Mendelian randomization in cardiometabolic disease: challenges in evaluating causality

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Abstract | Mendelian randomization (MR) is a burgeoning field that involves the use of genetic variants to assess causal relationships between exposures and outcomes. MR studies can be straightforward; for example, genetic variants within or near the encoding locus that is associated with protein concentrations can help to assess their causal role in disease. However, a more complex relationship between the genetic variants and an exposure can make findings from MR more difficult to interpret. In this Review, we describe some of these challenges in interpreting MR analyses, including those from studies using genetic variants to assess causality of multiple traits (such as branched-chain amino acids and risk of diabetes mellitus); studies describing pleiotropic variants (for example, C-reactive protein and its contribution to coronary heart disease); and those investigating variants that disrupt normal function of an exposure (for example, HDL cholesterol or IL-6 and coronary heart disease). Furthermore, MR studies on variants that encode enzymes responsible for the metabolism of an exposure (such as alcohol) are discussed, in addition to those assessing the effects of variants on time-dependent exposures (extracellular superoxide dismutase), cumulative exposures (LDL cholesterol), and overlapping exposures (triglycerides and non-HDL cholesterol). We elaborate on the molecular features of each relationship, and provide explanations for the likely causal associations. In doing so, we hope to contribute towards more reliable evaluations of MR findings.

The *raison d'être* for medical and scientific research is to understand disease aetiologies and identify opportunities for prevention and treatment. Observational epidemiological studies provide a wealth of information on associations between disease exposures and outcomes, but they cannot be interpreted as indicating causality, owing to limitations introduced by confounding and reverse causality[1,2]. Although randomized, controlled trials (RCTs) remain the gold-standard study design for inferring causality, they are exceedingly expensive and time-consuming efforts with high failure rates (>50% fail owing to lack of efficacy)[3,4]. In addition, RCTs might involve interventions that are pleiotropic (such as drugs that modify multiple biomarkers), which can challenge causal deductions for any individual biomarker. Finally, RCTs are not always feasible or ethical to conduct[5], such as when attempting to clarify the causal role of alcohol in cardiovascular disease[6–9].

Mendelian randomization (MR) is an established epidemiological approach that can provide information on causality and prioritize biomarkers for drug-target validation[10–14]. Grouping individuals in the population according to the possession of genetic variants that modify an exposure allows researchers to infer whether a biomarker is causally related to a disease (FIG. 1). This inference is permissible owing 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. In addition, reverse causality bias would not occur owing to the essentially nonmodifiable nature of the transmitted germline genome. Therefore, MR can help to strengthen causal inferences on the role of modifiable exposures, such as circulating biomarkers in disease risk. Genetic variants that are associated with LDL-cholesterol levels have been used to assess the causal role of LDL cholesterol in coronary heart disease (CHD)[15–18]. Irrespective of the genetic variants used as a proxy for LDL cholesterol, strong dose–response relationships have been identified[15]. This observation suggests that lowering LDL-cholesterol levels by any means would lead to a reduction in CHD, and

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Key points

- Mendelian randomization (MR) is a powerful tool that utilizes genetic information to inform about the likely causal relevance of an exposure to an outcome
- When performed rigorously, MR findings should be free from reverse causality bias, and only minimally affected by confounding
- The number of MR studies has been increasing in the past decade, providing important new insights into disease aetiology
- However, as MR studies become more common, and as increasingly complex gene-to-exposure and exposure-to-outcome relationships are investigated, reliable conduct and interpretation of MR analyses can be challenging
- Potential solutions to aid the conduct and interpretation of MR studies can be derived, for example, through use of emerging statistical approaches to investigate potential genetic pleiotropy that can distort the findings

further validates the linear dose–response relationship between LDL cholesterol and CHD risk identified from meta-analyses of RCTs assessing statins and other cholesterol-lowering interventions[19–21].

The use of MR analysis had led to several major findings and validations of cause–effect relationships in cardiometabolic disorders. In the absence of any RCTs that have assessed the efficacy of a specific C-reactive protein (CRP)-lowering therapeutic for CHD, MR studies have successfully shown that CRP is unlikely to have a major role in the development of CHD[22,23]. Similarly, whereas an RCT on the effect of alcohol consumption on cardiovascular health is unfeasible and unethical, MR analyses have shown that moderate alcohol consumption is unlikely to reduce CHD risk[24]. Increased adiposity was found to increase the risk of CHD in several MR studies[25–28], whereas the only RCT investigating this relationship was underpowered for assessing causality[29]. Furthermore, MR analyses validated findings from RCTs assessing drug targets, including 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase)[30,31], secretory phospholipase A_2-IIA[32,33], lipoprotein-associated phospholipase A_2 (REFS 34–38), and Niemann-Pick C1-like protein[39]. Finally, MR studies have also identified a potential increased risk of diabetes mellitus with pharmacological inhibition of PCSK9 (REFS 40–42). These studies 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.

Randomized, controlled trials (RCTs)
An interventional study in which individuals are randomized to an 'exposed' arm (for example, an active drug) or to a comparator control arm (for example, placebo) to test the efficacy of an intervention on an outcome (such as disease risk). Such trials are considered the gold standard for asserting causal relationships of an exposure to disease risk.

Mendelian randomization
A genetic epidemiological approach that aims to quantify the causal relationship between an exposure and an outcome, by using the properties of the genome (randomized owing to Mendel's second law, and invariant) to minimize the issues of confounding and reverse causality that can undermine traditional observational epidemiology.

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As with conventional observational, epidemiological, and interventional trials, MR analyses typically yield a quantitative association between a biomarker and disease risk. This approach is most robust when a variant is in or near a gene that is responsible for the synthesis of the protein under investigation, and associates with concentrations of the same protein without disrupting protein function (for example, using genetic variants around the *CRP* gene that are associated with concentrations of circulating CRP)[22]. However, there are various scenarios in which the accuracy of data derived from MR analyses becomes compromised. In this Review, we highlight scenarios in which the interpretation of MR analyses is not straightforward and, in each case, we elaborate on the molecular details and provide what we consider to be a more accurate interpretation (FIG. 2).

Multiple biomarkers on separate pathways

In general, a genetic variant used for MR should affect only a single pathway on which the exposure of interest lies (BOX 1). For example, the use of a genetic variant in MR that, through its association with the exposure of interest, associates with multiple biomarkers on a single biological pathway is valid and is termed vertical pleiotropy. By contrast, horizontal pleiotropy — when a genetic variant employed in MR associates with multiple biomarkers on discrete pathways (BOX 1; FIG. 2a) — can yield invalid causal estimates. Two related examples are discussed below: the first example involves the same genetic variant used incorrectly to assess causal relationships of multiple traits on discrete pathways, and the second example relates to the use of pleiotropic single nucleotide polymorphisms (SNPs) to assess the causal role of CRP in CHD without applying appropriate analytical checks.

Branched-chain amino acids and diabetes. Observational studies have reported an association between the branched-chain amino acids (leucine, isoleucine, and valine) and risk of incident diabetes[43–48]. In a genome-wide association study (GWAS) published in 2016, a genetic variant associated with these three amino acids was identified and used to conduct MR analyses[49]. At the *PPM1K* locus, the rs1440581 SNP was used to generate causal estimates for both leucine and valine, whereas a SNP in the same locus (rs7678928, in linkage disequilibrium with rs1440581 at $r^2 = 0.79$ and $D' = 1$) was used in combination with other SNPs for isoleucine. According to the investigators, "the association of genetic variants appeared highly specific", and they subsequently generated MR estimates for each of the three amino acids, and used these to implicate the role of branched-chain amino acid metabolism in the development of diabetes[49].

