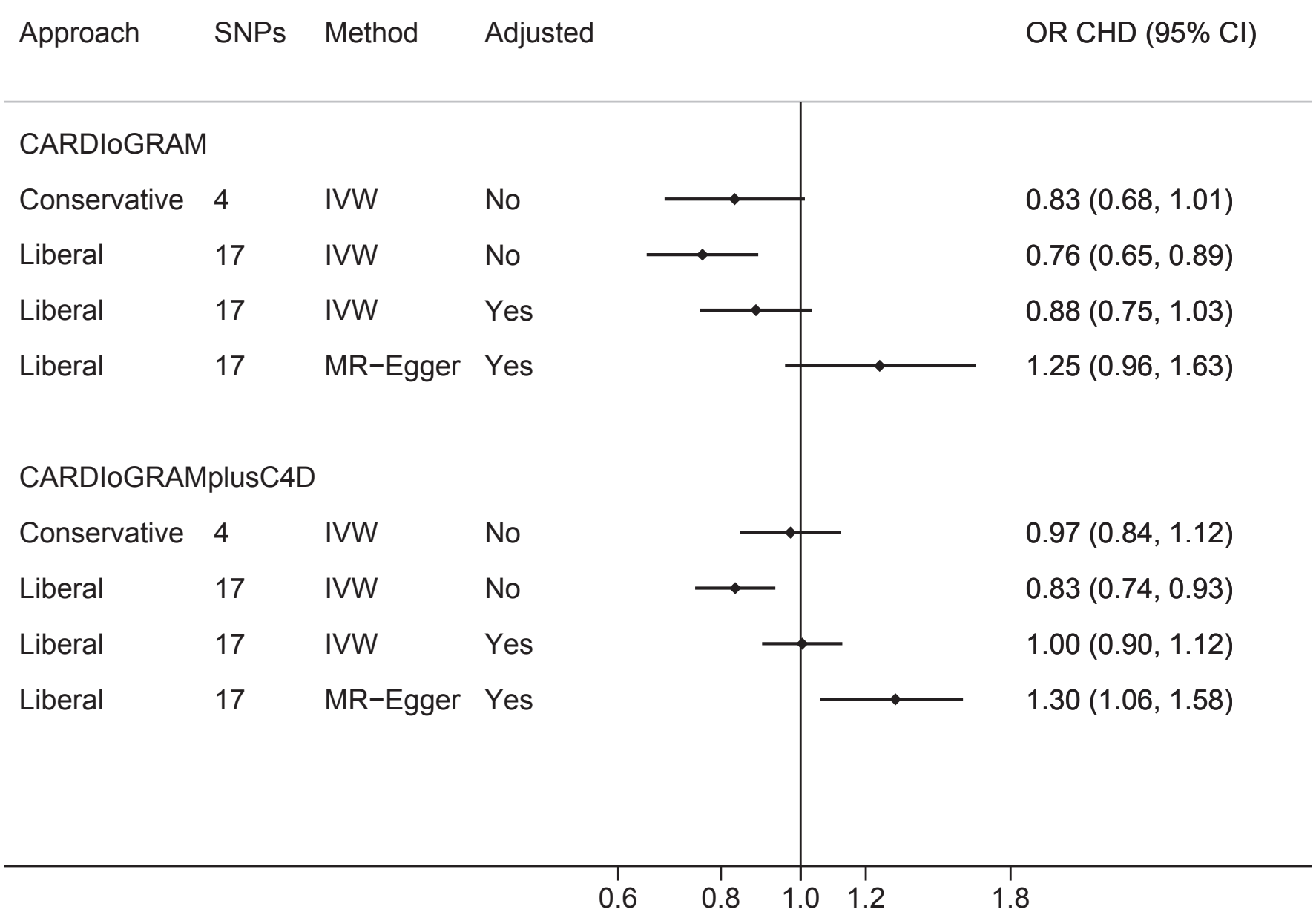
	Hb_{A1c}		Insulin		HDL-c		LDL-c		TAG		WC		BMI	
	β	p	β	p	β	p	β	p	β	p	β	p	β	p
rs1415293	0.004	0.513	-0.016	**$1*10^{-4}$**	0.015	0.009	-0.012	0.063	-0.014	0.019	-0.006	0.200	0.004	0.283
rs1108842	-0.003	0.586	-0.009	0.027	0.008	0.077	0.010	0.024	-0.009	0.037	-0.020	**$2*10^{-8}$**	0.011	0.001
rs6810075*	-0.011	0.123	-0.005	0.214	-0.003	0.813	0.005	0.562	-0.004	0.350	0.003	0.530	-0.001	0.884
rs16861209*	-0.002	0.888	-0.009	0.321	0.000	0.813	0.009	0.245	-0.001	0.610	-0.004	0.570	0.000	0.995
rs17366568*	0.000	0.984	-0.005	0.478	0.009	0.292	0.012	0.343	-0.004	0.587	-0.007	0.400	0.000	1.000
rs3774261*	0.001	0.932	-0.001	0.781	-0.006	0.108	-0.001	0.863	0.001	0.707	0.000	0.910	-0.005	0.100
rs998584	-0.014	0.045	-0.002	0.657	0.026	**$2*10^{-11}$**	-0.001	0.936	-0.029	**$3*10^{-15}$**	-0.029	**$6*10^{-15}$**	0.017	**$9*10^{-7}$**
rs2980880	0.014	0.030	0.000	0.967	0.043	**$1*10^{-26}$**	-0.040	**$6*10^{-22}$**	-0.067	**$2*10^{-82}$**	0.001	0.790	0.007	0.026
rs7955516	-0.013	0.051	0.001	0.910	0.019	0.001	-0.003	0.650	-0.007	0.096	0.006	0.210	0.007	0.069
rs601339	0.005	0.528	-0.011	0.036	0.030	**$3*10^{-6}$**	0.007	0.284	-0.016	0.013	-0.017	0.003	0.004	0.414
rs6488898	0.027	0.050	-0.004	0.642	0.026	0.007	0.016	0.198	-0.023	0.048	-0.007	0.430	0.021	0.005
rs7978610	0.000	0.959	-0.003	0.432	0.032	**$2*10^{-9}$**	-0.020	0.001	-0.029	**$2*10^{-8}$**	-0.021	**$3*10^{-6}$**	0.013	0.002
rs2925979	-0.001	0.853	-0.005	0.236	0.035	**$1*10^{-19}$**	0.003	0.630	-0.021	**$2*10^{-7}$**	-0.011	0.003	0.001	0.721
rs7200895	0.000	0.966	0.005	0.347	0.006	0.278	-0.002	0.985	0.005	0.720	-0.001	0.850	-0.002	0.697
rs8047711	-0.020	0.329	-0.005	0.649	0.010	0.887	-0.011	0.482	-0.001	0.678	-0.006	0.700	0.000	0.982
rs12929479	0.002	0.764	-0.006	0.141	-0.008	0.530	-0.010	0.088	-0.004	0.380	-0.011	0.013	-0.016	**$1*10^{-4}$**
rs731839	-0.007	0.288	-0.011	0.009	0.022	**$3*10^{-9}$**	0.002	0.517	-0.022	**$3*10^{-9}$**	0.007	0.059	0.007	0.038

