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Mendelian randomization analyses in cardiometabolic disease: the challenge of rigorous interpretations of causality

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Key points

- Mendelian randomization (MR) is a powerful tool that exploits genetic information to inform on the likely causal relevance of an exposure to an outcome, that should be free from reverse causality and minimizes confounding
- In the past 5-10 years, the number of MR studies appearing each year has increased, providing important new insights into disease aetiology.
- However, as MR studies become more common-place, and as increasingly complex gene-to-exposure and exposure-to-outcome relationships are investigated, the reliable conduct and interpretation of MR analyses can be challenging
- In this review, we highlight scenarios where MR analyses can be non-trivial and in each case, we elaborate on the molecular details and provide what we consider to be correct interpretations
- We conclude by providing some general themes to help guide the MR practitioner and reader and to facilitate robust conduct and interpretation of MR analyses

Abstract

Mendelian randomization (MR) is a burgeoning field that uses genetic variants to assess causal relationships between exposures and outcomes. MR studies can be straightforward, e.g. using genetic variants associated with protein concentrations to assess their causal role in disease. However, a more complex role of genetic variants in relation to an exposure can make findings from MR more difficult to interpret. We illustrate this with examples from recent literature, including using genetic variants: (i) to assess causality of multiple traits (e.g. branched chain amino acids and risk of diabetes); (ii) that are pleiotropic, e.g. for C-reactive protein in assessing its contribution to coronary heart disease (CHD); (iii) that disrupt normal function of an exposure (e.g. high-density lipoprotein cholesterol (HDL-C) or interleukin-6 and CHD); (iv) encoding enzymes responsible for the metabolism and consumption of an exposure (e.g. alcohol and blood pressure); (v) for a potentially time dependent exposure (e.g. extracellular superoxide dismutase and CHD); (vi) for a cumulative exposure (e.g. low-density lipoprotein cholesterol); and, (vii) for overlapping exposures (e.g. triglycerides and non-HDL-C). We elaborate on the molecular details in each and provide explanations for the likely causal relationships. In doing so, we hope to contribute towards more reliable interpretations of MR findings.

Introduction

A *raison d'être* for medical sciences is to understand disease aetiologies to identify opportunities for prevention and treatment. Observational epidemiological studies provide a wealth of information on associations between exposures and outcomes, but they cannot be interpreted as indicating causality, due to limitations introduced by confounding and reverse causality^{1,2}. While randomized controlled trials (RCTs) remain the gold standard study design for inferring causality, they are exceedingly expensive, long-term efforts with high risks (over 50% fail due to lack of efficacy)^{3,4}. RCTs may also use interventions that are pleiotropic (e.g. using drugs that modify multiple biomarkers), which can challenge causal deductions for any individual biomarker. Furthermore, it is not always feasible or ethical to conduct an RCT⁵, for example, in attempting to clarify the causal role of alcohol in cardiovascular disease⁶⁻⁹.

Mendelian randomization (MR)¹⁰⁻¹³ is an established genetic epidemiological approach that can provide information on causality, and prioritize biomarkers for drug target validation¹⁴. By grouping individuals in the population according to the possession of genetic variants that modify an exposure, it is possible to infer whether a biomarker is causally related to a disease (**Figure 1**). This is permissible due to the fundamental nature of the genome: genetic variants should be free from conventional confounding owing to the random independent assortment of DNA at meiotic segregation of alleles; and, reverse causality bias should be abolished due to the essentially non-modifiable nature of the transmitted germline genome. Thus, MR can help to strengthen causal inference regarding the role of modifiable exposures such as circulating biomarkers in the risk of disease. For example, genetic variants that associate with low-density lipoprotein cholesterol (LDL-C) have been used to

assess the causal role of LDL-C in CHD¹⁵⁻¹⁸. Irrespective of the genetic variants used to proxy LDL-C, strong, dose-response relationships have been identified¹⁵. This provides reliable evidence that (i) lowering LDL-C by any means would lead to reductions in CHD; and (ii) recapitulates the linear dose-response relationship between LDL-C and CHD risk identified from meta-analyses of RCTs of statins and other cholesterol lowering interventions¹⁹⁻²¹.

Key findings from MR analyses include: (i) identifying, in the absence of an RCT of a specific CRP-lowering therapeutic, that C-reactive protein is unlikely to play a major role in the development of CHD^{22,23}; (ii) that moderate alcohol consumption probably does not protect from the risk of CHD²⁴ in a scenario in which an RCT would be unfeasible and possibly unethical; (iii) that BMI increases risk of CHD²⁵⁻²⁷ even though the only RCT for this is underpowered²⁸; (iv) concordance of MR analyses of drug targets with findings from RCTs for HMG-CoA reductase^{29,30}, secretory phospholipase A2-IIA (sPLA2-IIA)^{31,32}, lipoprotein-associated phospholipase A2 (Lp-PLa2)³³⁻³⁷ and Niemann-Pick C1-like (NPC1L) protein³⁸, and (v) identifying, before adequate evidence has accrued from clinical trials, that pharmacological inhibition of PCSK9 may lead to increased risk of diabetes³⁹⁻⁴¹. These and other notable examples of MR analyses that have progressed our understanding of the aetiology of cardiovascular and metabolic diseases are outlined in **Table 1**.

As with conventional observational epidemiology and interventional trials, MR analysis can express associations with disease risk in relation to a particular difference in the biomarker that the genetic variants used in the MR study are instruments for. This approach is most robust when a variant is in or near a gene that is responsible for the synthesis of a protein under investigation and associates with concentrations of the same protein (but without

disrupting protein function), for example using genetic variants in the *CRP* gene that associate with concentrations of circulating C-reactive protein²². However, there are various scenarios where the assumptions of MR (see **Figure 1**) become compromised. In the following we discuss some potentially paradoxical interpretations in recent MR analyses.

MR using a genetic variant that associates with multiple biomarkers on separate pathways

In general, a genetic variant used for MR should only affect a single pathway on which the exposure of interest lies (see **Box 1**). When a genetic variant associates with multiple biomarkers on a pathway, through influencing an underlying phenotype, this is referred to as vertical pleiotropy, and the MR approach would be valid. In contrast, if a genetic variant associates with multiple biomarkers on discrete pathways, this is termed horizontal pleiotropy (see **Figure in Box 1** and **Figure 2, Scenario 1**), which can yield invalid causal estimates. We discuss two related examples below. One, where the same genetic variant is used, incorrectly, to assess causal relationships of multiple traits on discrete pathways. A second example relates to the use of pleiotropic SNPs in assessing the causal role of C-reactive protein in CHD, without applying appropriate analytical checks.

