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Mendelian Randomization: New Applications in the Coming Age of Hypothesis-Free Causality

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Abstract

Mendelian randomization (MR) is an approach that uses genetic variants associated with a modifiable exposure or biological intermediate to estimate the causal relationship between these variables and a medically relevant outcome. Although it was initially developed to examine the relationship between modifiable exposures/biomarkers and disease, its use has expanded to encompass applications in molecular epidemiology, systems biology, pharmacogenomics, and many other areas. The purpose of this review is to introduce MR, the principles behind the approach, and its limitations. We consider some of the new applications of the methodology, including informing drug development, and comment on some promising extensions, including two-step, two-sample, and bidirectional MR. We show how these new methods can be combined to efficiently examine causality in complex biological networks and provide a new framework to data mine high-dimensional studies as we transition into the age of hypothesis-free causality.

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INTRODUCTION

A central question in epidemiology concerns the degree to which an observational association between two quantities reflects a causal effect of one variable on the other. Unfortunately, observational studies are subject to confounding, reverse causation, and various biases that often make it impossible to know whether such an association reflects a causal relationship. Indeed, there is a long history of observational epidemiological studies purporting to show robust associations between a variety of risk factors and disease that upon subsequent investigation turned out to be noncausal, most probably owing to the presence of residual confounding. Some prominent examples include the observational associations between vitamin supplements and coronary heart disease (87, 104, 123), beta-carotene and lung cancer (86), and hormone replacement therapy and cardiovascular disease (106).

The gold standard for demonstrating causality between a medically relevant exposure and outcome is the randomized controlled trial (RCT). However, RCTs are expensive, are often of long duration, and are not always ethical or generalizable to populations outside the strictly controlled confines of the study. Mendelian randomization (MR) is an approach that uses genetic variants robustly associated with a modifiable exposure or biological intermediate of interest to estimate the causal relationship between these variables and a medically relevant outcome free from the influence of confounding. Although it was initially developed to examine the relationship between modifiable exposures/biomarkers and disease (35), its use has expanded to encompass applications in molecular epidemiology, systems biology, pharmacogenomics, and other areas. This is important because an increasing number of studies are investigating relationships between high-throughput molecular intermediates (e.g., DNA expression, gene methylation, metabolites, and metagenomic information), and these investigations suffer from all the same issues of confounding and reverse causality as more traditional measurements.

The purpose of this review is to introduce MR, the principles behind the approach, and its limitations. We consider some of the new applications of the methodology, including its growing role in drug development, and comment on some promising extensions to the approach, including two-step, two-sample, and bidirectional MR. We show how these new methods can be combined to efficiently examine causality in complex biological networks and provide a new framework to data mine high-dimensional studies as we transition into the age of hypothesis-free causality (33).

HISTORY AND PRINCIPLES OF MENDELIAN RANDOMIZATION

MR refers to the random segregation and assortment of genes from parent to offspring that occur during gamete formation and provides a method of using genetic variants in observational settings to make causal inferences regarding the relationship between exposures and outcomes. The basic principle utilized in the MR framework is that if genetic variants either alter the level of or mirror the biological effects of a modifiable exposure that itself alters disease risk, then these genetic variants should be related to disease risk to the extent predicted by their influence on exposure to the risk factor (35). This is qualitatively different from the traditional gene discovery paradigm typified in genome-wide association studies (GWAS), where the focus is on demonstrating a statistical association between a genetic variant and an outcome. Rather, in MR studies, the aim is to provide evidence for or against a causal relationship between a modifiable exposure variable and a disease or health-related outcome of interest (and, often, to estimate the magnitude of this causal relationship).

The influential statistician and geneticist Ronald Fisher (49) was well aware of the peculiar advantages afforded by genotypes and their potential use in scientific study designs. Indeed, genetic

variation has been utilized in various ways over the last few decades to leverage evidence about the potentially causal nature of exposure-disease associations (32). In 1986, Katan (67, 68) explicitly described the use of genetically influenced differences in a modifiable risk factor to avoid the problem of reverse causation when he suggested that polymorphisms of the *apolipoprotein E* (*ApoE*) gene could be used to interrogate the observational relationship between low cholesterol levels and risk of cancer. The general lack of confounding, absence of reverse causation, protection against measurement error, and avoidance of other biases in observational studies inherent in what is now termed the MR approach were formulated in the early years of this century (35). The ability to estimate the magnitude of the causal effect of the long-term exposure on the modifiable exposure of interest was also recognized (35). Since the formal presentation of the overall approach, there have been more than 350 mentions of the phrase "Mendelian randomization" in the title or scientific abstract of indexed peer-reviewed publications, with a steeply increasing trajectory (PubMed, accessed November 18, 2014). **Table 1** describes some recent MR studies.