As shown in FIG. 3a, the *PPM1K* locus encodes mitochondrial phosphatase that activates branched-chain α-ketoacid dehydrogenase (BCKD), which is responsible for the metabolism of leucine, isoleucine, and valine[49]. Therefore, by activating the enzyme responsible for the metabolism of these three amino acids, SNPs in the *PPM1K* locus associate with the concentrations of these amino acids. In MR analyses, the association of the SNPs

with risk of disease is scaled to the association of the SNP with the exposure to generate a single causal estimate for the relationship between the exposure and disease (FIG. 1). When the same SNP is used to make causal deductions on multiple exposures, there is only a single association of the SNP with risk of disease; therefore, this SNP-to-disease association is used multiple times, each time assuming that the exposure individually accounts for the disease association. In this scenario, the association of *PPM1K* at rs1440581 (or a SNP in linkage disequilibrium with rs7678928) with diabetes is used to generate individual MR estimates for each of the three branched-chain amino acids, ascribing a causal estimate that is scaled to the effect of the SNP on the amino acid[49] (TABLE 2). Crucially, this analysis makes the invalid assumption that each of the three amino acids in isolation would be causal, and is in violation of one of the principal rules of MR: that the instrument acts only 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 three different exposures, which is incoherent. Using this single locus, the MR analyses cannot clarify which, if any, of the three amino acids is actually driving the causal relationship with diabetes. Given that the researchers concluded that their findings are "consistent with a causal role of [branched-chain amino acid] metabolism in the aetiology of type 2 diabetes", readers might incorrectly interpret the quantitative MR estimates reported for each of the three branched-chain amino acids as evidence that each branched-chain amino acid is individually and independently related to type 2 diabetes[49]. The only information that can be inferred from this study is that the *PPM1K* locus activates an enzyme (BCKD) that has a range of substrates (which might not be limited to the three amino acids studied), and that one or more of these pathways leads to diabetes. Therefore, although we agree with the researchers regarding their results being consistent with the proposed causal role of branched-chain amino acid metabolism in diabetes, the presentation of the data could lead to misinterpretation[49].

APOE, C-reactive protein, and risk of CHD. In MR studies, incorrect analysis of a genetic variant in *APOE* that associates with circulating levels of CRP might erroneously imply a causal relationship between CRP levels and CHD risk[50]. However, this relationship is actually driven by the association of the *APOE* genotype with multiple biomarkers on discrete pathways, including LDL cholesterol, which is causally related to CHD (FIG. 3b). Use of such pleiotropic variants in isolation is, therefore, likely to lead to incorrect causal interpretations. An alternative approach would be to combine multiple SNPs across the genome into a genetic variant score for CRP[51]. Although pleiotropy in a gene score might 'balance out' in certain settings (so-called 'balanced horizontal pleiotropy'; BOX 1), the horizontal pleiotropy of the CRP gene score is likely to persist, resulting in potential biased estimates using conventional MR approaches (BOXES 2,3). Furthermore, when using a multilocus genetic variant score for CRP and removing SNPs on the basis of tests for heterogeneity, this approach can still yield biased results, because multiple SNPs that have pleiotropic effects might remain. Therefore, causal associations for disease that arise from horizontal pleiotropy when analysed using conventional MR methods might be biased (known as unbalanced horizontal pleiotropy)[52]. This approach could be improved by using SNPs that are either confined to the *CRP* locus (which are more likely to show specificity for the protein trait), or using SNPs across the genome identified from GWAS in combination with use of more novel approaches, such as MR-Egger (BOX 4), which allows relaxation of the instrumental variable assumption that there is no unbalanced horizontal pleiotropy[53,54]. Therefore, our recommendation would be to utilize a range of applicable methodologies as sensitivity analyses to test the robustness of the MR estimates against potential violations, including unbalanced horizontal pleiotropy.

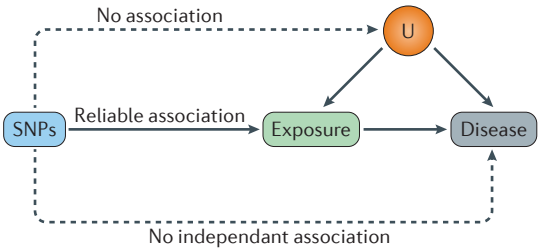


Figure 1 | **Instrumental variable analysis to generate causal estimates through Mendelian randomization.** The three principles of instrumental variable analysis are: the instrumental variable (in this case a genetic variant either in isolation or in combination with other variants) must associate with the exposure; the instrumental variable must not associate with confounders that are either known or unknown (U); and there is no pathway from the single nucleotide polymorphism (SNP) to disease that does not include the exposure of interest. This figure is a schematic representation and should not be interpreted as a formal directed acyclic graph.

Disrupting the function of the exposure

A paradoxical association between the levels of the biomarker and disease risk in MR (FIG. 2b) can take place if a SNP disrupts the normal function of a causal exposure (such as binding of the exposure to its target receptor). In this setting, the difference in biomarker concentrations from the genetic variant might be used to infer inaccurate directionality of causation. However, by perturbing the normal function of the biomarker, the genetic variant can paradoxically be linked with a biomarker that confers an opposite risk of disease in MR analysis to that observed in epidemiological studies.

SCARB1, *HDL cholesterol, and CHD risk.* In 2016, a rare variant in *SCARB1* was identified that results in the loss of function of the scavenger receptor B1 (SR-B1)[55]. Individuals carrying this variant have higher levels of circulating HDL-cholesterol levels and an elevated risk of CHD. The study investigators proposed that reduced hepatic SR-B1 function in humans causes impaired reverse cholesterol transport, which in turn leads to

Vertical pleiotropy
The association of a genetic marker (for example, a single nucleotide polymorphism [SNP] in isolation, or a genetic instrument consisting of multiple SNPs) with more than one phenotype on the same biological pathway.

Horizontal pleiotropy
The association of a genetic marker with more than one phenotype on discrete biological pathways.

Genome-wide association study (GWAS)
The hypothesis-free investigation of hundreds of thousands to millions of genetic variants (typically single nucleotide polymorphisms) for their association with a phenotype to characterize the underlying genetic architecture of the phenotype.

Linkage disequilibrium
The nonrandom assortment of genetic variants, meaning that when linkage disequilibrium between a pair of variants is high (for example, as measured by a r^2 value of > 0.80), a single nucleotide polymorphism (SNP) can be used as a 'proxy' for another SNP in the absence of this second SNP being directly genotyped.

Unbalanced horizontal pleiotropy
When horizontal pleiotropy is such that alternative pathways from the genetic marker to disease can lead to distortion of the association of the exposure under investigation. Unbalanced horizontal pleiotrophy is a violation of the exclusion restriction assumption of an instrumental variable.

Instrumental variable
A variable used as a proxy for an exposure of interest that is not associated with confounders and only associates with an outcome through the exposure of interest

increased risk of CHD despite elevated HDL-cholesterol levels[55]. These findings have been widely interpreted to mean that high levels of HDL cholesterol can actually contribute to the development of CHD, which is inconsistent with the well-established putative protective role of HDL cholesterol against cardiovascular risk[56,57].

The HDL-mediated transport of cholesterol from peripheral tissues to the liver (reverse cholesterol transport) is thought to result in a decrease in atheroma burden, and a commensurate reduction in the risk of CHD[58,59]. However, a critical component of HDL-mediated reverse cholesterol transport is the selective uptake of circulating HDL particles by the liver. After hepatic uptake, cholesterol is excreted in bile; the binding and uptake of HDL particles into the liver occurs principally through SR-B1 (REF. 60). Therefore, the increased risk of CHD associated with a genetic variant that disrupts normal function of SR-B1 potentially provides new evidence that HDL-mediated reverse cholesterol transport (through SR-B1) might have a role in preventing the development of CHD (FIG. 4a). However, to temper enthusiasm, whole-genome sequencing studies that investigated sequence variants within the *SCARB1* locus have identified additional links with other biomarkers, including lipoprotein-assoiated phospholipase A2 and vitamin E, suggesting the potential for horizontal pleiotropy (BOX 1). Furthermore, both RCTs[61–63] and MR studies[16–18] have failed to show that increasing HDL-cholesterol levels can reduce cardiovascular disease risk. Therefore, whether HDL metabolism has a causal role in the aetiology of CHD remains speculative, and studies are now evaluating the function of HDL particles, as opposed to measuring only circulating HDL-cholesterol concentrations[64–67].