SNPs within *ADIPOQ* gene (± 50kb) are identified by an asterisk (*). After Bonferroni correction, only p-values lower than $4.2*10^{-4}$ (0.05 ÷ 17 SNPs ÷ 7 phenotypes) were considered statistically significant (values in bold).









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The Role of Adiponectin in Coronary Heart Disease Risk: A Mendelian Randomization Study

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Inverse variance weighted (IVW) method

For unadjusted and adjusted Mendelian randomization analyses, the inverse variance weighted (IVW) method was used to derive the beta coefficient (log odds ratio of CHD per 1 natural log greater adiponectin level) and its standard error by using the following formulas:

$$\hat{\beta}_{\text{IVW}} = \frac{\sum_{k=1}^{K} X_k Y_k \, \sigma_{yk}^{-2}}{\sum_{k=1}^{K} X_k^2 \sigma_{yk}^{-2}} \qquad SE_{\hat{\beta}_{\text{IVW}}} = \sqrt{\frac{1}{\sum_{k=1}^{K} X_k^2 \sigma_{yk}^{-2}}}$$

Where X_k is the mean change in log adiponectin level per additional effect allele of SNP k and Y_k is the mean change in log odds of CHD per additional effect allele of SNP k with standard error σ_{Yk}.

The IVW method was also used to estimate the effect of adiponectin on cardiovascular risk factors (HbA1c, fasting insulin levels, HDL-c, LDL-c, TAG, BMI, and WC) (X_k: mean change in log adiponectin level per additional effect allele of SNP k, Y_k: mean change in the risk factor per additional effect allele of SNP k; σ_{Yk}: standard error of Y_k).

To estimate the association of genetically raised adiponectin and CHD in the model adjusted for cardiovascular risk factors, we used betas for SNP-CHD association as the dependent variable, betas for SNP-adiponectin and SNP-cardiovascular risk factors as independent variables and inverse variance weights (with no intercept). This method is equivalent to IVW method when there is only one independent variable [1].

MR-Egger regression method

The Egger regression has been used for almost two decades to detect small study bias (which may be due to publication bias) in meta-analyses of randomized clinical trials [2]. In this method, the ratio of the effect estimate by its standard error is regressed against the estimate's precision (the inverse of the standard error). Bowden et al. [3] recently proposed an adaptation of the Egger regression to test for bias from horizontal pleiotropy in Mendelian randomization studies.