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Table 1 Examples of recent Mendelian randomization studies

Study	Reference	Exposure	Outcome	Instrument	Description of results
Holmes et al. (2014)	60	Alcohol intake	Cardiovascular risk factors, CHD, stroke	Variant in *ADH1B*	Increased alcohol intake is associated with a worse cardiovascular risk profile, increased risk of CHD, and ischemic stroke.
Holmes et al. (2014)	61	BMI	Fasting glucose, fasting insulin, IL6, SBP, HDL, LDL, T2D, CHD, stroke	14 BMI-associated variants	BMI causally influences T2D, stroke, and a range of cardiometabolic traits, but a causal association with CHD risk is questionable.
Proitsi et al. (2014)	98	LDL, HDL, TG, TC	Late-onset Alzheimer's disease	157 SNPs associated with lipid levels	No strong evidence was found of a causal relationship between lipids and late-onset Alzheimer's disease.
Richmond et al. (2014)	102	BMI	Exercise	32 BMI-associated variants and a genome-wide allelic score related to physical activity	Bidirectional MR was performed using BMI variants and genome-wide score for physical activity. The results suggested that an increase in BMI causes a reduction in physical activity and not vice versa.
Smith et al. (2014)	116	LDL, HDL, TG	Aortic stenosis	Allelic scores of known variants from large genome-wide association studies	Increased LDL causally increases risk of aortic stenosis.
Thrift et al. (2014)	130	BMI	Barrett's esophagus, esophageal adenocarcinoma	29 BMI-associated variants	Increased BMI causally increases risk of Barrett's esophagus and esophageal adenocarcinoma.
Wium-Andersen et al. (2014)	145	CRP	Depression	4 variants in CRP-encoding gene	CRP is not causally associated with depression.

Abbreviations: BMI, body mass index; CHD, coronary heart disease; CRP, C-reactive protein; HDL, high-density lipoprotein cholesterol; IL6, interleukin 6; LDL, low-density lipoprotein cholesterol; SBP, systolic blood pressure; SNP, single-nucleotide polymorphism; T2D, type 2 diabetes; TC, total cholesterol; TG, triglycerides.

There are several reasons for using genetic variants as proxies for exposure variables rather than using the exposures themselves. First, genetic variants are less susceptible to confounding. As stated by Mendel's first law (the law of segregation), genetic variants segregate randomly and independently of environmental factors, and Mendel's second law (the law of independent assortment) suggests that genetic variants should also segregate independently of other traits (although with some exceptions—see below) (79). There is empirical evidence that this is indeed the case (subject to certain qualifications, described below). For example, one study showed that although 96 nongenetic characteristics showed substantial intercorrelation with one another, 23 genetic variants were associated with the same traits only at the level expected by chance (37).

Second—and in sharp contrast to retrospective observational study designs—provided that a genetic instrument has been appropriately chosen, reverse causality is not an issue in MR studies, because an individual's germline genotype must precede the outcome of interest. Third, MR studies are arguably less prone to bias from measurement error than other observational studies. Most genetic variants are measured with a high degree of precision. By contrast, exposure variables are often subject to considerable measurement error in traditional observational association studies. Random error in measuring the exposure variable can bias estimates of the regression coefficient in ordinary least squares regression toward the null and can contaminate other forms of analysis, including mediation and path analyses, producing erroneous inferences (52, 90, 94, 118, 119). Lastly, and related to the point above, an individual's genotype can be thought of as providing a lifelong exposure in contrast to a one-off measurement, which again may be subject to error.