Table 1 | **Notable Mendelian randomization studies in cardiometabolic disease**

Exposure	Outcome	Interpretation	Importance	Refs
BMI	Metabolites	BMI causally influences many circulating metabolites	Supports the interpretation that BMI might influence cardiometabolic disease through its effect on metabolites	139
HMGCR/ statins	Metabolites	Causal	Shows consistency of observational data on statins versus predicted MR effects on metabolites	30
Adiposity (BMI and waist–hip ratio)	CHD	BMI and waist–hip ratio (adjusted for BMI) causally increases risk of CHD	No trial has yet shown this causal relationship[29]	25–28
CRP	CHD	No causal relationship	No trial of a therapy specific to CRP for CVD events has been conducted	22,23,50
LDL cholesterol	CHD	Dose–response relationship irrespective of locus	Suggests LDL-cholesterol lowering by any means is beneficial, consistent with trials involving statin and other cholesterol-lowering interventions[19,21,108]	15
HDL cholesterol	CHD	No causal effect	Contradicts observational data[140], but supports findings from recent RCTs[61–63]	16–18
Triglycerides	CHD	Causal	Precedes trial data of a triglyceride-lowering agent	16,18,141
$sPLA_2$-IIA	CHD	Noncausal	Published at a similar time to the VISTA-16 trial[33] of a $sPLA_2$-IIA-lowering drug that did not have beneficial effects on CVD	32
Lp-PLA_2	CHD	Noncausal	Many resources were spent on trials[35,62] that showed therapeutic lowering of Lp-PLA_2 level does not lower risk of CVD; some MR studies were published before the reporting of RCT results	34,36,37, 142
NPC1L1/ ezetimibe	CHD	Causal	MR studies preceded RCT data[143], that showed lowering of LDL-cholesterol level via inhibition of *NPC1L1* results in reduced risk of CVD	39,144
PCSK9, lipoprotein(a), and ANGPTL4	CHD	Causal	Drugs developed for CVD prevention on basis of genetic findings, some of which have since shown cardiovascular benefit in phase III RCTs[145]	146–148
LDL cholesterol	Diabetes	Causal	Suggests LDL-cholesterol lowering might generally lead to increased risk of diabetes mellitus, and has potential ramifications for drugs that lower LDL-cholesterol level	18
HMGCR/ statins	Diabetes	Causal	Indicates that the (albeit small) diabetogenic effects of statins (that are outweighed by the cardiovascular benefits[149]) seen in RCTs are on-target[150]	31
PCSK9/PCSK9 inhibitors	Diabetes	Causal	Suggests PCSK9 inhibition might increase risk of diabetes	40–42
Alcohol	CVDs (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]; findings are important for public-health policy[151]	24,83,152

ANGPTL4, angiopoietin-related protein 4; CHD, coronary heart disease; CRP, C-reactive protein; CVD, cardiovascular disease; Lp-PLA_2, lipoprotein-associated phospholipase A_2; MR, Mendelian randomization; NPC1L1, Niemann–Pick C1-like protein 1; RCT, randomized, controlled trial; $sPLA_2$-IIA, secretory phospholipase A_2-IIA.