While the IVW estimate is equivalent to the slope of the best fitting line through the observations that pass through the origin, the MR-Egger estimate would be the best fitting line through the observations in a model that allows the intercept to vary. In this method, the intercept will reflect the average pleiotropic effect across genetic variants (e.g. log odds CHD per allele when difference in adiponectin per allele is zero) and the slope coefficient will provide an estimate of the causal effect provided that the InSIDE (Instrument Strength Independent of Direct Effect) assumption holds, which requires that there is no correlation between SNP-exposure association and direct effects of SNP on outcome.

Bootstrapping (10,000 iterations) was used to derive corrected 95% confidence intervals for MR-Egger intercept and slope using the percentile method [3].

Positive control analysis

The positive control consisted of a Mendelian randomization analysis in which LDL-c was the biomarker of interest and CHD risk was the outcome. 58

SNPs were reported as strongly associated with LDL-c in GLGC consortium [4]. Of there, 38 could be found in both Cardiogram and CARDIoGRAMplusC4D Metabochip dataset and, thus, were used as the instrumental variable for LDL-c. The crude IVW method was used to estimate the association of LDL-c with CHD risk. Since many SNPs were also associated with other lipid traits (ex: HDL-c, TAG and total cholesterol), the MR-Egger method was also used.

Online Tables

Online Table I. Characteristics of the data sources

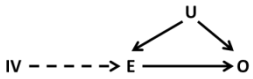
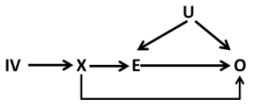
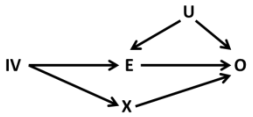
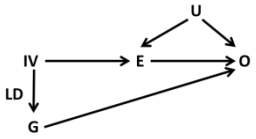
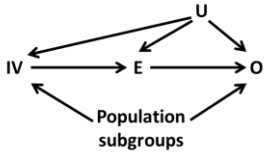
Consortium	Use	Studies	Study population	Imputation	QC criteria†	Model	Adjustments	Data download
ADIPOGen	SNP-log adiponectin	16 cohort studies with GWAS data	29,347 individuals of European ancestry	IMPUTE, MACH, BIMBAM or Beagle (reference: Phase II CEU HapMap population)	Call rate > 0·95; MAF > 0·01; $p_{HWE} > 10^{-6}$; and quality measures for imputed SNPs ($r^2 \geq 0{\cdot}3$, or proper info ≥ 0·4)	additive	Age, sex, BMI, principal components of genomic ancestry, study site (where appropriate), family structure (one family-based study) and genomic control inflation factor (λ)	https://www.mcgill.ca/genepi/adipogen-consortium
CARDIoGRAM§	SNP-log odds CHD	14 case-control or cohort studies with GWAS data	22,233 CHD cases and 64,762 controls of European ancestry	IMPUTE, MACH or BIMBAM (reference: CEU HapMap population)	Sample cal rate > 0·97-0·98; SNP call rate > 0·95-0·99; MAF > 0·01; $p_{HWE} \leq 10^{-3}$- 10^{-6}; ethnic outliers. Quality measures for imputed SNPs: NR*	additive	Age, sex and genomic control inflation factor (λ)	http://www.cardiogramplusc4d.org/downloads/
CARDIoGRAMplus C4D Metabochip§	SNP-log odds CHD	48 case-control or cohort studies with GWAS data	63,746 CHD cases and 130,681 controls of European (~91%) and Asianancestry	NA, Minimac or IMPUTE (reference: HapMap 2/3 or 1000 Genomes Project phase 1)	Sample call rate > 0·98; MAF > 0·01; $p_{HWE} > 10^{-4}$; and other study-specific criteria	additive	Age, sex and genomic control inflation factor (λ)	http://www.cardiogramplusc4d.org/downloads/
MAGIC	SNP-HbA_{1c} (%)	23 cohort studies with GWAS data	35,920 individuals of European ancestry	IMPUTE or MACH (reference: CEU HapMap population)	Sample call rate > 0·95-0·97; SNP call rate > 0·90-0·95; MAF > 0·01; $p_{HWE} > 10^{-4}$-10^{-6}; sex mismatch between genotyped and reported sex; outliers as assessed by population structure analysis; and quality measures for imputed SNPs ($r^2 \geq 0{\cdot}3$, or proper info ≥ 0·4, and MAF > 0·01)	additive	Age, sex, other cohort-specific variables as applicable, and genomic control inflation factor (λ)	http://www.magicinvestigators.org/downloads/
	SNP-log fasting insulin	20 cohort studies with GWAS data	38,238 individuals of European ancestry	IMPUTE, MACH or BIMBAM (reference: CEU HapMap population)	Sample call rate > 0·94-0·99; SNP call rate > 0·90- 0·95; MAF > 0·01-0·05; $p_{HWE} > 10^{-4}$-10^{-7}; and quality measures for imputed SNPs ($r^2 \geq 0{\cdot}3$, proper info ≥ 0·4 or observed/expected variance ratio > 0·3)			
GLGC	SNP-HDLc SNP-LDLc SNP-TAG	60 cohort and case control studies with GWAS or Metabochip data	188,577 European-ancestry individuals	MACH (reference: CEU HapMap population)	Quality control: NR*	additive	Age, sex, principal components of genomic ancestry (some studies), and genomic control inflation factor (λ). Individuals taking lipid-lowering medications were excluded.	http://csg.sph.umich.edu/abecasis/public/lipids2013/
GIANT	SNP- BMI	114 studies of multiple designs with GWAS or Metabochip data	up to 322,154 individuals of European ancestry	IMPUTE, MACH or BIMBAM (reference: Phase II CEU HapMap population)	Sample cal rate > 0·80-0·98; SNP call rate > 0·90-0·99; MAF > 0·01-0·05; $p_{HWE} > 10^{-3}$-10^{-7}; and quality measures for imputed SNPs ($r^2 \geq 0{\cdot}3$, proper info ≥ 0·4, or no filtering)	additive	Age, age2, and study specific variables (e.g. principal components of genomic ancestry), and genomic control inflation factor (λ)	http://www.broadinstitute.org/collaboration/giant/index.php/GIANT_consortium_data_files