ANALOGY BETWEEN MENDELIAN RANDOMIZATION AND RANDOMIZED CONTROLLED TRIALS

In RCTs, the process of randomization ensures that in large samples the distribution of known and unknown confounders is balanced evenly across the different treatment arms. Assuming that the trial is conducted competently and compliance is perfect, differences between the groups at the end of the trial should reflect only the different treatments received, enabling the investigators to draw conclusions regarding the effectiveness of the treatment protocol. In MR, the segregation of alleles during meiosis is analogous to the randomization process in RCTs, except that in MR, the randomization occurs at conception and the causal effects estimated in the analysis represent the long-term effects of lifelong exposures. This implies that MR estimates of causal effects may be larger than the benefits seen following treatment, because the latter will generally be for only a fraction of the lifetime.

More formally, MR analysis uses genetic variants as instrumental variables (IVs) to provide evidence of causality between an exposure and outcome. IVs are factors that are associated with the exposure of interest but do not suffer from confounding, reverse causality, or other biases that typically plague traditional observational studies. To be used as an IV, a genetic variant [or an allelic combination of such variants (24)] must satisfy three core assumptions (40): (*a*) The instrument must be associated with the exposure of interest, (*b*) the instrument must not be associated with confounders of the exposure-outcome association, and (*c*) the instrument must not affect the outcome except possibly through the exposure variable (see **Figure 1**).

The first of the core assumptions can be easily verified by examining the strength of association between the genetic instrument and the exposure. Single genetic variants can be used as instruments, or the effect of several polymorphisms could be used in the form of a weighted or unweighted allelic score to increase power (24). Note that the genetic variant does not have to be a true functional variant that produces a downstream effect on the exposure; rather, it needs only

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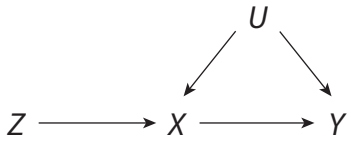

Figure 1

Directed acyclic graph showing the assumptions of the Mendelian randomization methodology. The different nodes in the graph represent the genetic instrument (Z), the modifiable exposure variable (X), the outcome (Y), and the effect of all possible confounding variables on variables X and Y (U). Causal effects are denoted by arrows; the absence of an arrow indicates no direct causal effect. (Note that for the graphical model to be directed, there must be no feedback loops in the system—i.e., a variable cannot be a direct or indirect cause of itself.) For Z to be used as an instrumental variable, it must be associated with X (core assumption one, denoted by the arrow between Z and X), must not be related to confounders of the exposure-outcome relationship (core assumption two, denoted by a lack of paths between Z and U), and must be related to the outcome only through the exposure of interest (core assumption three, denoted by an absence of paths from Z to Y except the one that passes through X).

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to be a marker in linkage disequilibrium with a functional variant. Intuitively, one can think of the IV as dividing the sample into subgroups that differ with respect to the exposure of interest and any causal descendants, but not with respect to confounding variables. Because one does not require knowledge of the functional variant to do this, a variant in linkage disequilibrium suffices for this purpose.

Although it is technically impossible to prove that the second assumption holds true for all possible confounders, it is possible at least to examine the relationship between the genetic instrument and a range of likely measured confounders (and this should be done wherever possible). The absence of a statistical association between the two can increase confidence (but never prove) that the second core assumption is satisfied.

The third core assumption is possibly the most problematic for MR and can never be proved for certain, in that it is always possible that the instrument could affect the outcome via a biological pathway other than the exposure of interest. To minimize this possibility, a promising strategy is to use genetic variants in genes where the functions of those genes and their relationship to the exposure are well understood. If the intermediate of interest is a protein, then the best strategy is generally to use a genetic variant in the gene coding for the protein itself. When multiple instruments are available, it may be possible to fit a series of models involving different instruments (34, 88). If the different analyses yield heterogeneous estimates of the causal effect, then this suggests that core assumption three has been violated, and genetic pleiotropy (see **Table 2**) is likely to be an issue.

Finally, making valid causal inferences from MR analysis requires a further structural assumption—specifically, that intervening on the exposure does not affect any of the other terms in the joint probability density function of the directed acyclic graph (40). In other words, it is assumed that naturally induced changes in the exposure variable produce the same change in the outcome variable as an experimenter-directed alteration. If all of these assumptions are satisfied, then a statistical test of the association between the genetic instrument and the outcome is a valid assessment of the presence of a causal relationship between the exposure and the outcome (40). However, a simple test of association does not provide a causal estimate of the effect of the exposure on the outcome, and consequently it is difficult to know whether a negative test result represents a true finding or is simply due to lack of power.