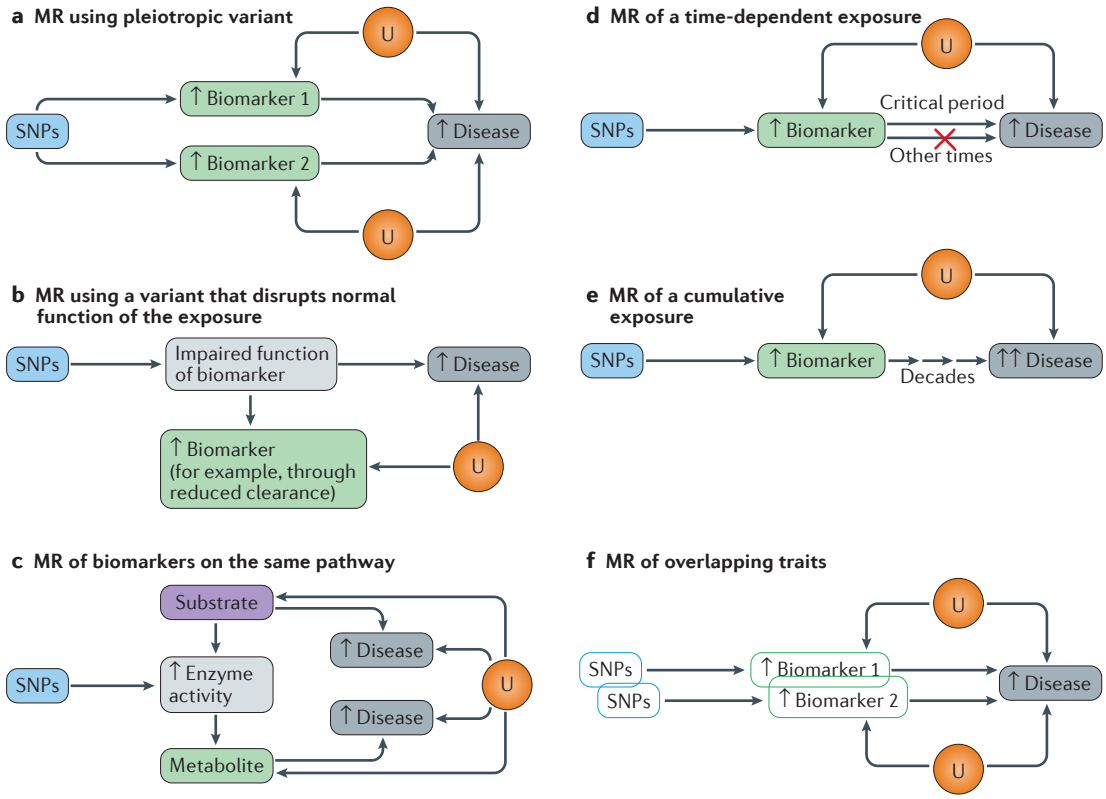


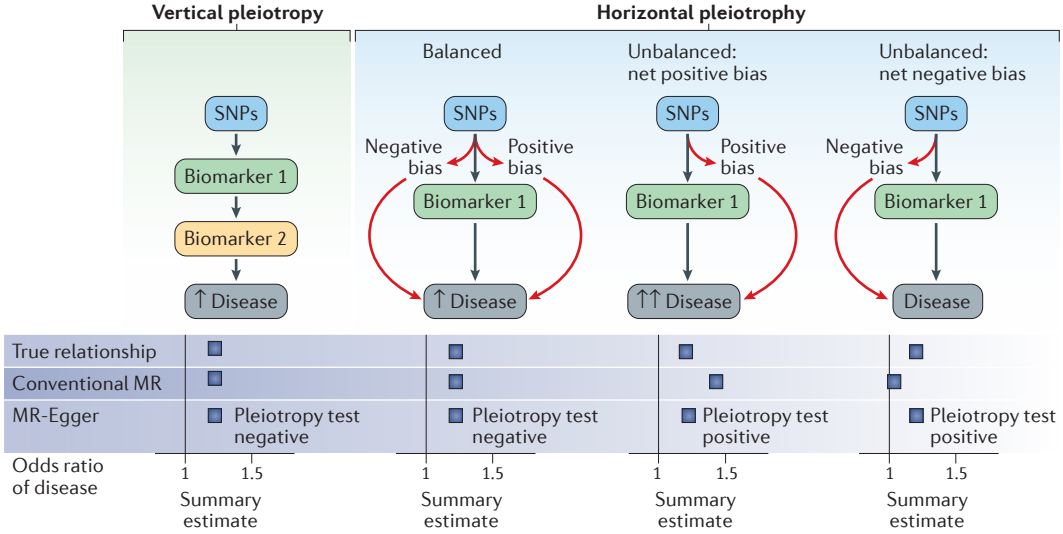
Figure 2 | **Paradoxical scenarios in Mendelian randomization (MR). a** | An example of MR using a pleiotropic variant. The genetic variant associates with multiple biomarkers on separate biological pathways. Generating separate causal estimates for biomarkers 1 and 2 is invalid as they ascribe the same single nucleotide polymorphism (SNP)–disease effect to each biomarker. Furthermore, if only one of the biomarkers is causal, then using the SNP to make causal inferences on the noncausal biomarker can generate an erroneous conclusion. **b** | An example of MR using a variant that disrupts normal function of the exposure. Possession of the genetic variant might lead to increased concentration of the exposure (for example, owing to impaired clearance), but paradoxically lead 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. **c** | An example of MR involving biomarkers on the same pathway, in which the genetic variant encodes an enzyme that metabolizes a substrate into a metabolite. If the substrate and metabolite have contrasting roles in the development of diseases, this discrepancy might lead to complexity in the interpretation of findings. **d** | An example of MR involving a time-dependent exposure. If the biomarker is causal for disease only during a critical time period, MR might show evidence of a protective effect. However, intervening on the biomarker during the noncritical time period will not alter risk of disease. **e** | An example of MR of a cumulative exposure, in which the exposure is causal for disease, but has a long latency. For example, the disease might typically present after decades of exposure-induced subclinical disease development. **f** | An example of MR involving overlapping traits. MR of overlapping biomarkers can lead to paradoxical findings as the overlapping nature of the traits might lead to a diminution of their apparent causal effect on multivariate analyses. These figures are schematic representations and should not be interpreted as formal directed acyclic graphs. U, unknown.

IL-6 signalling and risk of CHD. IL-6 is a proinflammatory cytokine produced by stromal and immune cells that circulates in the blood and binds to plasma membrane receptor complexes. IL-6 can exert its biological effect via two signalling mechanisms: classical signalling and trans-signalling. Classical signalling involves the binding of IL-6 to cellular membranes that express both the IL-6 receptor (IL6R) and glycoprotein-130 (REF. 68). Most cells express glycoprotein-130, but only a limited number express IL6R. In trans-signalling, a soluble form of the IL6R binds to circulating IL-6 in the blood, and this IL-6/IL6R complex 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 IL-6 signalling is thought to have a more prominent role in the development of systemic diseases, whereas trans-IL-6 signalling might be more involved in local tissue inflammation[69].

Using a nonsynonymous SNP (rs8192284) in the gene encoding IL6R, two studies have reported that variants associated with increased concentrations of circulating IL-6 are related to a reduction in CHD risk[70,71]. A naive interpretation, and one that the researchers explain is not the case, would be that IL-6 signalling is protective against CHD. However, this deduction would be at odds with the well-established role of IL-6 as an inflammatory cytokine[72].

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 a biomarker with disease risk. Vertical pleiotropy refers to when genetic variants associate with multiple biomarkers that are on the same pathway from exposure through to disease, and does not invalidate the findings as long as the primary phenotype (most proximal to the genotype) that is influenced by the genetic variant is understood. Horizontal pleiotropy refers to when a genetic variant associates with traits on discrete pathways that are also causal in disease. When using multiple genetic variants in combination, horizontal pleiotropy can 'balance out' and have no net effect on the association of the exposure and disease risk. Balanced horizontal pleiotropy should not bias the causal effect derived from Mendelian randomization (MR), even when using conventional approaches; however, it does lead to increased variance in the effect estimation, and thus less-precise confidence intervals. By contrast, unbalanced horizontal pleiotropy distorts the association between the exposure and the outcome, and the effect estimate from conventional MR approaches can be exaggerated or diminished, depending on the direction of the pleiotropy. The presence of unbalanced horizontal pleiotropy can be formally assessed by using MR-Egger[53], provided certain assumptions are satisfied. MR-Egger provides a valid MR estimate that takes into account presence of unbalanced horizontal pleiotropy. Other approaches include median and weighted median MR[54], which provide a valid MR estimate as long as the majority of SNPs (or the majority of the statistical weight contributed by the SNPs) in the instrument are valid, and the modal estimate[122], which assumes that the most common effect estimate among a set of instruments is the one most likely to be valid. Each of these approaches has their own assumptions[54]. When possible, investigators should perform these sensitivity analyses when conducting conventional (inverse variance-weighted) MR analyses. Figure adapted from White, J. *et al.* Association of lipid fractions with risks for coronary artery disease and diabetes. *JAMA Cardiol.* **1**, 692–699 (2016), with permission from the American Heart Association.



A genetic variant in *IL6R* (rs8192284) has also been shown to increase generation of soluble IL6R through increased proteolytic cleavage of membrane-bound IL6R, leading to a subsequent reduction in membrane-bound IL6R[73,74]. This reduction in turn leads to diminished IL-6-mediated classical signalling, and a shift from classical signalling to trans-signalling, effectively attenuating downstream classical signalling of IL-6. Consequently, decreased classical IL-6 signalling increases circulating IL-6 (owing to a reduction in membrane-bound IL6R and an increase in the circulating IL-6/IL6R complex), but a reduction in CRP levels (as classical IL-6 signalling is impaired; FIG. 4b)[75,76]. Of note, the association of SNPs in *IL6R* with CRP concentrations in this setting reflects vertical pleiotropy. Although in no way does this observation indicate that CRP is causal for CHD, the association of SNPs in *IL6R* with CRP does not invalidate the use of *IL6R* in MR because, unlike in FIGS 3a,b, CRP is downstream of the same pathway as IL-6. One way to dissect these relationships would be to use separate genetic instruments for IL-6 and CRP, and construct a causal framework involving molecular intermediates using mediation analysis[77].

Multiple biomarkers on the same pathway

If a variant is associated with multiple dependent traits on the same pathway, and if those biomarkers have different roles in disease, paradoxical situations can arise (FIG. 2c).

ALDH2 *and alcohol consumption.* Alcohol is metabolized in tissues and in the liver by the enzymes alcohol dehydrogenase 1B (ADH1B) and acetaldehyde dehydrogenase 2 (ALDH2). Metabolism of alcohol by ADH1B yields acetaldehyde, a group 1 human carcinogen[78], which is rapidly metabolized by ALDH2 into acetate. When ADH1B and ALDH2 function normally, systemic 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 such as flushing, nausea, and headache upon consumption of alcohol. A naturally occurring genetic

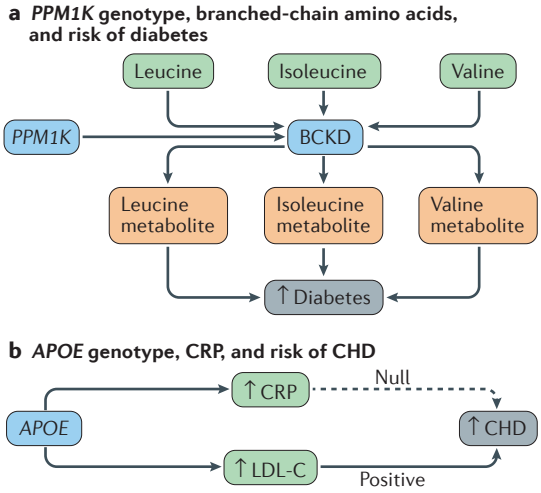


Figure 3 | **Mendelian randomization (MR) using a genetic variant that associates with multiple biomarkers on separate pathways. a** | Using single nucleotide polymorphisms (SNPs) in *PPM1K* (which encodes a mitochondrial phosphatase that activates branched-chain α-keto acid dehydrogenase [BCKD], responsible for the rate-limiting step of metabolism of the branched-chain amino acids) to infer causality of three separate amino acids yields an erroneous conclusion, as this inference 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). The three amino acids are initially catabolized prior to the enzymatic action of BCKD. **b** | Using SNPs in *APOE* to infer causality of C-reactive protein (CRP) yields an erroneous conclusion, as the SNP is pleiotropic for CRP and LDL cholesterol (LDL-C). These figures are schematic representations and should not be interpreted as formal directed acyclic graphs. CHD, coronary heart disease.