Online Table I. Characteristics of the data sources (continued)

SNP-BMI-adjusted WC	101 studies of multiple designs with GWAS or Metabochip data	up to 210,088 individuals of European ancestry	IMPUTE, MACH or Beagle (reference: Phase II CEU HapMap population)	Sample cal rate > 0·85-0·98; SNP call rate > 0·90-0·99; MAF > 0·00-0·01; $p_{HWE} > 10^{-3}$-10^{-7}; and quality measures for imputed SNPs ($r^2 \geq 0{\cdot}3$, proper info ≥ 0·4, or no filtering)	Age, age2, BMI and study specific variables (e.g. principal components of genomic ancestry), and genomic control inflation factor (λ)

† Quality control criteria varied across studies; * NR: not reported in the main consortium publication. § CHD was defined as the presence of coronary artery disease or myocardial infarction. Detailed criteria for CHD definition for each study can be found in the Supplementary material of the main publications [5-7]. QC: quality control; GWAS: genome-wide association study; CEU: Centre d'Etude du Polymorphisme Humain collected in Utah; CHD: coronary heart disease; MAF: minor allele frequency; SNP: single nucleotide polymorphism; BMI: body mass index; WC: waist circumference; NR: not reported; NA: not applicable. CARDIoGRAM: Coronary ARtery DIsease Genome-wide Replication And Meta-analysis; MAGIC: Meta-Analyses of Glucose and Insulin-Related Traits Consortium; GLGC: Global Lipids Genetics Consortium; GIANT: Genetic Investigation of ANthropometric Traits.

Online Table II. Core instrumental variable assumptions and strategies to address them

Assumption	*Graphical examples of assumption violation*[*]	*Consequences of potential violation*	*Validation of assumption in the current analysis*
1. IV should be (strongly) associated with the exposure		A weak association between the IV and E can reduce precision and introduce weak instrument bias, which tends to bias the causal estimate towards the OLS estimate in one-sample MR	- The strength of SNPs-adiponectin association was explored using the F-statistic (F > 20 for every SNP) - In two-sample MR studies with non-overlapping datasets, any bias from weak instruments would be in the direction of the null and, thus, should not result in false positive findings
2. IV should only affect the outcome through the exposure	  	Bias in MR estimate can result from horizontal pleiotropy (e.g. genetic variant itself or a correlated variant is associated with multiple pathways independent of the exposure); the direction and magnitude of this bias will depend on the direction and magnitude of the association path from IV to O that is not via E	Issues of horizontal pleiotropy were addressed by three different strategies: - The association of SNPs with known CHD risk factors was tested. In case of evidence of potential pleiotropy, this was accounted for in the analyses - By comparing the conservative and the liberal approach. In the conservative approach, horizontal pleiotropy is less likely given that variants in the ADIPOQ gene are more plausible valid instrumental variables for adiponectin levels. In the liberal approach, there is an increased likelihood of horizontal pleiotropy but also increased power, since more variants can be selected by this approach - Using methods that account for unknown directional pleiotropy (MR-Egger method)
3. IV should be independent of exposure-outcome confounders		In cases of population stratification, there could be an spurious association between IV and phenotypes	- We cannot test for the absence of exposure-outcome confounders relating to the IV when summary-level data are used, but there is empirical evidence that this is unlikely[8] - To reduce the possibility of bias due to population stratification, the analyses were restricted to only (or predominantly) European-ancestry individuals - All consortia adjusted for genomic control inflation factor

IV: instrumental variable; E: exposure; O: outcome; U: unknown confounder; X: other phenotype: G: other genetic variant in LD; LD: linkage disequilibrium; CHD: coronary heart disease. A dashed arrow was used to indicate weak association between IV and E. [*]Adapted from Vanderweele [9].