The IV core assumptions can be violated in multiple ways, and these are summarized in **Table 2** together with other potential complicating factors of MR analysis and methods to control or mitigate these problems. Pleasingly, though, it appears that the results of many published MR

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Table 2 Potential pitfalls in Mendelian randomization studies

Phenomenon	Description/explanation	Strategy for detecting or reducing influence
Genetic instrument unavailable	Sometimes genetic variants are unavailable to proxy the exposure or intermediate of interest.	Genome-wide association studies are discovering an increasing number of variants related to exposures or intermediates; sequencing studies have potential to discover low-frequency variants; genome-wide allelic scores might have some utility, but their use is controversial and likely premature (43).
Weak instruments	Analyses performed with instruments that show weak association with the exposure (typically a regression F ratio < 10) bias the instrumental variable estimate toward the observational association (23, 122).	Increase sample size; combine genetic variants into an allelic score to increase instrument strength; use the limited information likelihood estimator, which is close to median unbiased (5, 38), although some discourage its use (57).
Low power	Mendelian randomization analyses have low power (17, 92).	Increase sample size; combine genetic variants into allelic score (92).
Pleiotropy	The genetic variant is associated with a risk factor for the outcome that is not on the same causal path as the exposure under study, or the genetic variant affects the outcome through a pathway other than the exposure (139).	Use variants in genes where the function of the gene is well understood (or directly codes for the intermediate, if possible); test the relationship between the instrument and potential confounding variables to detect violations of assumptions; use different genetic instruments and test for heterogeneity of causal effect estimates; show that the association between the genetic instrument and outcome is present only in individuals positive for the exposure (although this may be possible only in some situations).
Linkage disequilibrium	Another variant may be in linkage disequilibrium with the genetic instrument, and this affects the outcome through a pathway other than the exposure (similar to pleiotropy).	Similar to above for pleiotropy.
Population stratification	The sample consists of different subpopulations and/or admixed individuals who also differ in the frequency or mean of their exposure and/or outcome. This can induce spurious associations among the genetic instrument, exposure, and outcome.	Restrict analysis to ethnically homogeneous individuals; include ancestry-informative principal components in the analysis; perform an analysis within families.
Canalization/ developmental compensation	An individual adapts in response to a genetic change so that the effect of that genetic change is reduced or absent (138). This may produce estimates that are not representative of modifying the exposure.	No strategy known (although knowledge of underlying biology may suggest whether this likely to be a problem).
Complexity of association	Inadequate knowledge regarding the underlying biology of the instrument, exposure, and outcome may produce misleading inferences.	Increase the biological understanding of the relationship between genotype, intermediate, and phenotype.

studies have shown good concordance with the findings from RCTs of the same exposure and outcomes, suggesting that the method is valid and of considerable utility if it is used appropriately and the limitations of the approach are kept in mind (**Table 3**).

Prizment et al. (97) illustrated some of the difficulties faced in interpreting the results of MR studies, particularly those that combine several single-nucleotide polymorphisms (SNPs) into a single genetic score. The authors examined the relationship between a genetic risk score consisting of 20 SNPs robustly related to increased levels of C-reactive protein (CRP) and risk of

Table 3 Alignment between Mendelian randomization (MR) studies and randomized controlled trials (RCTs)

Exposure	Outcome	Comments on MR study	Comments on RCT
HDL	MI	It is difficult to find variants that are specific for one blood lipid fraction, potentially complicating interpretations of MR studies (147). However, variants specific for HDL, such as the loss-of-function coding SNP in the endothelial lipase gene (*LIPG* N396S), are not strongly related to risk of CHD, suggesting that HDL has either a small or no causal effect on MI risk (137).	RCTs involving a range of drug treatments that increase HDL levels, including CETP inhibitors (11, 26, 112), combination therapy of statin plus fibrates (1), and niacin treatment (62), do not decrease risk of MI.
LDL	CHD	There is strong evidence that genetic variants that increase LDL also proportionately increase risk of CHD (46).	HMGCR inhibitors, which decrease LDL, have well-documented efficacy in reducing risk of CHD (9).
LDL	Late-onset Alzheimer's disease	Genetic risk scores proxying LDL show no relationship with late-onset Alzheimer's disease (98).	There is no evidence that RCTs of LDL-lowering statins have any effect on risk of Alzheimer's disease (45, 76).
Maternal folate	Neural tube defects	Mothers who have the TT *MTHFR* C677T genotype are at increased risk of having children with neural tube defects (148), but this is not the case for fathers (50), suggesting that increased maternal folate consumption is protective against neural tube defects.	RCTs indicate that maternal folate supplementation decreases the risk of neural tube defects in offspring (31).
Statins	T2D	Variants in the *HMGCR* gene are associated with increased risk of T2D (125).	A meta-analysis of RCTs of statins showed a dose-dependent increase in T2D for individuals on statins compared with those on a placebo (96, 108).