variation in *ALDH2* (rs671, in which carriage of the variant that influences enzyme activity is referred to as *2) that is present in East-Asian individuals, but not in white individuals (*ALDH2**1*1) results in loss-of-function of ALDH2 in a dose-dependent fashion. Individuals who are homozygous for the normal variant can consume alcohol in the usual way, whereas those who are heterozygous can still consume alcohol, although they experience symptoms of acetaldehyde toxicity. However, individuals who are homozygous for the *ALDH2**2 variant tend to consume almost no alcohol[79], given the symptoms experienced by this population. Individuals who carry one copy of the *ALDH2**2 variant experience a less severe flushing response as compared with individuals who carry two copies of the *2 variant[80], and this is evidenced by differences in blood acetaldehyde levels for a given dose of alcohol[81].

ALDH2 *and hypertension.* Given that alcohol consumption is associated with hypertension[82], the *ALDH2**2 variant can be used as a genetic instrument to assess the causal role of alcohol consumption on blood pressure levels[83]. As each additional *ALDH2**2 allele reduces alcohol consumption in a dose-dependent manner (FIG. 5), a conventional MR study using a per-allele genetic model for the *2 allele of *ALDH2* will produce a valid causal estimate[83]. Among East-Asian individuals, women generally consume considerably lower amounts of alcohol compared with men; therefore, 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[84,85]. Given that the genetic variant should associate with blood pressure only in the presence of alcohol, a larger association of the genetic variant with blood pressure should be seen in men versus women (because men consume more alcohol); this pattern has been reported in several studies[83,86]. The interaction between sex and *ALDH2* genotype can be used as the instrumental variable to estimate the causal effect of alcohol on outcomes, and is a robust analytical strategy that circumvents the instrumental variables assumption of no pleiotropy[79].

ALDH2 and oesophageal carcinoma. When investigating the association between *ALDH2* and oesophageal carcinoma, a different interpretive framework is required. Individuals who drink the most (that is, those who are homozygous for the wild-type gene) might be expected to have the highest risk of oesophageal carcinoma; however, individuals who carry one copy of the *2 allele actually have the highest risk[87]. Although it might be tempting to interpret this observation as meaning that moderate drinkers having the highest risk of oesophageal carcinoma (a paradoxical scenario that is at odds with dose-dependent increase in risk with alcohol

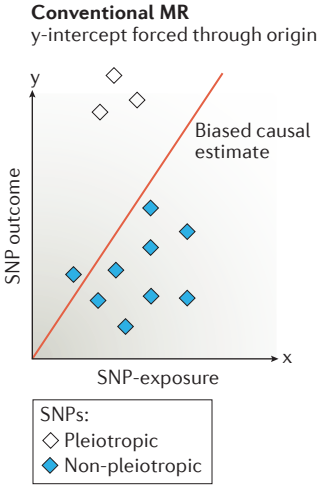
Table 2 | **MR of discrete biomarkers when using identical or highly correlated genetic variants**

BCAA	SNP in *PPM1K*	Linkage disequilibrium (r^2) with rs1440581	Effect/ other allele	EAF (%)	Standardized beta (SE) of metabolite level per allele	*P* value for BCAA	OR (95% CI) for T2DM per allele	*P* value for T2DM	MR estimate of T2DM 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)	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}

Branched-chain amino acids (BCAAs) are arranged according to the effect size of the single nucleotide polymorphism (SNP) on each trait (sorted from smallest to largest). Given that the association of each SNP with type 2 diabetes mellitus (T2DM) is identical (or near-identical when using rs7678928, in linkage disequilibrium with rs1440581 at $r^2 = 0.79$), the MR estimate is scaled to this effect. Therefore, on Mendelian randomization (MR) analysis, leucine has the greatest magnitude of association with risk of T2DM, and valine has the lowest magnitude of association. This analysis reflects how the MR estimates are generated by dividing the SNP–T2DM estimate by SNP–BCAA. When the SNP–BCAA estimate is the smallest (as for leucine), the MR point estimate is the greatest. Critically, none of the MR estimates for the three BCAAs is valid because they all assume that each BCAA individually is causal. EAF, effect allele frequency. Adapted from Lotta, L. A. *et al.* Genetic predisposition to an impaired metabolism of the branched-chain amino acids and risk of type 2 diabetes: a Mendelian randomisation analysis. *PLoS Med.* **13**, e1002179 (2016).

Box 2 | **Conventional MR approaches**

When using multiple single nucleotide polymorphisms (SNPs) for Mendelian randomization (MR) in summary-level data, inverse variance-weighted analysis was, until recently, the most common analytical approach. In this form of regression, each SNP contributes a data point on the x-axis (SNP-to-exposure association) and y-axis (SNP-to-disease association). However, inverse-variance-weighted MR forces the y-intercept through the origin. In the setting of balanced pleiotropy, bias should not be evident and the slope of the regression line can be reliably interpreted as the causal effect of the exposure on the outcome. However, in the presence 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. To overcome this issue, investigators previously relied on approaches such as 'manual pruning' of SNPs that they considered pleiotropic. However, this approach relies on the availability and precision of SNP estimates with multiple biomarkers to inform on the presence of such pleiotropy; many SNP associations that do not meet conventional significance thresholds in genome-wide association studies (GWAS) are false-negatives, owing to the stringent α values used to avoid false-positives[133,134]. Second, this approach can be subjective as one investigator might consider a trait to be indicative of vertical pleiotropy and retain SNPs associated with the trait, whereas another could consider the same trait to be evidence of horizontal pleiotropy and remove SNPs showing association with the trait. This approach would lead to different genetic instruments and potentially inconsistent results between studies. Furthermore, reasons for exclusion can be nontransparent and differ by study, further reducing the degree of objectivity. Finally, manual pruning can lead to a genetic instrument that is no longer biologically meaningful (BOX 3).



intake), this relationship can be explained by the effect of *ALDH2* on both alcohol consumption and circulating concentrations of acetaldehyde[81] (FIG. 5).

Individuals who are heterozygous for *ADLH2* rs671 (that is, carriers of the *1*2 allele) have the highest circulating concentrations of acetaldehyde (FIG. 5b), despite the fact that they do not consume the greatest amount of alcohol (ALDH2 *1 homozygotes consume the greatest amounts; FIG. 5a), nor do they have the genetic variant (like that carried by *ALDH2* *2 homozygotes[81]) that conveys highest concentrations of acetaldehyde for a given amount of alcohol (FIG. 5c). The highest absolute concentration of circulating and tissue acetaldehyde by genotype results in an increased risk of oesophageal carcinoma in *1*2 allele group compared with either the *1*1 allele or *2*2 allele groups. In this setting, the genetic variant influences both alcohol consumption and, among alcohol drinkers, acetaldehyde levels. For these reasons, a per-allele analytical model would be inappropriate for evaluation of causality[88].

To summarize, the way in which a genetic variant might serve as an exposure measure for different traits depends on the outcome under investigation. For blood pressure, which seems not to be influenced in the long term by acetaldehyde levels, the variant is an instrument for causal inferences with respect to alcohol intake. For oesophageal cancer, the variant is an additional instrument for assessing the causal effects of acetaldehyde among consumers of alcohol. Without a good understanding of the biological basis of the effects of the genetic variant, misleading interpretations could be drawn from MR analyses.