Online Table III. Power simulations for the Mendelian randomization analyses

Data source	Sample size	Proportion of cases	Type-I error rate (α)	Original OR	Equivalent standardized OR	R^2_{x-z}	Power
CARDIoGRAM	86,995	25.6%	0.05	0.70	0.80	5%	100%
CARDIoGRAM	86,995	25.6%	0.05	0.80	0.87	5%	97%
CARDIoGRAM	86,995	25.6%	0.05	0.90	0.94	5%	42%
CARDIoGRAMplusC4D Metabochip	194,427	32.8%	0.05	0.70	0.80	5%	100%
CARDIoGRAMplusC4D Metabochip	194,427	32.8%	0.05	0.80	0.87	5%	100%
CARDIoGRAMplusC4D Metabochip	194,427	32.8%	0.05	0.90	0.94	5%	81%

OR: Assumed true odds ratio of CHD risk per standard deviation of the exposure variable

Conversion of original (per log adiponectin) to equivalent standardized OR (per standard unit of log adiponectin) was made using an external source of individual level data (1982 Pelotas Birth Cohort) with similar adiponectin distribution (adiponectin levels in ADIPOGen consortium: mean = 9.8 µg/ml (SD = 5.6); adiponectin levels in 1982 Pelotas Birth Cohort: mean = 9.3 µg/ml (SD = 5.7)).

R^2_{x-z}: proportion of variance explained for the association between the genetic instrument (Z) and adiponectin levels (X). Values approximate findings from Dastani et al [10] and Yaghootkar et al [11].

Calculations were performed in the power calculator for Mendelian Randomization studies, available at http://cnsgenomics.com/shiny/mRnd/, based on the publication by Brion et al 2013 [12].

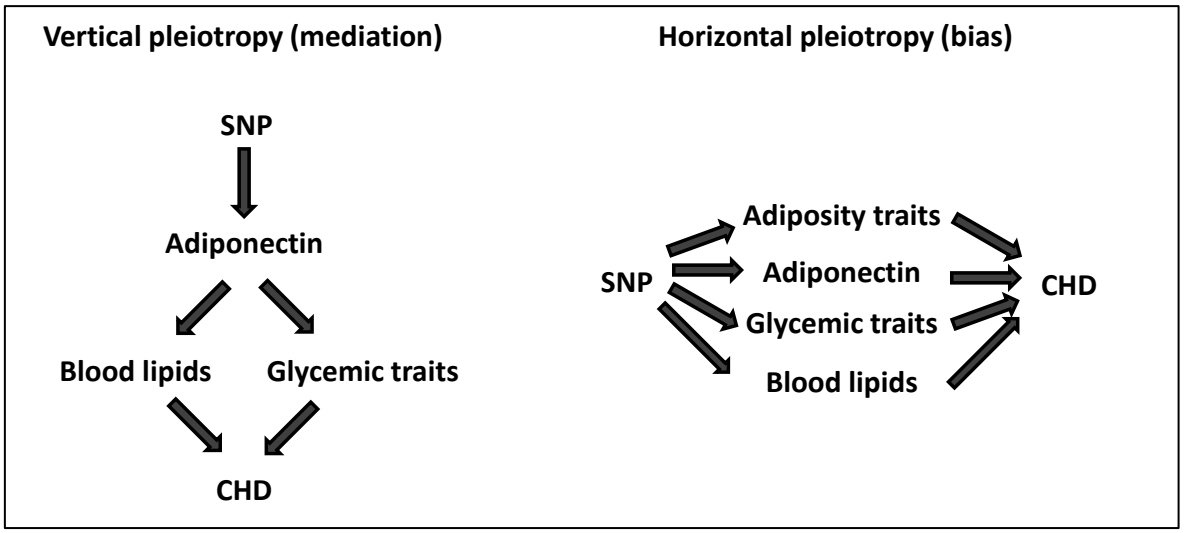
Online Figures

Online Figure I. Graphical illustration of scenarios of (A) vertical pleiotropy (mediation) and (B) horizontal pleiotropy (bias) by CHD risk factors in the relation among SNPs, adiponectin levels and CHD risk. CHD: coronary heart disease; SNP: single nucleotide polymorphism.