Abbreviations: CETP, cholesteryl ester transfer protein; CHD, coronary heart disease; HDL, high-density lipoprotein cholesterol; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDL, low-density lipoprotein cholesterol; MI, myocardial infarction; SNP, single-nucleotide polymorphism; T2D, type 2 diabetes.

several cancers, including colorectal cancer. They found that the score was related to increased risk of colorectal cancer, which they interpreted as supporting a causal effect of CRP on cancer risk. However, the difficulty in interpreting the results in this fashion is that it is possible that many of the SNPs in the risk score may exert pleiotropic effects on CRP through pathways directly upstream of CRP [e.g., interleukin 6 receptor (IL6R)], through adiposity, or via pathways unrelated to inflammation. Indeed, the association between the CRP risk score and colorectal cancer in this study was only marginally attenuated after adjusting for plasma CRP, consistent with the notion that at least some of the CRP SNPs might exert their effect on cancer risk through pathways independent of CRP. Indeed, the authors tried to discount this possibility by examining the association between different subsets of SNPs and cancer risk and showing that the association was present in each of the analyses—although this of course does not address the possibility that pleiotropy was an issue in each of the subsets of SNPs analyzed.

Another strategy to minimize the possibility of horizontal pleiotropy is to examine the association between variants within the *CRP* gene itself and risk of cancer. There are several variants within *CRP* that influence the level of serum CRP and are not located in the coding sequence of the gene and do not lead to alternative splicing, isoforms, etc., and so it could be argued that these polymorphisms are less likely to influence risk of colorectal cancer except through CRP itself. Allin et al. (2) found no relationship between several of these variants and risk of cancer (including colorectal cancer), suggesting that the relationship between the two was not due to a

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causal effect of CRP. However, a more recent study that used a similar set of SNPs did find a positive association between the variants in *CRP* and risk of colorectal cancer (84). Clearly, the jury is still out on whether the association between CRP and colorectal cancer reflects a causal relationship, and larger studies will need to be conducted, keeping in mind the complexities of utilizing the myriad of variants related to CRP levels.

ESTIMATING THE SIZE OF CAUSAL EFFECTS

Estimating the size of causal effects requires additional assumptions that depend on the nature of the exposure and outcome. For example, in the case of continuous exposures and outcomes, linearity is typically assumed between the variables, as is the absence of statistical interaction between the exposure and unmeasured confounders. Under these assumptions, the simplest way to estimate the causal effect of the exposure on the outcome ($\hat{\beta}_{\mathrm{IV}}$) is via the Wald method (140), which is simply the ratio of the linear regression coefficient of the outcome on the instrument ($\hat{\beta}_{\mathrm{Y|G}}$) to the regression coefficient of the exposure on the instrument ($\hat{\beta}_{\mathrm{X|G}}$):

$$\hat{\beta}_{\mathrm{IV}} = \frac{\hat{\beta}_{\mathrm{Y|G}}}{\hat{\beta}_{\mathrm{X|G}}}.$$

Standard errors can be calculated in a variety of ways, including via the delta method (129), Fieller's theorem (48), and bootstrapping (41). Several other methods of IV estimation are also available, including two-stage least squares, which is useful when multiple genetic instruments are fit simultaneously to the data, and limited information likelihood (LIML), which may have advantages if the investigator can use only weak instruments (5, 38) (see also **Table 2**). In the case of binary outcomes, where the assumption of linearity between the exposure and outcome is more tenuous, various forms of semiparametric estimation that do not make strong assumptions about the form of the relationship between exposure and outcome can be used to estimate causal effects, including G-estimation and the generalized method of moments (for a review of these methods, see 89).