Box 3 | **Effects of manual pruning on interpretation of MR analysis**

As mentioned in BOX 2, manual pruning of single nucleotide polymorphisms (SNPs) that are considered pleiotropic might result in a genetic instrument that is no longer biologically meaningful, as SNPs that are retained might not be indicative of any tangible entity. For example, if we have SNPs in 70 loci associated with HDL cholesterol from genome-wide association studies (GWAS) using conventional *P*-value thresholds (that is, $P<5\times10^{-8}$)[135], and manually prune out the SNPs associated with LDL cholesterol or triglycerides (associated with either LDL cholesterol or triglycerides at $P<5\times10^{-8}$, or by using a more relaxed *P*-value threshold) in order to obtain an instrument that is more 'specific' for HDL cholesterol, this approach will retain a smaller number of SNPs in the genetic instrument. However, by removing SNPs that account for the underlying genetic architecture of HDL cholesterol acquired in a hypothesis-free GWAS study, the remaining SNPs might not be representative of features that are biologically meaningful of HDL cholesterol. Furthermore, manual pruning can exacerbate the problem when removal of SNPs occurs on the basis of vertical pleiotropy. For example, pruning SNPs associated with fasting glucose for their association with type 2 diabetes mellitus to evaluate whether fasting glucose is associated with coronary heart disease independently of diabetes might inadvertently lead to SNPs being selected on the basis of pleiotropy (as a fasting glucose SNP that is not associated with diabetes might associate with pathways that counterbalance the glucose association, such that the overall association of the SNP with diabetes is null)[136]. Therefore, an MR analysis using manually pruned SNPs in a genetic instrument can lead to findings that are very challenging to interpret.

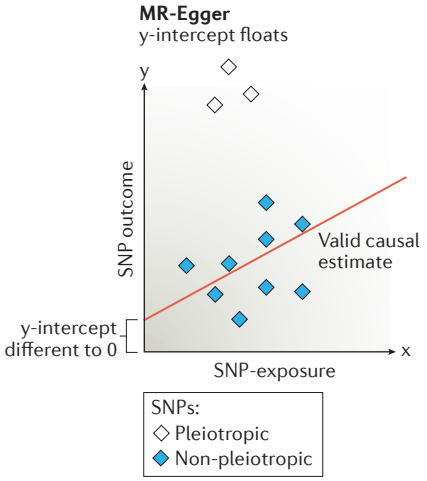
Time-dependent exposures

If an exposure is time-dependent (for example, if an exposure is influential only during a period of development, such as during adolescence), then despite MR results suggesting a causal effect, modification of the exposure in later life will not necessarily alter the risk of disease (FIG. 2d).

Vitamin D and multiple sclerosis. A MR study published in 2015 suggested a causal role of vitamin D in the aetiology of multiple sclerosis[89]. Although this example does not pertain directly to cardiovascular disease, we use it to introduce a general issue in MR analysis that might also apply for studies in cardiovascular disease. Previous observational studies of migration and risk of multiple sclerosis have consistently identified a time-dependent

Box 4 | **New MR approaches**

In contrast to conventional Mendelian randomization (MR), MR-Egger[53] takes the approach of Egger regression[137] (introduced in the context of small study bias evaluation in clinical trials) and allows the y-intercept to float, which achieves two outcomes. First, this flotation provides a statistical test for the presence of unbalanced horizontal pleiotropy; evidence that y is different to 0 when $x = 0$ is suggestive of the 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[53]) from the slope of the regression line. Therefore, the advantages of MR-Egger are manifold, as it obviates the need for manual pruning of SNPs, the intercept can inform on the presence of unbalanced pleiotropy, and the slope can provide a valid causal estimate even in the presence of such pleiotropy (as long as certain conditions are met). One disadvantage of MR-Egger is that, for a given sample size, power is reduced compared with inverse variance-weighted MR, although newer extensions to MR-Egger, such as MR-Egger with SIMEX[138], seek to increase power.



relationship between these two variables, indicating that sunlight exposure (and perhaps vitamin D levels) during early life, but not during adulthood, is associated with the risk of multiple sclerosis[90–93]. In this scenario, a 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 multiple sclerosis (FIG. 6a). However, modifying vitamin D levels after the critical time period (infanthood up to early adulthood) would not reduce the risk of multiple sclerosis. If this time period were proven to be critical for disease development, RCTs would need to be commenced in at-risk individuals (perhaps identified through family history or genetic risk scores) during childhood, as interventions commenced after this critical period would not be expected to influence disease risk.

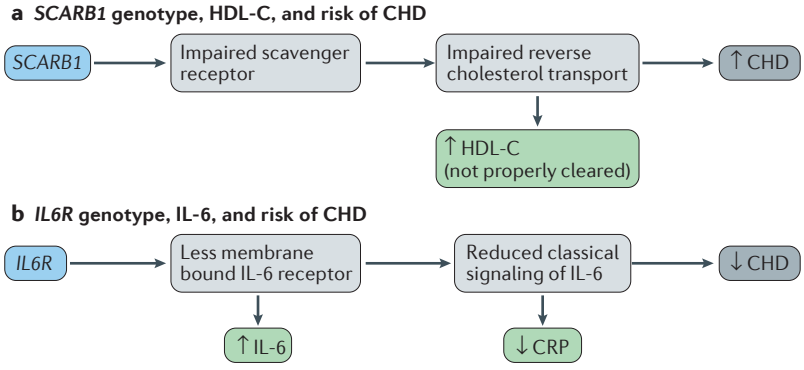


Figure 4 | **Mendelian randomization (MR) using a variant that disrupts normal function of the exposure. a** | Reduced hepatic uptake of HDL particles through the scavenger receptors leads to the accumulation of circulating HDL cholesterol (HDL-C) and increased risk of coronary heart disease (CHD). However, this observation does not indicate that HDL-C is harmful, but provides some support for the notion that appropriate function of reverse cholesterol transport might be beneficial to cardiovascular health. **b** | A variant in *IL6R* leads to reduced membrane-bound IL-6, which in turn results in increased levels of circulating IL-6, disruption of classical IL-6 signalling, reduced C-reactive protein (CRP) levels, and a reduction in risk of CHD. These figures are schematic representations and should not be interpreted as formal directed acyclic graphs.

Extracellular superoxide dismutase and CHD. Antioxidants have long been hypothesized to prevent CHD[94]; however, evidence from major RCTs conducted in adults have yielded largely neutral findings[95,96]. Extracellular superoxide dismutase (ecSOD) protects the nitric oxide released from smooth muscle cells from degradation by superoxide[97,98]. In preserving the function of nitric oxide, ecSOD facilitates the vasodilatation of arterioles, allowing maintenance of normotension[99]. Therefore, ecSOD is considered an endogenous antioxidant that has an important role in the development of vascular disease. A large population-based cohort study assessing a genetic variant that encodes a missense mutation (R231G in the *ECSOD* gene) reported that, contrary to expectations, the variant was associated with elevated levels of circulating ecSOD and linked to an increase in CHD risk[100]. The genetic variant altered the heparin-binding domain of ecSOD, affecting the binding capacity of ecSOD to the external membrane of endothelial cells (FIG. 6b). As a result, ecSOD plasma levels increase, but the ecSOD cannot protect nitric oxide from degradation by superoxide anions[98]. Reduced bioavailability of nitric oxide can lead to hypertension and increased risk of CHD. This scenario is, therefore, another example of a seemingly paradoxical association between the concentration of the biomarker (ecSOD) in relation to its purported role in disease development, and supports the protective role of antioxidants in CHD.