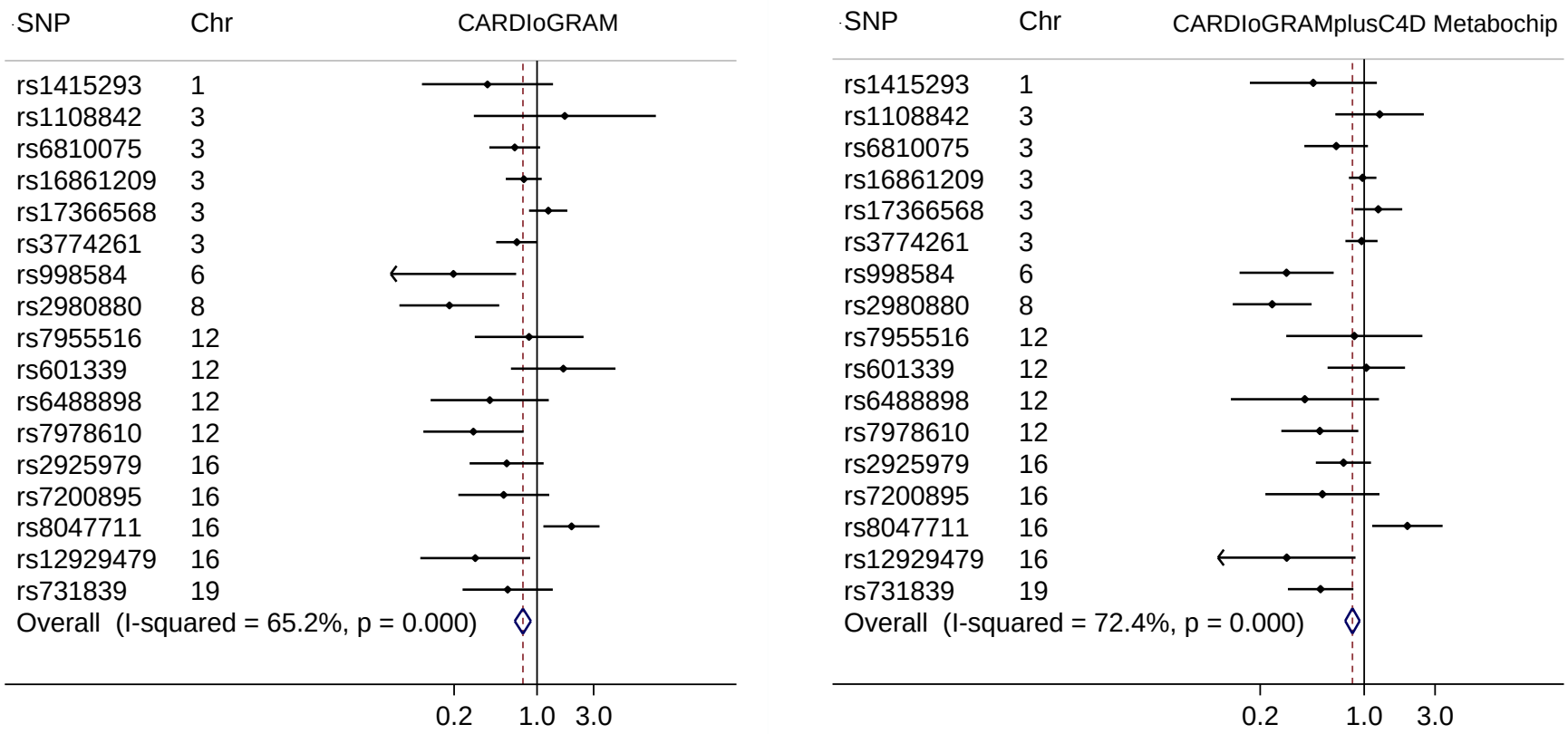
Online Figure II. Meta-analysis and heterogeneity analysis of Mendelian randomization estimates of each SNP for the association of blood adiponectin levels with CHD risk. CHD: coronary heart disease; SNP: single nucleotide polymorphism, Chr: chromosome.

Online Figure III. Pooled odds ratio (and 95%CI) of coronary heart disease risk per unit increase in log adiponectin levels omitting one SNP at a time (influence meta-analysis) estimated by the IVW method and by the MR-Egger method. CHD: coronary heart disease; IVW: inverse-variance weighted method; MR-Egger: Mendelian randomization-Egger method; OR: odds ratio; SNP: single nucleotide polymorphism.

Online Figure IV. Log odds ratio of coronary heart disease and mean increase in log adiponectin levels per adiponectin raising allele in CARDIoGRAM and CARDIoGRAMplusC4D Metabochip. Each data point represents betas for SNP-log OR CHD (Y axis) and SNP-adiponectin (X axis) association (N = 17 SNPs). CHD: coronary heart disease; IVW: inverse-variance weighted method; MR-Egger: Mendelian randomization-Egger method; OR: odds ratio; SNP: single nucleotide polymorphism



Online Figure I.