NOVEL APPLICATIONS OF MENDELIAN RANDOMIZATION

Table 1 lists some recent examples of MR studies of behavioral factors and biomarkers as potential causes of a variety of health outcomes. Such conventional MR approaches are now widely used and have been reviewed in both general and domain-specific fashions (34, 53, 65). Here, we describe some exciting recent extensions to the MR methodology that are different from the traditional uses of MR that have focused on the causal effect of environmental exposures on medically relevant outcomes, and have been discussed in several other reviews.

Using Mendelian Randomization to Inform Drug Development

Although GWAS have been extremely successful over the last decade in identifying common genetic variants associated with complex traits and diseases, many critics of the approach have argued that the results from such studies have been slow to translate to the clinic in the form of drug therapies (136). However, this overlooks the fact that many GWAS discoveries could be used immediately to guide the process of drug development using the principles of MR. A major concern in the pharmaceutical industry is late-stage failure, where novel compounds that show considerable potential in preclinical and animal studies turn out to lack efficacy or have important toxicological side effects in phase III clinical trials (6). By this stage, of course, millions of dollars and many years of research have often been wasted developing the compound. There is no guarantee that a

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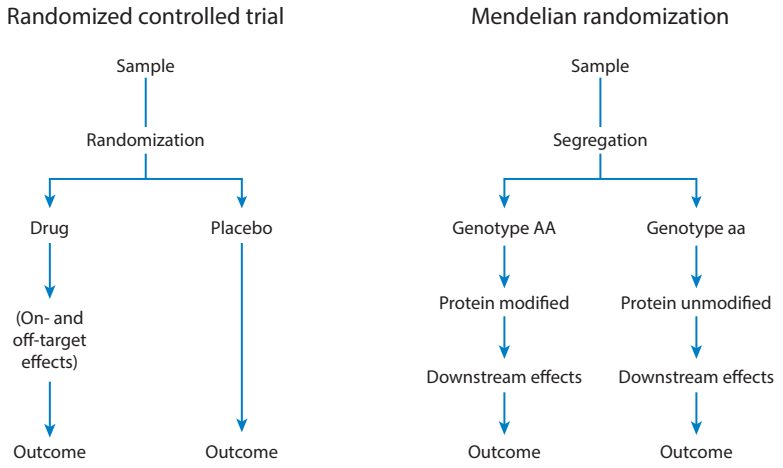

Figure 2

Similarities and differences between a randomized controlled trial of a drug and a placebo compared with Mendelian randomization of a genetic variant in a drug target. In the randomized controlled trial, the drug may produce both on- and off-target effects. By contrast, genetic variants in the gene of the target of interest should produce only on-target effects.

compound that works well in vitro or in animal models will succeed in clinical trials, and indeed, the vast majority of compounds that undergo development are never approved for clinical practice (7). Part of this inefficiency is a direct consequence of the traditional model of drug development: Pharmaceutical companies must commit substantial time and financial resources to following up a new compound before embarking on large-scale phase III clinical trials, which have no guarantee of success. MR can assist in this process by providing a useful adjunct (although not a substitute) at several points in the drug development pipeline (95).

The basic idea is that variants in a gene encoding a specific drug target that alters the level of activity of the encoded protein can be used to mimic the consequences of targeting the same protein pharmacologically using an idealized compound free from off-target effects (i.e., effects of a compound that are realized through mechanisms independent of the intended drug target). This approach is slightly different from usual applications of MR in that the focus is on the drug target itself rather than on biomarkers, exposures, or intermediates that may be downstream of the target. MR could therefore be used to assess not only the probable efficacy of pursuing a particular target, but also whether unintended side effects of the drug are likely due to on- or off-target effects (117) (see **Figure 2**).

MR could be used advantageously at several stages in the drug development pipeline. Early in the process, it could be used to ascertain whether time and money are likely to be well spent developing compounds targeting a particular protein. If MR studies indicate that variants within the gene encoding the protein have little effect on disease risk, then targeting other proteins might be preferable. The same principle could also be applied to choosing which compounds to prioritize for development in small-molecule libraries.