Interestingly, other studies have shown that this same genetic variant associated with higher CHD risk is also associated with lower risk of lung disease[101–103], albeit with some mixed findings[104]. However, this observation can be explained by findings from animal studies; the R231G SNP in the *ECSOD* gene has been shown to reduce ecSOD concentration in blood vessels, while concomitantly increasing ecSOD levels in alveolar fluids. Therefore, this variant might be associated with detrimental effects on the vasculature, but beneficial effects for lung function[105]. Notably, antioxidants might be important only at critical times during the development of vascular disease (unlike LDL cholesterol,

Friedewald equation
The estimation of LDL-cholesterol levels in the absence of its direct measurement, from total cholesterol, high-density lipoprotein cholesterol, and triglycerides

which we discuss below). This critical time effect is consistent with other MR studies that have provided some, admittedly weak, evidence for a causal role for vitamin C deficiency in CHD development[106], and might explain the possible discrepancy between these findings and the null findings from RCTs assessing vitamin C supplementation and risk of vascular disease in later life[107].

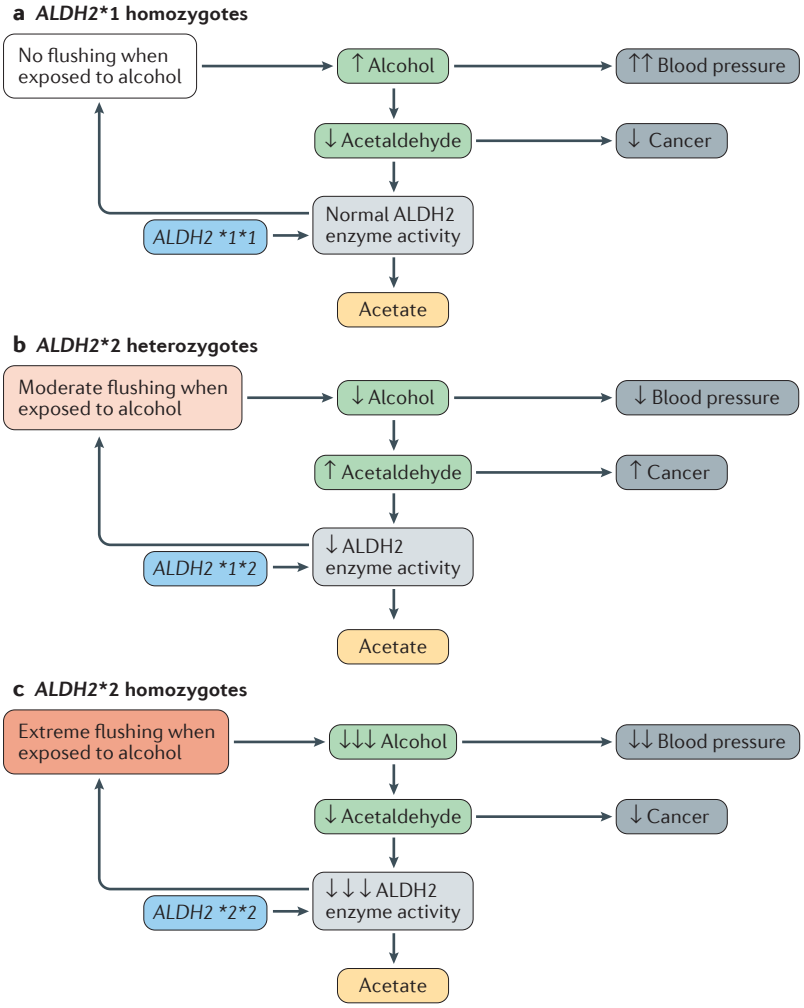


Figure 5 | **Mendelian randomization of biomarkers on the same pathway.** **a** | Individuals who are homozygous for the *ALDH2**1 variant can consume normal amounts of alcohol without symptoms of flushing and nausea, which can lead to increased alcohol intake and subsequent high blood pressure. However, because acetaldehyde is efficiently cleared by aldehyde dehydrogenase (ALDH2), the risk of oesophageal cancer is low. **b** | Individuals who are heterozygous for *ALDH2**2 are likely to consume lower amounts of alcohol than those who are homozygous for the *ALDH2**1 variant, given their symptoms of moderate flushing. This lower alcohol consumption leads to lower blood pressure. Reduced functioning of ALDH2 leads to increased acetaldehyde levels, which in turn results in increased risk of oesophageal cancer. **c** | Individuals homozygous for *ALDH2**2 consume almost no alcohol, given the severe symptoms and, therefore, blood pressure levels are expected to be lower than in those who are heterozygous for *ALDH2**2 or homozygous for the *ALDH2**1 variant. Similarly, acetaldehyde levels in individuals homozygous for *ALDH2**2 are also expected to be lower than in individuals heterozygous for the *ALDH2**2 variant, resulting in lower risk of oesophageal cancer (and similar to or lower than carriers of *ALDH2**1*1, depending on alcohol consumption). These figures are schematic representations and should not be interpreted as formal directed acyclic graphs.

Cumulative exposures

If an exposure accumulates to cause disease over many years, MR analyses might generate a causal estimate that is larger than that from a RCT (which alters exposure for only a limited time period) or from observational epidemiology (which generally captures the effect of the exposure for only a particular time period). In general, MR findings should be interpreted to reflect a lifelong exposure to a biomarker (FIG. 2e), although this inference changes if an exposure occurs only after a certain age (for example, with alcohol or smoking, in which case the genetic instrument influences exposure only after the habit has been taken up)[11]. Furthermore, MR studies should demonstrate that the SNP associates with the biomarker across the lifetime, to allow appropriate interpretations to be made.

LDL cholesterol and CHD risk. An MR study using multiple independent genetic loci that influence concentrations of LDL cholesterol reported that a decrease in LDL-cholesterol level of 1 mmol/l results in >50% reduction in the risk of CHD[39]. This projection is approximately double the estimated effect reported in RCTs for a similar reduction in LDL-cholesterol level (25% reduction in the risk of a major coronary event for each 1 mmol/l reduction with statins)[108]. As such, this magnitude of effect from the MR study could be considered an overestimation[109]. However, the causal estimate from MR depicts lifelong exposure to a harmful trait (FIG. 6c). Given that atherosclerosis is a disease that accumulates over a lifetime[110,111] and the clinical effects of CHD generally present at advanced ages[112], genetic variants provide an insight into the expected effect sizes if interventions were initiated from early childhood to reduce circulating LDL-cholesterol levels. Therefore, effect sizes from MR analyses should not be considered equivalent to those from an RCT of a short-term intervention. Differences in estimates from MR and RCTs can inform about disease latency and critical exposure periods.

Overlapping exposures

An emerging approach in MR analyses is the combination of multiple traits and genetic instruments into one model to try to tease out independent causal effects (so-called 'multivariable MR' (REF. 113)). However, multivariable MR for discrete traits (for example, BMI and blood pressure in relation to cardiovascular disease) has several limitations, such as collider bias, in which the conditioning of a mediator between the exposure and outcome can induce new confounding by factors that are not related to the MR instrument before such conditioning[114,115]. This situation becomes more complex if the traits themselves overlap (that is, they contain the same element in their total value; FIG. 2f).