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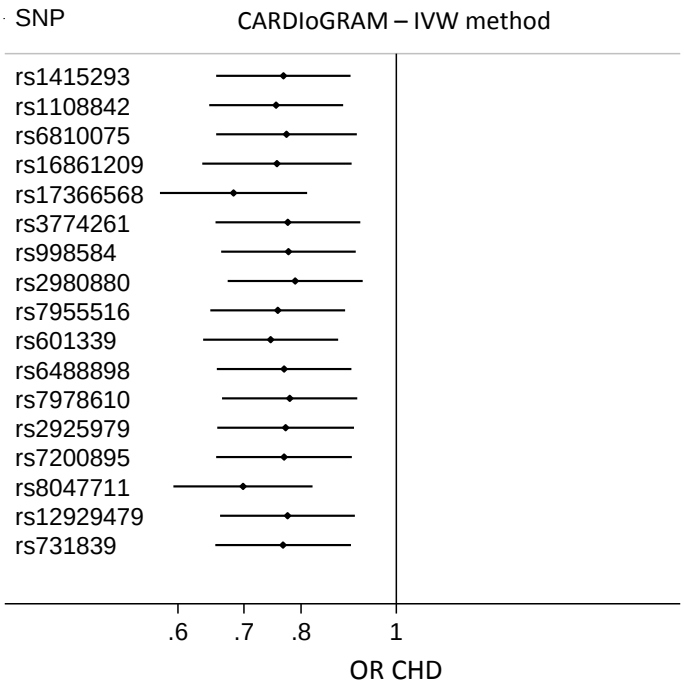
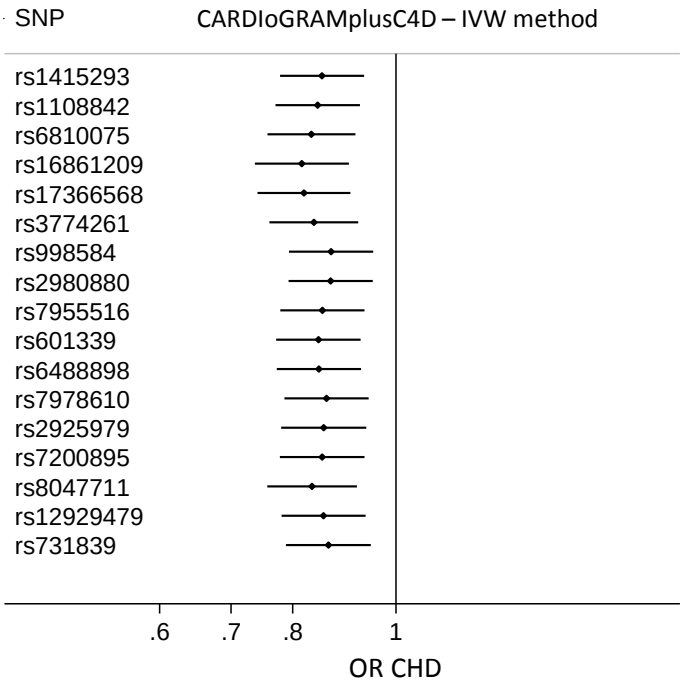
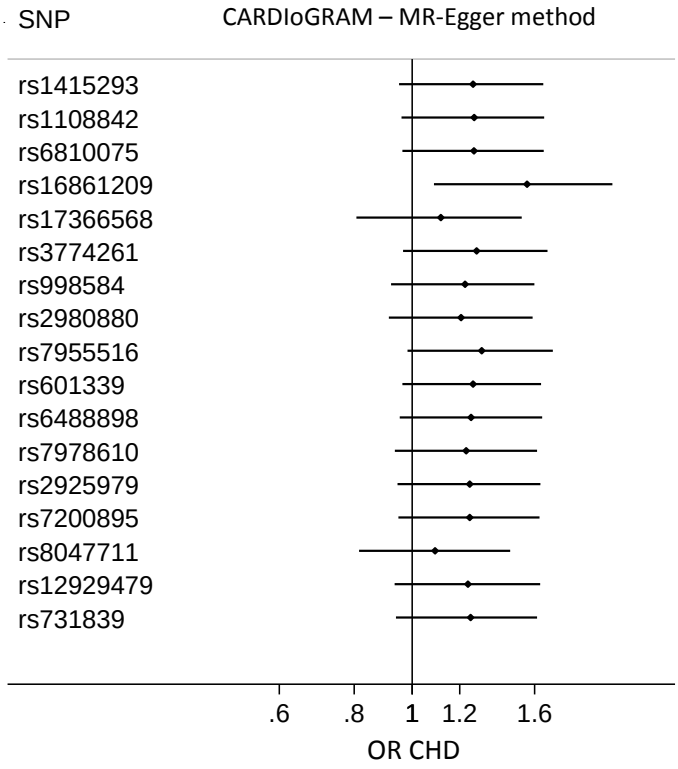