***PPM1K*, branched chain amino acids and risk of diabetes**

Observational studies identify that the branched chain amino acids (leucine, isoleucine and valine) associate with risk of incident diabetes⁴²⁻⁴⁷. In a recent genome-wide association study (GWAS) genetic variants associating with these three amino acids were identified and used to conduct MR analyses⁴⁸. The same locus (*PPM1K*) was a GWAS hit for all three amino

acids, and the authors proceeded to use the exact same SNP in isolation (rs1440581 in *PPM1K*) to generate causal estimates for both leucine and valine, and a SNP in the same locus (rs7678928 in *PPM1K*, in linkage disequilibrium (LD) with rs1440581 at $r^2=0.79$ and $D'=1$) in combination with other SNPs for isoleucine. The authors state that “associations of genetic variants appeared highly specific”, generate MR estimates for each of the three amino acids and use this to implicate BCAA metabolism in the development of diabetes.

As shown in **Figure 3A**, the *PPM1K* locus encodes an enzyme (branched-chain α -keto acid dehydrogenase, BCKD) that is responsible for the metabolism of leucine, isoleucine and valine to their derivatives (and therefore, by encoding the enzyme responsible for the metabolism of these three amino acids, SNPs in the *PPM1K* locus associate with concentrations of all 3 amino acids). In MR analyses, the SNP to disease estimate is scaled to the association of the SNP to the exposure to generate a single causal estimate for the exposure to risk of disease (**Figure 1**). When the *same* SNP to disease (in this case *PPM1K* rs1440581, or a SNP in LD rs7678928, to diabetes) association is used to generate MR estimates for each of the three branched-chain amino acids, this ascribes a causal estimate that is scaled to the effect of the SNP on the amino acid (see **Table 2**). Crucially, this makes the invalid assumption that each of the three amino acids *in isolation* would be causal. This is in violation of one of the three principal rules of MR: that the instrument only acts on the outcome through the exposure of interest. Furthermore, the genetic variant association with diabetes is triple-counted, in that the full effect is attributed to 3 different exposures, which is incoherent. Using this single locus, it is not possible to clarify which, if any, of the 3 amino acids is actually driving the causal relationship with diabetes. Thus, while the authors conclude that their findings are “consistent with a causal role of BCAA metabolism in the

aetiology of type 2 diabetes”, a reader may interpret the quantitative MR estimates that are reported for each of the 3 BCAA as providing evidence that each BCAA is individually and independently causally related to T2D, which would be incorrect. All that can be inferred is that the *PPM1K* locus encodes an enzyme (BCKD) that has a range of substrates (which may not be limited to the three amino acids studied), and that one or more of these pathways leads to diabetes. Thus, while we agree with the authors regarding their results being “consistent with a causal role of BCAA metabolism” we feel that the presentation of the data could lead to misinterpretation.

***APOE*, C-reactive protein and risk of CHD**

Naïve use of a genetic variant in *APOE* that associates with circulating levels of C-reactive protein (CRP) may give the impression that CRP would be causal for CHD⁴⁹. However, this is driven by the associations of the *APOE* genotype with multiple biomarkers on discrete pathways, including LDL-C, which is causally related to CAD (**Figure 3B**). Use of such pleiotropic variants in isolation is thereby likely to lead to incorrect causal interpretations.

An alternative approach would be to combine multiple SNPs across the genome into a gene score for CRP⁵⁰. While in some cases, pleiotropy in a gene score may ‘balance out’ (so-called “balanced horizontal pleiotropy”; see **Box 1**), for the CRP gene score, this is not likely to be the case and horizontal pleiotropy could result in biased estimates using conventional MR approaches. Furthermore, when using a multilocus gene score for CRP and removing SNPs on the basis of tests for heterogeneity, this approach can still yield a biased results as multiple SNPs may remain in the instrument that have pleiotropic effects, thus yielding biased causal associations for disease that arise from horizontal pleiotropy when analysed using conventional MR methods (see “unbalanced horizontal pleiotropy”; **Box 1**)⁵¹. A more

optimal approach would be one where SNPs are either confined to within the *CRP* locus (which should show specificity for the protein trait), and/or using SNPs across the genome identified from GWAS in combination with use of more novel approaches such as MR-Egger (described in **Box 2**), which allows relaxation of the instrumental variable assumption that there is no unbalanced horizontal pleiotropy^{52,53}. Our recommendation would therefore be to utilise a range of applicable methodologies, in the spirit of sensitivity analysis.

MR using a variant that disrupts normal function of the exposure

If a SNP disrupts the normal function of an exposure, such as binding of the exposure to its target receptor, and if that exposure plays a causal role in the aetiology of a disease, this can lead to a paradoxical direction in association between the SNP and level of the biomarker in comparison to the direction of association in relation to risk for that disease (**Figure 2, Scenario 2**). A naïve interpretation would be to use the difference in biomarker concentrations from the instrumental variable analysis to infer directionality of causation. However, by perturbing the normal function of the biomarker, the genetic variant can paradoxically lead to the biomarker being linked with a directionally opposite risk of disease in MR analysis as compared to observational epidemiology.

***SCARB1* variants, HDL-C and risk of CHD**

In a recent work⁵⁴, a team of researchers identified a rare variant in *SCARB1* that results in loss of function in scavenger receptor B1 (SR-B1). Individuals carrying this variant had higher levels of circulating HDL-C, and an elevated risk of CHD. The authors suggested that reduced hepatic SR-B1 function in humans causes impaired reverse cholesterol transport, which leads to increased risk of CHD despite elevation in HDL-C levels.⁵⁴ These findings have been widely

reported^{55,56} as HDL-C being harmful with respect to CHD in some circumstances, and interpreted as questioning the protective role that HDL may have in CHD.

The HDL-mediated transport of cholesterol from peripheral tissues to the liver, so-called reverse cholesterol transport^{57,58}, has been expected to lead to a reduction in atheroma burden, and a commensurate reduction in the risk of CHD. However, a critical component of the HDL-mediated reverse cholesterol transport is the selective uptake of circulating HDL particles by the liver. Following hepatic uptake, cholesterol is excreted in bile. This binding to, and uptake of HDL particles into the liver, is principally through SR-B1⁵⁹. Thus, the increased risk of CHD that accompanies a genetic variant that disrupts normal function of SR-B1 potentially provides new evidence that normal function of HDL-mediated reverse cholesterol transport (through SR-B1) may have a role in preventing the development of CHD (**Figure 4A**).

However, to temper enthusiasm, a recent study used whole-genome sequencing to investigate sequence variants within the *SCARB1* locus and identified associations with traits including lipoprotein-phospholipase and vitamin E (meaning there is potential for horizontal pleiotropy; **Box 1**). Furthermore, RCTs of HDL-C raising therapies⁶⁰⁻⁶² and several MR studies¹⁶⁻¹⁸ have not shown benefit for CVD, thus whether HDL metabolism is causal in the aetiology of CHD remains speculative, and studies are now moving to investigate multiple functions of HDL particles (as opposed to only measuring circulating HDL-C concentrations)⁶³⁻⁶⁶. In summary, the association of variants in *SCARB1* with higher HDL-C and higher risk of CHD does not indicate HDL-C being harmful to CHD, but, rather, introduces the possibility

that disruption of normal reverse cholesterol transport may be deleterious to cardiovascular health.