One example concerns recently developed monoclonal antibodies to the proprotein convertase subtilisin/kexin type 9 serine protease (PCSK9). PCSK9 is synthesized primarily in the liver and enters the circulation, where it binds to low-density lipoprotein (LDL) receptors on the surface of the liver. The process decreases the capacity of the liver to remove LDL cholesterol from circulation and results in increased LDL cholesterol levels. Genetic association studies have shown

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that variants in *PCSK9* that are relatively selectively associated with reduced LDL cholesterol levels are also associated with reduced risk of coronary heart disease (27). Phase I clinical trials of monoclonal antibodies to PCSK9 are now under way, and have shown that the compound is well tolerated and significantly reduces LDL cholesterol in healthy volunteers in conjunction with statin therapy (124). Although it remains to be seen whether these compounds also reduce the risk of coronary heart disease, results to date show considerable promise.

More recently, there has been considerable excitement over the identification of low-frequency loss-of-function mutations within the *APOC3* gene that are associated with low levels of triglycerides (66, 127). APOC3 is a glycoprotein synthesized chiefly in the liver that is present on ApoB-containing triglyceride-rich particles [chylomicrons and very-low-density lipoproteins (VLDLs)] and to a lesser extent on high-density lipoprotein (HDL) particles. It has a variety of functions, including inhibiting lipoprotein lipase (an enzyme responsible for the hydrolysis of triglyceride-rich particles), reducing the liver's uptake of triglyceride-rich lipoproteins, and promoting triglyceride and VLDL synthesis and secretion by the liver (14, 55). Low-frequency loss-of-function variants in *APOC3* that are related to decreased levels of triglycerides are also associated with reduced risk of ischemic cardiovascular disease (66, 127). Although the precise mechanism by which the variants reduce risk of coronary heart disease is uncertain and may be mediated by factors other than reduced triglycerides (28), second-generation antisense oligonucleotides that selectively inhibit APOC3 are already in development and show considerable promise in initial studies (56). Other promising examples of MR's potential utility in the field of cardiovascular pharmacology are also appearing (82).

Finally, at the other end of the spectrum, another example concerns recently developed oligonucleotide inhibitors of CRP (85). CRP is an inflammatory biomarker that is inflated in many lifestyle diseases, including coronary heart disease and metabolic syndrome, and it has often been suggested as a target for pharmacologic intervention. However, MR studies utilizing variants within the *CRP* gene that affect levels of the protein have consistently failed to find any evidence of a causal association with coronary heart disease (25) or metabolic phenotypes (131), suggesting that this compound is unlikely to be useful in treating these conditions and should perhaps be targeted toward other disorders.

MR can also be useful in investigating on- and off-target drug effects, possible side effects, and whether the balance of favorable and unfavorable side effects is likely to be acceptable (141). An example is the cholesteryl ester transfer protein (CETP) inhibitor torcetrapib, which was designed to increase HDL cholesterol levels by inhibiting the CETP molecule but exhibited unexpected blood-pressure-raising effects in clinical trials (11). MR could be used to examine the relationship between functional variants within the *CETP* gene and blood pressure. A positive association would suggest a class effect and that all CETP inhibitors might be expected to show an effect on blood pressure (117). The lack of such an association would suggest that the effect is specific to the torcetrapib molecule and unlikely to be shared by other members of the class. Indeed, this seems to be the case, as RCTs of other CETP inhibitors do not appear to show an increase in blood pressure (26).

Another recent example concerns the observation in RCTs that statin treatment appears to increase the risk of type 2 diabetes in a dose-dependent fashion (96, 108). The question, however, is whether this represents an on- or off-target effect—that is, is this effect mediated through inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) or explained by one of the proposed pleiotropic mechanisms of statins? A recent meta-analysis showed that two variants in the *HMGCR* gene that were related to decreased LDL cholesterol were also related to a host of metabolic measures and increased risk of type 2 diabetes (125), suggesting that statins do indeed causally increase risk of type 2 diabetes and that this is mediated through an on-target effect of the

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drugs. Indeed, MR studies that have revealed an association between genetic variants in a drug target and a range of different biomarkers suggest that it would be wise to monitor these biomarkers in upcoming clinical trials. For example, the P446L variant in the *glucokinase regulator* (*GCKR*) gene is associated not only with lower plasma glucose levels but also with higher triglyceride levels (12), suggesting that additional monitoring of triglyceride levels might be advisable in future clinical trials of glucokinase activators (22).