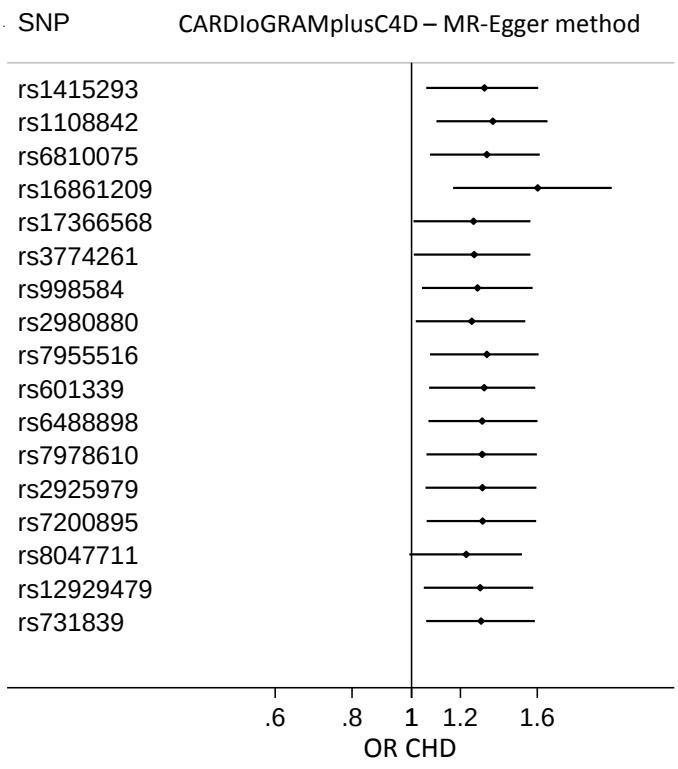
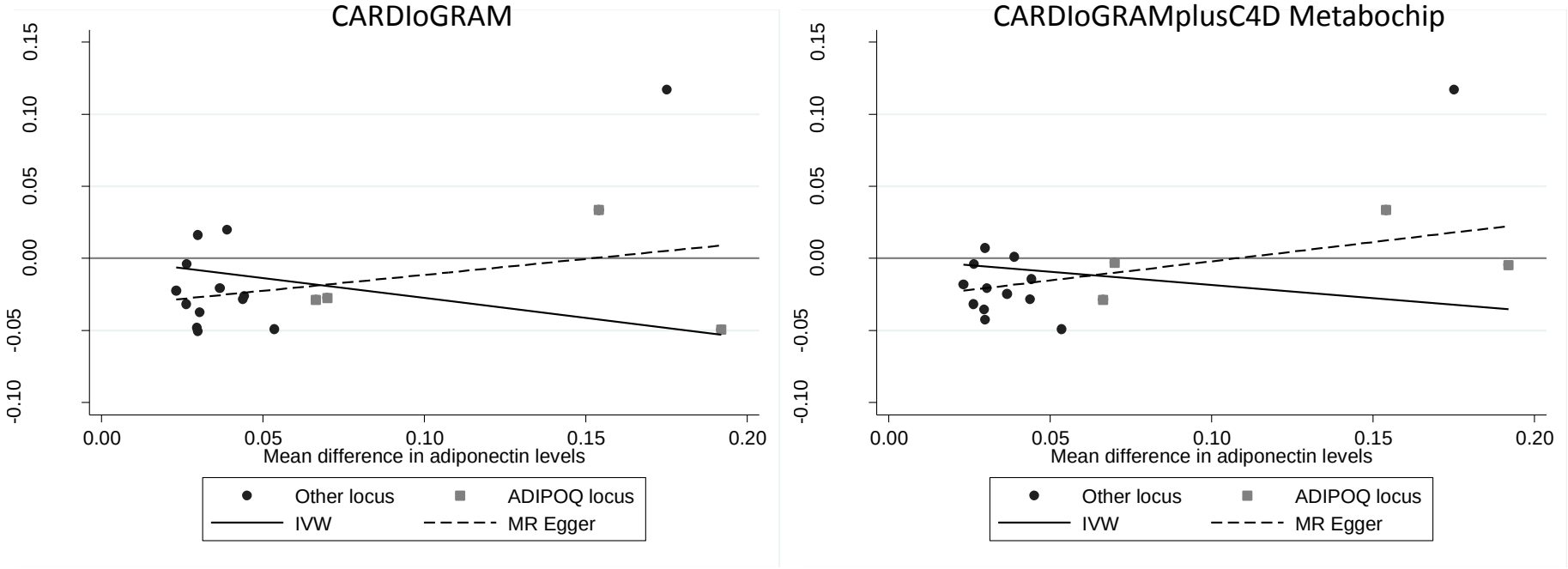








Online Figure III.



Online Figure IV.

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