Genetic variants in *IL6R*, interleukin-6 and risk of CHD

Interleukin 6 (IL6) is a pro-inflammatory cytokine produced by stromal and immune cells that circulates in the blood and binds to plasma membrane receptor complexes. There are two signalling mechanisms by which IL6 can exert its biological effect: classical and trans-signalling. Classical signalling is the situation in which IL6 binds to cellular membranes that express both IL6-receptor (IL6R) and glycoprotein-130. Most cells express glycoprotein-130 but only a limited number express IL6R. In the trans-signalling, a soluble form of IL6R binds to circulating IL6 in the blood, and this complex of IL6-IL6R can then bind to any cell expressing glycoprotein-130. Since glycoprotein-130 is ubiquitous, trans-signalling can involve many more cell types than classical signalling. Classical IL6 signalling is thought to have a more prominent role in the development of systemic diseases, whereas trans-IL6 signalling may be more involved in local tissue inflammation⁶⁷.

Using a non-synonymous SNP (rs8192284) in the *IL6R* gene encoding the IL6-receptor, two studies reported that variants associated with increased concentrations of circulating IL-6 related to a reduction in risk of CHD^{68,69}. A naïve interpretation, and one that the authors explain is not the case, would be that IL6 is protective of CHD. However, this would be at odds with prior considerations regarding the aetiology of CHD, since IL-6 is an inflammatory cytokine⁷⁰.

The genetic variant in *IL6R* (rs8192284) increases generation of soluble IL6R (through increased proteolytic cleavage of membrane-bound IL6R^{71,72}). This reduction in membrane-bound IL6R leads to reduced IL-6 mediated classical signalling, resulting in a shift from classical to trans-signalling, and effectively attenuating downstream classical signalling of IL-6. Decreased classical IL6-signalling results in an increase in circulating IL-6 (owing to a reduction in membrane bound IL6R and an increase in circulating IL6-IL6R complex), yet a reduction in CRP (as classical IL6 signalling is impaired), as shown in **Figure 4B**^{73,74}. Of note, the association of SNPs in *IL6R* with CRP concentrations in this scenario reflects vertical pleiotropy (see **Box 1**). While in no way does this indicate that CRP is causal, the association of SNPs in *IL6R* with CRP does not invalidate the use of *IL6R* in MR as, unlike in **Figures 3A** and **3B**, CRP is downstream on the same pathway as IL-6. One particular way to tease this out (i.e. that IL6 is causal and CRP non-causal) would be to use separate genetic instruments for IL6 and CRP, as in MR for mediation⁷⁵.

MR of biomarkers on the same pathway

If a variant associates with multiple traits on the same pathway (vertical pleiotropy; see **Box 1**), and if those biomarkers have differing roles in disease, paradoxical situations can arise (**Figure 2, Scenario 3**).

***ALDH2* genotype, alcohol and risk of hypertension and oesophageal carcinoma**

Alcohol is metabolized in tissues and in the liver by the enzymes alcohol dehydrogenase 1B (ADH1B) and aldehyde dehydrogenase (ALDH2). Metabolism of alcohol by ADH1B yields acetaldehyde, a Group 1 human carcinogen⁷⁶, which is rapidly metabolized by ALDH2 into acetate. Normal function of ADH1B and ALDH2 means that circulating and tissue

concentrations of acetaldehyde are low. However, when ADH1B enzymatic function is increased, or when ALDH2 enzymatic function is impaired, acetaldehyde concentrations rise, resulting in symptoms of flushing, nausea and headache, which is unpleasant to the person. Naturally occurring genetic variation in *ALDH2* (rs671), present in Asians (but not in white Europeans), results in loss of function of ALDH2 in a dose-dependent fashion. Individuals that are homozygous wild-type can consume alcohol normally, those that are heterozygous can still consume some alcohol although they experience symptoms of acetaldehyde toxicity (nausea, flushing), while those that are homozygous for the *2 variant tend to consume almost no alcohol, given the symptoms drinking alcohol causes amongst them.

Alcohol consumption is associated with higher blood pressure⁷⁷. The *ALDH2* *2 variant can be used as a genetic instrument to assess the causal role of alcohol consumption in blood pressure⁷⁸. As each additional carriage of the *ALDH2* *2 allele reduces alcohol consumption in a dose-response relationship (**Figure 5**), a conventional MR using a per-allele genetic model for the *2 allele of *ALDH2* will derive a valid causal estimate²⁴. A particular nuance is that *ALDH2* is monomorphic in Europeans (i.e. individuals are *ALDH2* *1*1) and polymorphic in East Asians. In Asians, women have generally consumed considerably lower amounts of alcohol compared to men, thus stratifying the MR analysis by sex can test one of the fundamental principles of MR, that the genetic instrument is acting through the exposure of interest^{79,80}. Since the genetic variant should only associate with blood pressure in the presence of alcohol, a larger association of the genetic variant on blood pressure should be seen in men vs. women (as men consume more alcohol) and this is precisely what is seen^{78,81}. The interaction between sex and *ALDH2* genotype can be used as the instrumental

variable to estimate the causal effect of alcohol on outcomes, an analysis strategy robust to some pleiotropic violations of the instrumental variables assumption⁸².

However, when investigating the association with oesophageal carcinoma, a different interpretive framework is required. A naïve expectation would be that individuals that drink the most (i.e. those homozygous for the wild-type at *ALDH2* rs671) would have highest risk of oesophageal carcinoma. However, the highest risk is seen in those individuals that carry one copy of the *2 allele⁸³. While it might be tempting to interpret this as moderate drinkers having the highest risk of oesophageal carcinoma (a paradoxical scenario that goes against a dose-response relationship for alcohol), it can be explained by the impact of *ALDH2* on alcohol consumption and circulating concentrations of acetaldehyde (**Figure 5**)⁸⁴.

When examining circulating levels of acetaldehyde by *ALDH2* genotype, individuals that are heterozygous for *ADLH2* rs671 (i.e. *1*2) have the highest concentrations (**Figure 5B**), even though they do not consume the greatest amount of alcohol (that would be *ALDH2* *1 homozygotes, **Figure 5A**), nor do they have the genetic variant that conveys highest concentrations of acetaldehyde for a given amount of alcohol (this would be *ALDH2* *2 homozygotes⁸⁴, **Figure 5C**). The highest absolute concentration of circulating and tissue acetaldehyde by genotype results in an increased risk of oesophageal carcinoma in *1*2 group compared to both *1*1 and *2*2. In this scenario, the genetic variant influences both alcohol consumption and, among alcohol drinkers, acetaldehyde. In the latter case a per-allele analytical model would be inappropriate⁸⁵.

In this example a genetic variant will serve as an exposure measure for different features depending on the outcome under investigation. For blood pressure, which appears not to display a long-term influence from acetaldehyde levels, the variant instruments for alcohol intake. For oesophageal cancer the variant additionally instruments for acetaldehyde among consumers of alcohol. Without good understanding of the biological basis of the effects of the genetic variant, misleading interpretations could be drawn.