Non-HDL cholesterol and triglycerides. In a study by Helgadottir and colleagues, gene scores for non-HDL cholesterol and triglycerides were used to determine whether triglycerides have an independent causal role in CHD[116]. The researchers suggested that although LDL cholesterol (calculated using the Friedewald equation[117])

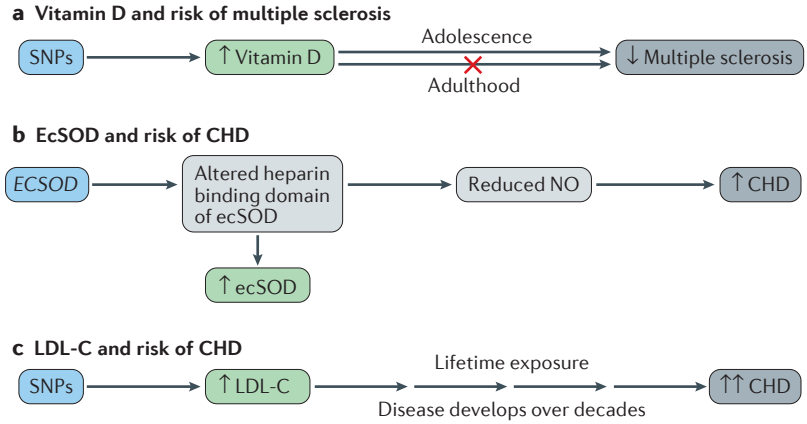


Figure 6 | **Mendelian randomization of a time-dependent and cumulative exposure.** **a** | The genetic variant-to-disease (multiple sclerosis) association reflects lifetime associations (including causal effects mediated through vitamin D that only occur during adolescence). Therefore, Mendelian randomization might provide evidence of a causal effect when this effect actually only occurs during a critical time period. **b** | The genetic variant alters the heparin-binding domain of extracellular superoxide dismutase (ecSOD), meaning that it cannot bind to the external membrane of endothelial cells, and cannot prevent nitric oxide (NO) from being degraded by superoxide anions. Less NO results in vasoconstriction and increased risk of coronary heart disease (CHD). **c** | Genetic variants instrumenting LDL cholesterol (LDL-C) have large effects on risk of CHD. Given that 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-cholesterol levels. These figures are schematic representations and should not be interpreted as formal directed acyclic graphs.

does not include cholesterol from triglyceride-rich lipoproteins (TRLs), non-HDL cholesterol does include this class of lipoproteins. However, estimation of LDL cholesterol via the Friedewald equation also includes intermediate-density lipoproteins[118], and given that intermediate-density lipoprotein particles are semi-enriched in triglycerides, LDL cholesterol estimated via the Friedewald equation should also contain TRL-related cholesterol. A MR analysis that included both non-HDL cholesterol and triglycerides showed that although the association of non-HDL cholesterol with risk of CHD remained largely unaltered, the association of triglycerides with CHD after adjusting for non-HDL cholesterol diminished towards the null[116].

In this scenario, non-HDL cholesterol and triglycerides should not be considered as discrete entities, but as overlapping entities (FIG. 7). Therefore, adjustment of the triglyceride genetic instrument for non-HDL cholesterol adjusts for overlapping components, and the diminution of the triglyceride score should not be interpreted as meaning that triglycerides have no causal effect (that is, in effect, the multivariable analysis has adjusted triglycerides for a component of triglycerides contained within non-HDL cholesterol). By contrast, although non-HDL cholesterol consists of cholesterol in TRLs, it also contains LDL cholesterol, which has a triglyceride-independent effect on CHD[18]. Therefore, a lack of diminution of the association of the non-HDL cholesterol gene score with risk of CHD after adjustment for triglycerides provides no additional information beyond what is already understood about the causal role of LDL cholesterol in CHD, and is not useful for assessing the causality of triglycerides in CHD. By contrast, MR for correlated, but nonoverlapping traits can be highly informative. For example, separate genetic instruments (albiet not using a multivariable MR framework) for CRP and IL-6 show that whereas IL-6 upregulates CRP[119], causality for CHD is limited to IL-6 (REFS 22,23,50,70,71).

Solutions for rigorous interpretations of MR

Although each example highlighted above is unique, they can be categorized into general themes, each of which has potential solutions to aid interpretation. First, when a single genetic variant associates with multiple traits on discrete pathways (horizontal pleiotropy), the use of this individual genetic variant to generate causal estimates for each individual trait is invalid, because 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 BMI[120]) should be undertaken cautiously owing to the high likelihood of horizontal pleiotropy. This notion is especially true for nonprotein (complex) traits, because no single genetic variant will tag a direct pathway to the exposure under investigation.

When an MR analysis generates associations that directly contradict what has been established from observational, epidemiological studies, investigators should consider whether the genetic variants used in the instrument disrupts the normal function of an exposure. If so, the association might arise owing to the biomarker not exerting its normal biological effect, and levels of this biomarker might be elevated despite this lower functional effect. An alternative explanation in this setting might be negative bias owing to unbalanced horizontal pleiotropy. By contrast, if the magnitude of effect from MR is directionally consistent but larger in magnitude than that seen in observational studies, an alternative explanation might be cumulative exposure, given that the genetic variant proxies a lifetime exposure. Alternative explanations include measurement error in the observational analysis (leading to regression dilution bias, from which MR analysis is protected) or a positive bias induced by horizontal pleiotropy.

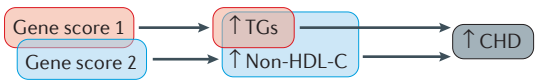


Figure 7 | **Mendelian randomization of overlapping exposures.** As triglycerides (TGs) overlap with non-HDL cholesterol (non-HDL-C), adjusting the association of TGs with risk of coronary heart disease (CHD) for non-HDL-C diminishes the causal effect of TGs to null. By contrast, non-HDL-C contains the entire cascade of apolipoprotein B-containing lipoproteins, including intermediate-density lipoprotein cholesterol and LDL cholesterol, 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 of TG, because it adjusts for an overlapping trait. This figure is a schematic representation and should not be interpreted as a formal directed acyclic graph.

Two-sample Mendelian randomization
The single nucleotide polymorphism (SNP)-to-exposure estimate is obtained from a separate dataset to that of the SNP-to-outcome estimate.

In these examples, pleiotropy can seriously perturb estimates derived from MR analyses. Uncovering the presence of horizontal pleiotropy when using a single genetic variant is challenging. Detailed knowledge about the function of the variable, or access to large cohorts in which a phenome-wide association scan can reveal associations that might be indicative of unknown pleiotropy can guide interpretation. By contrast, when multiple SNPs are used in combination as genetic instruments, approaches now exist (such as MR-Egger; BOX 4) to quantify and assess the presence of pleiotropy, and can provide valid causal estimates even in the presence of pleiotropy (although additional assumptions are required)[53,54,79,113,121–123]. These tests for pleiotropy should be used as sensitivity analyses in addition to conventional MR approaches.

When an MR study provides evidence of causality that has not been recapitulated in RCTs, one explanation is that the biomarker might be causal only during a particular time period of life. Therefore, the evidence obtained from MR studies might not translate into equivalent benefit if the intervention to modify the biomarker is at a different period of the life course from when it has its causal effect.

Finally, when assessing multiple traits in combination, if the traits are overlapping (that is, they contain elements of each other in their individual measures), then MR might not allow reliable individual assessment of which trait is causal. In this scenario, MR analyses are fraught with the same issues as conventional observational, epidemiological studies whereby adjusting for an overlapping trait diminishes associations of traits in the model with risk of disease.

Conclusions

In this Review, we have sought to illustrate and provide explanations for potentially paradoxical and implausible findings from MR analyses. As MR studies are increasingly performed to clarify causal relationships of the expanding number of traits that are measurable (such as '-omics' analyses, including metabolomics[44,124], lipidomics[125], and proteomics[126]), 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 utilizing readily available data increases, the reliability of such studies are likely to decrease, through both methodological errors and through publication bias influencing which results are deemed 'of interest' (REF. 127). Despite these caveats, with increasing large-scale genetic data becoming available to facilitate two-sample MR[128], together with resources such as MR-Base[129], LD Hub[130], and PhenoScanner[131], MR promises to provide an efficient and pragmatic means to identify traits that are likely to be causal in cardiometabolic and other diseases, and to help to prioritize drug targets to take forward into therapeutic clinical trials[132]. Such drug-target prioritization might result in fewer failed multibillion dollar clinical trials and can contribute to improving the drug-development pipeline.

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