MR of a time dependent exposure

If an exposure is time-dependent: e.g. if an exposure is only influential during a period of development (e.g. adolescence), then although MR results may suggest a causal effect, it does not necessarily mean that modification of the exposure in later life will alter the risk of disease (**Figure 2, Scenario 4**).

Vitamin D and multiple sclerosis

A recent MR study points to vitamin D potentially playing a causal role in the aetiology of multiple sclerosis (MS)⁸⁶. However, previous observational studies of migration and risk of MS have consistently identified a time-dependent relationship – i.e. that sunlight exposure (and thus perhaps vitamin D) during early life and not during adulthood associates with the risk of subsequent MS⁸⁷⁻⁹⁰. In this scenario, an MR study utilizing genetic variants associated with circulating levels of vitamin D would yield evidence in support of a protective role of vitamin D in the aetiology of MS (**Figure 6A**). However, modifying vitamin D levels after the critical period would not reduce the risk of multiple sclerosis. If this time critical period were true, RCTs (perhaps identifying people at risk of multiple sclerosis through family history or

genetic risk scores for MS) would need to be commenced in childhood as intervention commenced after this critical period would not be expected to influence MS risk.

Extracellular superoxide dismutase and CHD

There has long been the hypothesis that antioxidants may prevent CHD⁹¹. Evidence from major randomized clinical trials, conducted in adulthood, have been negative^{92,93}.

Extracellular superoxide dismutase (ecSOD) is responsible for protecting nitric oxide released from smooth muscle cells from degradation by the reactive oxygen species superoxide^{94,95}. In preserving the function of nitric oxide, ecSOD facilitates the vasodilatation of arterioles, allowing the maintenance of normotension⁹⁶. Thus ecSOD can be thought of as an endogenous antioxidant that could play an important role in vascular disease.

Studying a genetic variant (R231G) in *ECSOD* that encodes a missense mutation in ecSOD, a large population-based cohort study reported that a genetic variant associated with elevated levels of circulating ecSOD associated with an increase in risk of CHD, contrary to expectations⁹⁷. The genetic variant resulted in an alteration in the heparin-binding domain of ecSOD, and the normal structure of this ecSOD domain is important for ecSOD to bind to the external membrane of endothelial cells (**Figure 6B**). As a result, in the presence of this variant, ecSOD plasma levels increase, yet ecSOD is unable to effect its role to protect nitric oxide from degradation by superoxide anions⁹⁵. Reduced bioavailability of nitric oxide can lead to hypertension and thereby increase risk of CHD. This is therefore another example of paradoxical concentrations of the biomarker (ecSOD) in relation to its purported role in

disease development and findings from the genetic study, showing that disrupted function of ecSOD leads to increased CHD risk, supports the protective role of antioxidants in CHD.

Interestingly, other studies have shown that the same genetic variant (associated with higher CHD risk) is associated with a reduction in risk from lung disease⁹⁸⁻¹⁰⁰, although another study casts this into doubt¹⁰¹. However, this can be explained by animal studies that used knock in mice¹⁰² to show that the R231G SNP in *ECSOD* results in reduced ecSOD in blood vessels and increased ecSOD in alveolar fluids, thus resulting in detrimental effects to vascular disease, and beneficial effects for lung disease. However, it may be that antioxidants are only important at critical times in vascular disease (unlike LDL-cholesterol, which we discuss later). A critical time effect of antioxidants in vascular disease is in keeping with other MR studies that have suggested that vitamin-C may play a causal role in CHD development¹⁰³ and could explain the possible discrepancy between such MR studies and the null findings from randomized controlled trials of vitamin C supplementation and risk of vascular disease in later life¹⁰⁴. Similar issues could apply with respect to other antioxidants, such as beta carotene, which MR studies suggest does not protect against diabetes, in contradistinction to observational studies^{105,106}. With respect to CHD a similar disjunction between observational studies and RCTs has been seen^{92,107}, which could arise from a causal effect operational only during a critical developmental period.

MR of a cumulative exposure

If an exposure causes disease over decades, MR may result in a causal estimate that is larger than that from a randomized controlled trial (which will only alter exposure for a limited period) or from observational epidemiology (which will generally only capture exposure for

particular periods). The MR finding should generally be interpreted to reflect a lifelong exposure to a biomarker (**Figure 2, Scenario 5**), although this varies if an exposure only occurs after a certain age (e.g. with alcohol or smoking, in which case the genetic instrument only influences exposure from after the habit has been taken up)¹¹. Furthermore, for biomarkers, it is important to demonstrate that the SNP does associate with the biomarker across the lifetime, to allow appropriate interpretation to be made.

LDL-C and the risk of CHD

MR studies using multiple independent genetic loci influencing concentrations of LDL-C identify that a 1 mmol/l lower LDL-C results in a >50% reduction in the risk of CHD³⁸. This is approximately double the effect estimate reported in RCTs for a similar reduction in LDL-C (25% reduction in the risk of major coronary event per 1 mmol/l reduction by statins)¹⁰⁸. As such, this magnitude of effect from MR could be considered exaggerated¹⁰⁹. However, the causal estimate from MR depicts lifelong exposure to a harmful trait (LDL-C) (**Figure 6C**). Given that atherosclerosis is a disease that accumulates over a lifetime^{110,111} and results in clinical symptoms of CHD typically at older ages¹¹², genetic variants provide an insight into expected effect sizes if we were to intervene from childhood to reduce the levels of circulating LDL-C. Thus, effect sizes from MR analyses should not generally be considered equivalent to those from an RCT of a short-term intervention. Differences in estimates from MR and RCTs can thus be informative about disease latency periods.

MR of overlapping exposures

Finally, emerging MR approaches include combining multiple traits and genetic instruments into one model to try to tease out independent causal effects (so-called multivariate MR¹¹³).

When multivariate MR for discrete traits (e.g. BMI and blood pressure in relation to cardiovascular disease) holds its own challenges, such as collider bias (where conditioning on a mediator between the exposure and outcome can induce new confounding)^{114,115}, the situation becomes more complex if the traits themselves overlap (**Figure 2, Scenario 6**), i.e. they contain the same element in their total value.

Non-HDL-C and triglycerides

In a recent study gene scores for non-HDL-C and triglycerides (TGs) were used in order to try and tease out whether TGs have an independent causal role in CHD¹¹⁶. The authors suggested that while LDL-C (calculated using the Friedewald equation¹¹⁷) does not include cholesterol from triglyceride-rich lipoproteins (TRLs), mostly consisting of very-low-density and intermediate-density lipoproteins (VLDL and IDL, respectively), non-HDL-C does. However, estimation of LDL-C via the Friedewald equation also includes IDL-C¹¹⁸. Since IDL particles are semi-enriched in TG, LDL-C estimated via the Friedewald equation also contains TRL-related cholesterol. A multivariate MR analysis including both non-HDL-C and TG showed that while the association of non-HDL-C with risk of CHD remained largely unaltered, the association of TG with CHD, after adjusting for non-HDL-C, diminished to null¹¹⁶.

It is important to realise here that non-HDL-C and TGs are not discrete entities (**Figure 7**) but overlap. Hence, adjustment of the TG instrument for non-HDL-C adjusts for overlapping components, and the diminution of the TG score is not interpretable as meaning that TG has no causal effect (as in effect the analysis has adjusted for a component of that trait). In contrast, while non-HDL-C contains cholesterol in TRLs, it also contains LDL-C (which has a

TG-independent effect on CHD¹⁸). Thus, a lack of diminution of the non-HDL-C gene score with risk of CHD after adjustment for TG provides no additional information beyond what is understood about the causal role of LDL-C in CHD and is not useful in assessing the causality of TGs in CHD. In contrast, MR for correlated, but non-overlapping traits can be highly informative e.g. separate genetic instruments for CRP and IL-6 show that while IL-6 up regulates CRP¹⁹, causality for CHD is limited to IL6^{22,23,49,68,69}.

Potential solutions for rigorous interpretations of MR analyses

While each case we have highlighted above is unique, they fit into general themes, each of which has potential solutions to aid interpretation. First, when a single genetic variant associates with multiple traits on distinct pathways (i.e. horizontal pleiotropy), it is invalid to use this individual genetic variant to generate causal estimates for each individual trait as it makes assumptions that each trait alone accounts for the causal effect. Furthermore, ascribing causal effects when using a single genetic variant to instrument a complex phenotype (such as *FTO* for BMI¹²⁰) should be undertaken cautiously owing to the high likelihood of (horizontal) pleiotropy (see **Box 1**). This is especially true for non-protein (complex) traits as no single genetic variant will account for the exposure under investigation.

When an MR analysis gives associations that are directionally opposite to the observational epidemiology, investigators should consider whether the genetic variants used in the instrument disrupts the normal function of an exposure. If so, the association may arise due to the biomarker not being able to exert its normal biological effect, and levels sometimes

being elevated despite this lower functional effect. An alternative explanation here may be negative bias due to unbalanced horizontal pleiotropy (**Box 1**). In contrast when the magnitude of effect from MR is directionally consistent but larger in magnitude to the observational literature, this may be due to cumulative exposure (since the genetic variant proxies a lifetime exposure). Alternative explanations include measurement error in the observational analysis (leading to regression dilution bias; which the MR analysis is protected from) and/or a positive bias induced by horizontal pleiotropy (**Box 1**).

In these examples, pleiotropy can seriously perturb estimates derived from MR. Uncovering the presence of horizontal pleiotropy when using a single genetic variant is challenging unless there is detailed knowledge about its function and/or access to large cohorts where a phenome-wide association scan can reveal associations that may be indicative of unknown pleiotropy. In contrast, when multiple SNPs are used in combination as genetic instruments, approaches now exist (such as MR-Egger) to quantify and assess the presence of pleiotropy, and indeed can provide valid causal estimates even in the presence of pleiotropy (although additional assumptions are required)^{52,53,113,121}. These tests for pleiotropy should be employed as sensitivity analyses in addition to conventional MR approaches.

When an MR study provides evidence of causality that has not been recapitulated in randomized controlled trials, it may be that the biomarker is only causal during a particular time period of life. Thus, the evidence obtained from MR may not translate into equivalent benefit if the intervention to modify the biomarker is at a different period of the life course than when it has its causal impact.

Finally, when assessing multiple traits in combination, if the traits are overlapping (i.e. contain elements of each other in their individual measures) then a multivariate MR may not allow reliable individual assessment of which trait is causal: in this scenario, MR suffers the same issues as conventional observational epidemiology whereby adjusting for an overlapping trait diminishes associations of traits in the model with risk of disease.

Conclusion

Here we sought to illustrate and provide explanations for potentially paradoxical and implausible findings from MR analyses. As MR studies are increasingly conducted to clarify causal relationships of the expanding number of traits that are measurable (e.g. “-omics” including metabolomics^{43,122}, lipidomics¹²³, proteomics¹²⁴ and others), these scenarios are likely to become more commonplace, highlighting the need for careful application and critical appraisal of MR findings. Indeed, as the relative ease of performing two-sample MR studies utilising readily available data increases, it is probable that the reliability of studies will decrease, through both methodological errors and through publication bias influencing which results are deemed “interesting”¹²⁵. Despite these caveats, with increasing large-scale genetic data becoming available to facilitate two-sample MR¹²⁶, together with resources such as MR-Base¹²⁷, LD Hub¹²⁸ and PhenoScanner¹²⁹, MR promises to provide an efficient and pragmatic means to identifying traits that are causal in cardiometabolic and other diseases, and to help prioritize drug targets to take forward into therapeutic clinical trials. Such drug target prioritization may avoid late stage failure of multi-billion dollar clinical trials and offers optimism to revamping the flailing drug development pipeline.

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Conflicts of interest

The authors do not report any disclosures.

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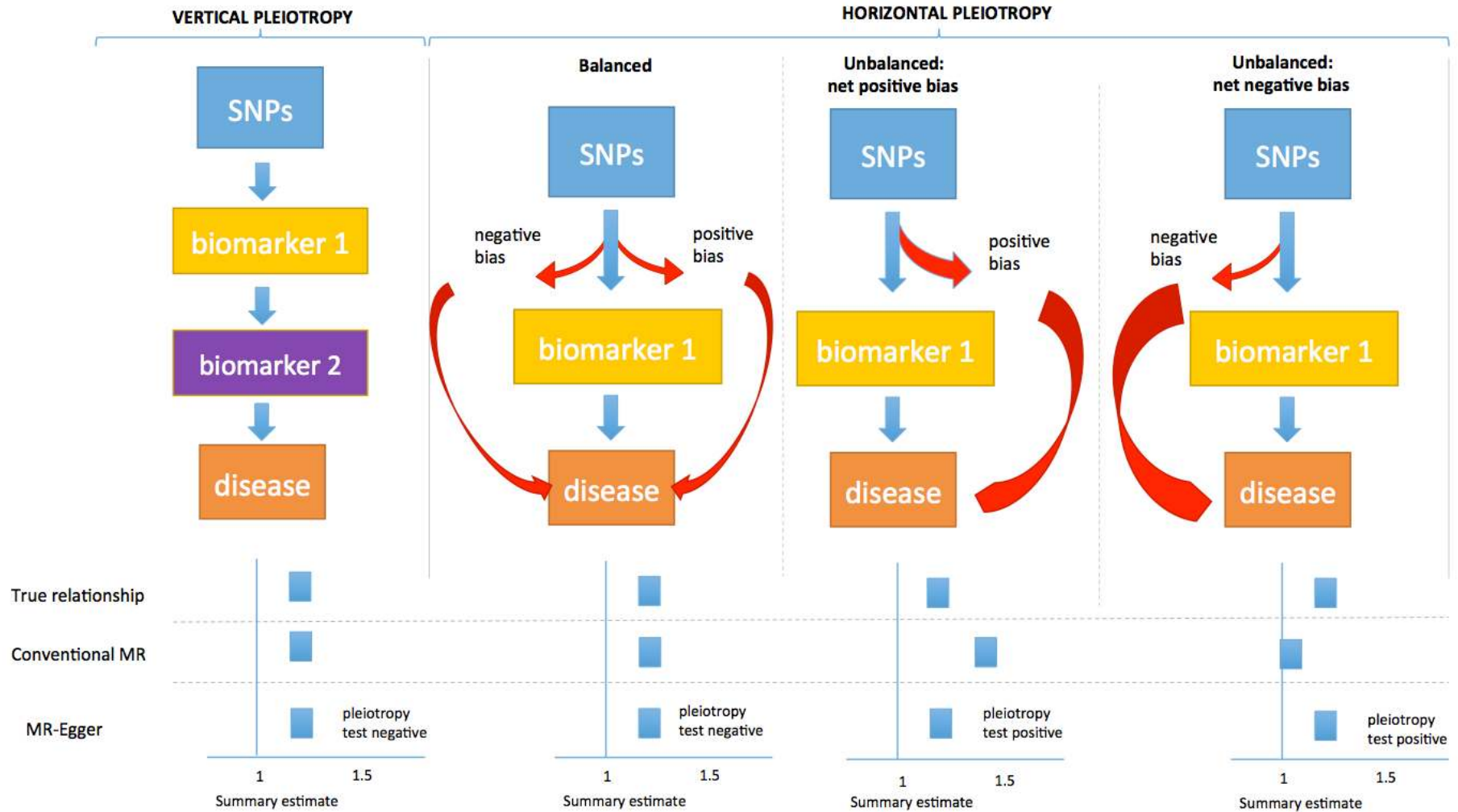
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Boxes

Box 1. Pleiotropy in MR and implications for causal deduction: Single nucleotide polymorphisms (SNPs) can be used in isolation or combination as genetic instruments to assess the causal role of an exposure or biomarker with risk of disease. When genetic variants associate with multiple biomarkers that are on the same pathway, this is termed vertical pleiotropy and it does not invalidate the findings from MR. Vertical pleiotropy is also known as ‘mediated’, ‘spurious’ and ‘type II’ pleiotropy¹⁰. In contrast, when a genetic variant (or genetic variants in combination) associate with traits on discrete pathways that are also causal in disease, this is termed horizontal pleiotropy. Alternative names for horizontal pleiotropy include ‘directional’, ‘type I’ and ‘biological’. When using multiple genetic variants in combination, horizontal pleiotropy of multiple variants can ‘balance out’ and have no net effect on the association of the exposure under investigation and risk of disease; this is termed ‘balanced horizontal pleiotropy’, and does not bias the causal effect derived from MR, even when using conventional MR approaches, such as inverse-variance weighted MR, although it does lead to increased variance in the effect estimation, and thus less precise confidence intervals. However, when conducting an MR using a single variant or multiple variants in combination, if horizontal pleiotropy distorts the association between the exposure and outcome, it is termed ‘unbalanced’. In unbalanced horizontal pleiotropy, the effect estimate from conventional MR approaches can be exaggerated or diminished, depending on the direction of the pleiotropy. E.g. if the genetic variant(s) associates with pleiotropic pathways that are positively associated with disease risk, this will exaggerate the causal association of the biomarker and risk of disease from MR analyses. In contrast, if the genetic variant(s) associates with pathways that are negatively associated with disease risk, this will diminish the MR estimate of the biomarker and risk of disease. For example, when

using variants in *APOE* as an instrument for C-reactive protein, the association of *APOE* with LDL-C results in unbalanced horizontal pleiotropy (as LDL-C causes CHD). In contrast, in using *APOE* as an instrument for LDL-C, the association of *APOE* with CRP does not result in horizontal pleiotropy as CRP is non-causal in the aetiology of CHD^{22,23,49}. Presence of unbalanced horizontal pleiotropy can be formally assessed through use of MR-Egger⁵² (if certain assumptions are satisfied) and furthermore, MR-Egger provides a valid MR estimate that takes into account presence of unbalanced horizontal pleiotropy (see Figure in Box). Other approaches include median and weighted median MR⁵³, which provide a valid MR estimates as long as the majority of SNPs (or the majority of the statistical weight contributed by the SNPs) in the instrument are valid. Each of these approaches (inverse-variance weighted MR, MR-Egger and weighted median MR) has their own assumptions, which are described further in⁵³ and, when possible, investigators should run MR-Egger, weighted median MR and other forms of sensitivity analyses when conducting conventional (inverse-variance weighted) MR analyses.

Figure to accompany Box 1

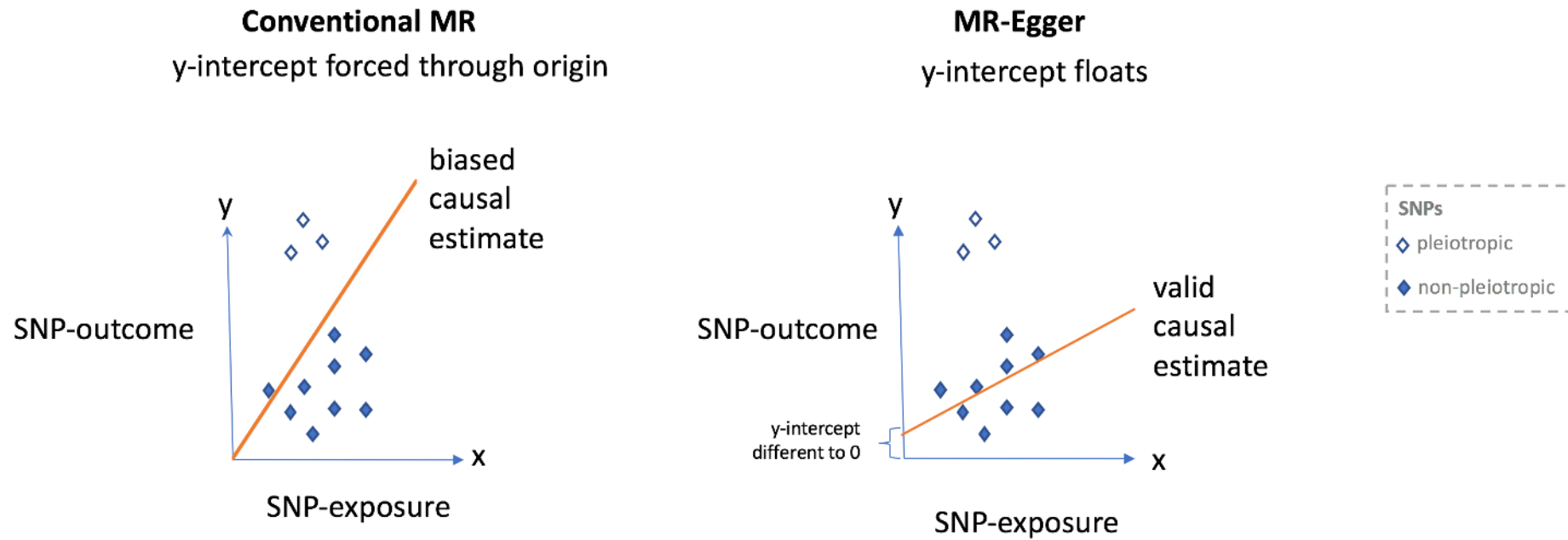


Box 2. Conventional and new approaches to MR: When using multiple SNPs for MR in summary-level data, a conventional (inverse variance weighted) analysis up till recently was the state of the art. However, this approach forces the y-intercept through the origin. In the context of no unbalanced pleiotropy, this should not lead to bias and the slope of the regression line can be reliably interpreted as the causal effect of the exposure on the outcome. However, in the context of unbalanced horizontal pleiotropy, conventional MR analysis can lead to bias as the y-intercept, being forced through the origin, means that the directional bias influences the regression slope (see Figure to Box 2, left panel). To overcome this issue, investigators relied on approaches such as ‘manual pruning’ of SNPs that they considered to be pleiotropic. However, this approach is suboptimal as: (i) it relies on availability and precision of SNP to trait estimates to inform on presence of such pleiotropy (e.g. many SNP associations that do not meet conventional significance thresholds in GWAS are false negatives owing to the stringent alpha values used to avoid false positives^{130,131}); (ii) it is subjective as one investigator may consider a trait to represent vertical pleiotropy (i.e. part of the pathway from exposure through to disease) and another investigator could consider the same trait to be evidence of horizontal pleiotropy (i.e. on a pathway distinct to that of the exposure); (iii) reasons for exclusion can be non-transparent and differ by study, thus removing objectivity from the study; (iv) while use of all SNPs identified from a GWAS of a trait is collectively informative and meaningful on the underlying genetic architecture of a trait, such manual pruning can lead to a genetic instrument that is no longer biologically meaningful of the exposure (i.e. the SNPs that are retained in the instrument may not be informative of any tangible entity).

In contrast to conventional MR, MR-Egger⁵² takes the approach of Egger regression¹³² (in the context of small study bias evaluation in clinical trials) and allows the y-intercept to

float. This flotation of the y-intercept does two things. First, it provides a statistical test for presence of unbalanced horizontal pleiotropy (when there is evidence that y is different to 0 when $x=0$, this suggests presence of unbalanced horizontal pleiotropy). Second, by absorbing the pleiotropic effects into the y-intercept, MR-Egger can provide a reliable estimate for the underlying causal effect (when certain additional assumptions are satisfied) from the slope of the regression line (see Figure to Box 2, right panel). Thus, the advantages of MR-Egger are manifold as it obviates the need for manual pruning of SNPs (that can be subjective), the intercept can inform on presence of unbalanced pleiotropy and the slope can provide a valid causal estimate even in the presence of such pleiotropy. The disadvantages are that for a given sample size, power in MR-Egger is reduced (compared to inverse variance weighted MR), although newer extensions to MR-Egger, such as MR-Egger with SIMEX¹³³, seek to increase power.

Figure to accompany Box 2



Legend: Each diamond represents a single SNP plotted so that the SNP to exposure estimate is on the x-axis and the SNP to outcome estimate is on the y-axis. Filled diamonds = non-pleiotropic variants and open diamonds = pleiotropic variants. In MR using summary level data, the regression slope provides an estimation of the causal effect of the exposure on the outcome.

Table 1: Notable Mendelian randomization studies in cardiometabolic disease

Exposure	Outcome	Interpretation	Importance	Refs
<i>Biomarkers and drug targets</i>				
BMI	Metabolites	BMI causally influences many circulating metabolites	Supports the interpretation that BMI may influence cardiometabolic disease through its influence on metabolites	134
<i>HMGCR/</i> Statins	Metabolites	Casual	Shows consistency of observational data on statins vs predicted MR effects on metabolites	29
BMI	CHD	BMI causally increases risk of CHD	No trial yet to show this ²⁸	25-27
C-reactive protein	CHD	No causal relationship	No trial of a therapy specific to CRP for CVD events has been conducted	22,23,49
LDL-C	CHD	Dose-response relationship irrespective of locus	Suggests LDL-C lowering by many means beneficial, consistent with statin and other cholesterol lowering trials ^{19,21,108}	15
HDL-C	CHD	No causal effect	Counter to observational data ¹³⁵ but supportive of RCTs ⁶⁰⁻⁶²	16-18
TGs	CHD	Causal	Precedes trial data of a TG-lowering agent	16,18,136
sPLA2-IIA	CHD	Non-causal	Published at a similar time to	31

			negative VISTA-16 trial ³²	
Lp-PLA-IIA	CHD	Non-causal	\$bn spent on trials that showed therapeutic lowering of Lp-PLA-IIA does not lower risk of CVD ^{34,61} ; some MR studies were published prior to RCTs	33,35,36,137
<i>NPC1L1</i> / Ezetimibe	CHD	Causal	Preceded trial data that showed lowering of LDL-C via inhibition of NPC1L1 results in reduced risk of CVD ¹³⁸	38,139
PCSK9, Lipoprotein (a) and ANGPTL4	CHD	Causal	Causal. Drugs developed for CVD prevention on basis of genetic findings	140-142
LDL-C	Diabetes	Causal	Suggests LDL-C lowering in general may lead to increased risk of diabetes and has ramifications for drugs that lower LDL-C	18
<i>HMGCR</i> / Statins	Diabetes	Causal	Indicates that the diabetogenic effects of statins seen in RCTs ¹⁴³ are on-target	30
PCSK9	Diabetes	Causal	Suggests PCSK9 inhibition may increase risk of diabetes	39-41
<i>Exogenous exposures</i>				

Alcohol	Cardiovascular diseases (including blood pressure, coronary artery calcification and CHD)	Causal	Suggests alcohol is harmful to cardiovascular health at all doses of consumption, contrary to decades of observational data ⁶ & important for public health policy ¹⁴⁴	24,78,85,145
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Table 2. Mendelian randomization of discrete biomarkers when using identical or highly correlated genetic variants.

Branched chain amino acid (BCAA)	SNP in <i>PPM1K</i>	Linkage disequilibrium with rs1440581	Effect/other allele	EAF	Beta (SE) of Metabolite Level per Allele	P-value for BCAA	OR (95% CI) for Type 2 Diabetes per Allele	P-value for T2D	MR estimate of diabetes per 1-SD higher BCAA	P-value for MR estimate
Leucine	rs1440581	1.0	C/T	53%	0.08 (0.013)	3.9×10^{-25}	1.04 (1.02–1.07)	0.00034	1.85 (1.41–2.42)	7.3×10^{-6}
Isoleucine	rs7678928	0.8	T/C	46%	0.09 (0.013)	5.6×10^{-19}	1.03 (1.01–1.05)	0.0055	1.40 (1.10–1.78) _ψ	5.5×10^{-3}
Valine	rs1440581	1.0	C/T	53%	0.10 (0.013)	4.4×10^{-24}	1.04 (1.02–1.07)	0.00034	1.54 (1.28–1.84)	4.2×10^{-6}

Legend: Adapted from Lotta et al⁴⁸. BCAAs are arranged according to the effect size of the SNP on each trait (sorted smallest with lightest green shading to largest with deepest green shading). Given that the association of each SNP with diabetes is identical (or near-identical when using rs7678928, in LD with rs1440581 at $r^2=0.79$; equal blue shading) the MR estimate is scaled to this effect. Thus leucine has the highest association with risk of T2D (deepest orange shading) and valine the lowest (lightest orange shading). This reflects precisely how the MR estimates are generated – by dividing the SNP-T2D estimate by SNP-BCAA: thus when the SNP-BCAA estimate is the smallest (as for leucine), the MR estimate is the highest. However, critically, none of the MR estimates for the three BCAA is valid as they all assume that each BCAA individually is causal. ψ taken from Table S6 in Lotta et al⁴⁸.

Figure captions

Figure 1. Instrumental variable analysis to generate causal estimates through Mendelian randomization. The three principles of an instrumental variable (IV) are: (i) the IV (in this case a genetic variant either in isolation or in combination with other variants) must associate with the exposure; (ii) the IV must not associate with confounders that are both known and unknown (represented by “U” in the figure); (iii) there is no pathway from the SNP to disease that does not include the exposure of interest. SNP= single nucleotide polymorphism; U= unknown/unmeasured confounders.

Figure 2. Paradoxical scenarios in Mendelian randomization.

Scenario 1: MR using a pleiotropic variant. The genetic variant associates with multiple biomarkers on separate biological pathways. It is invalid to generate separate causal estimates for biomarkers 1 and 2 as they ascribe the same SNP-disease effect to each biomarker. Furthermore, if only one of the biomarkers is causal, then using the SNP to make causal inference on the non-causal biomarker may draw an erroneous conclusion.

Scenario 2: MR using a variant that disrupts normal function of the exposure. Here, the genetic variant encodes impaired function of the exposure. Possession of the genetic variant may lead to increased concentration of the exposure (e.g. due to impaired clearance) yet paradoxically leads to an increased risk of disease (if the normal function of the biomarker would be protective of disease) or *vice versa* if the normal function of the biomarker increases risk of the disease.

Scenario 3: MR of biomarkers on the same pathway. Genetic variant encodes an enzyme that metabolises a substrate into a metabolite. If the substrate and metabolite have contrasting roles in the development of diseases, this may lead to considerable complexity in the interpretation of findings.

Scenario 4: MR of a time dependent exposure. The biomarker is only causal during a critical period; thus MR may yield evidence of a protective effect, however intervening on the biomarker during the non-critical period will not alter risk of disease

Scenario 5: MR of a cumulative exposure. The exposure is causal in disease but has a long latency – e.g. the disease typically presents after decades of exposure-induced subclinical disease development.

Scenario 6: MR of overlapping traits. MR of overlapping biomarkers can lead to paradoxical findings as the overlapping nature of the traits is responsible for diminishing their causal effect on multivariate analyses.

Figure 3. Mendelian randomization using a genetic variant that associates with multiple biomarkers on separate pathways.

A: *PPM1K* genotype, branched chain amino acids and risk of diabetes. Using SNPs in *PPM1K* to infer causality of three separate amino acids yields an erroneous conclusion as this ascribes a causal estimate to each amino acid from the same *PPM1K*-diabetes association that is scaled to the *PPM1K*-amino acid estimate (**Table 2**). BCKD: branched-chain α -keto acid dehydrogenase

B: *APOE* genotype, C-reactive protein and risk of CHD. Using SNPs in *APOE* to infer causality for C-reactive protein yields an erroneous conclusion, as the SNP is pleiotropic for CRP and LDL-C.

Figure 4. Mendelian randomization using a variant that disrupts normal function of the exposure.

A: *SCARB1* genotype, HDL-C and risk of CHD. Reduced hepatic uptake of HDL particles through the scavenger receptors leads to the accumulation of circulating HDL-C and increased risk of CHD. However, this does not point to HDL-C being harmful, but, on the contrary, supports the notion that appropriate function of reverse cholesterol transport may be beneficial to cardiovascular health.

B: *IL6R* genotype, IL-6 and risk of CHD. The variant in *IL6R* leads to reduced membrane-bound *IL6R*, which leads to (i) increased levels of circulating IL6, (ii) disruption of classical IL6 signaling with reduced CRP, and (iii) a reduction in risk of CHD.

Figure 5. Mendelian randomization of biomarkers on the same pathway; *ALDH2* genotype, alcohol, blood pressure and risk of cancer.

A: *ALDH2*1* homozygotes: Individuals consume normal amounts of alcohol which leads to higher blood pressure but since acetaldehyde is efficiently cleared by *ALDH2*, the risk of oesophageal cancer is low.

B: *ALDH22 heterozygotes:** Individuals consume lower amounts of alcohol which leads to higher blood pressure. Reduced functioning of *ALDH2* leads to increased acetaldehyde levels leading to increased risk of oesophageal cancer.

C: *ALDH22 homozygotes:** Individuals consume almost no alcohol thus blood pressure levels are lower. Acetaldehyde levels are also lower since alcohol consumption is near zero. Hence risk of oesophageal cancer is lower too (and similar or lower than *ALDH2* *1/*1, depending on alcohol consumption among *ALDH2* *1/*1 carriers).

Figure 6. Mendelian randomization of a time dependent and cumulative exposure.

A: Vitamin D and risk of MS. As the SNP-disease association reflects lifetime associations (including causal effects that only occur during adolescence), an MR may show evidence of a causal effect when in fact this only occurs during a time critical period.

B: Extracellular SOD and risk of CHD. The genetic variant alters the heparin binding domain of extracellular superoxide dismutase. This means that ecSOD cannot bind to the external membrane of endothelial cells, and cannot preserve nitric oxide (NO) from degradation by superoxide anions. Less NO results in vasoconstriction and increased risk of CHD.

C: LDL-C and risk of CHD. Genetic variants instrumenting LDL-C have large effects on risk of CHD, which reflects that the variants are proxying lifetime exposure. Since CHD is a disease that develops over decades, the effect estimates are equivalent to the estimates that would be derived from lifelong lowering of LDL-C.

Figure 7. Mendelian randomization of overlapping exposures; TG, non-HDL-C and risk of CHD. As TGs are overlapping with non-HDL-C, adjusting the TG association for non-HDL-C diminishes the causal effect of TG to null. In contrast, non-HDL-C contains the entire cascade of apolipoprotein B-containing lipoproteins, including IDL-C and LDL-C, meaning that an association persists between non-HDL-C and CHD on adjustment for TGs. The attenuation of the TG-CHD association does not provide any information about the causality as it is adjusting for an overlapping trait.