Finally, MR could be used to better target existing agents that have been licensed for other conditions through the process of drug repurposing or repositioning (7, 95, 107). An advantage of drug repositioning over the traditional pipeline is that the repurposed drug has already passed initial studies on its toxicity and could therefore proceed more quickly through the approval process. Although few existing therapies have been repurposed successfully all the way through clinical trials based on GWAS results, several promising candidates are currently undergoing trials or being considered for this purpose (95). Examples include trials of eculizumab, which targets the immune complement system in age-related macular degeneration (149). The importance of this pathway in disease pathogenesis was identified in one of the very first GWAS (69). Likewise, a nonsynonymous variant in the *IL6R* gene is associated with increased levels of circulating IL6, consistent with the action of the IL6R blocker tocilizumab, which has been approved in treating rheumatoid arthritis (54). The same variants are also associated with reduced risk of coronary heart disease events, suggesting that tocilizumab might be a promising candidate for coronary heart disease prevention (64).

Using MR to inform the drug development process has many obvious advantages. As outlined above, it can lead to substantial efficiencies and cost savings. Information can be leveraged from existing cohort studies and GWAS meta-analyses. MR should not be seen as a substitute for RCTs but merely as an adjunct for assisting at various points in the developmental pipeline. RCTs will continue to be required because novel compounds may have actions that cannot be modeled genetically, and the effect of the intervention needs to be quantified reliably to ensure that it is cost effective and to precisely quantify the balance between benefits and any harms (59).

The use of MR in the drug discovery pipeline has all the limitations of traditional MR analyses as well as some others that are specific to this paradigm. First, one must have a functional genetic variant that affects the drug target of interest (i.e., genetic variants that do not have functional consequences are less likely to be useful from the perspective of MR). Fortunately, the overwhelming majority of drug targets are proteins, many of which have *cis*-acting variants that at least affect gene expression (although perhaps not protein levels or function), suggesting that such instruments may be plentiful. The second difficulty is that sometimes it is not possible to know whether a genetic association with disease suggests that an agonist or antagonist would be better suited to treating the condition. A final consideration is that it is by no means certain that genes (and their products) implicated by GWAS in the etiology of disease will have clinical utility as therapeutic targets once the disease process has been initiated. In other words, for some conditions, once the disease process has begun, it may not be treatable by targeting the processes responsible for its development. Many cancers are likely to fall into this category. For example, in the case of lung cancer, even though variants in the *CHRNA5-CHRNA3* gene cluster may contribute to risk of lung cancer, most likely by influencing smoking intensity (81), it is highly unlikely that modifying this gene or its product, or indeed stopping smoking, will have any direct beneficial effect on preexisting cancer in individuals who already suffer from the disease. Conversely, there are without doubt diseases at the other end of the spectrum, for which discovering predisposing genes and targeting their products will result in therapies that offer a cure, reversal, remission, or at least treatment of the underlying condition.

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NOVEL EXTENSIONS OF MENDELIAN RANDOMIZATION

Two-Sample and Subsample Mendelian Randomization

In many situations, the exposure of interest, the outcome, and the genetic instrument have all been measured in the same sample, and it is possible to perform ordinary MR analysis. In other circumstances, however, these quantities have been ascertained in different samples or sometimes in a limited number of overlapping individuals. This situation is common in studies involving molecular intermediates, where it is often expensive to assay the measures on all the samples. In these circumstances, it is still possible to estimate the causal effect of the exposure on the outcome by employing two-sample IVs or (in the case of partially overlapping samples) split-sample estimators (4, 63, 93).

Under the usual IV assumptions, and assuming that the two samples have been drawn from the same population (i.e., if not, then the two-sample estimator will result in a biased estimate of the causal relationship between the two variables), two-sample estimators can be calculated by first regressing the outcome on the genotype in one sample ($\hat{\beta}_{GY}$) and then regressing the exposure on the genotype in the other ($\hat{\beta}_{GX}$). The ratio of these two quantities is the two-sample IV estimator ($\hat{\beta}_{TSIV}$):

$$\hat{\beta}_{TSIV} = \frac{\hat{\beta}_{GY}}{\hat{\beta}_{GX}}.$$

The standard error for the two-sample IV estimator must take into account that both the numerator and denominator are estimated. Standard errors can be estimated in several ways (e.g., Fieller's theorem), as discussed in detail elsewhere (63, 93).

Pierce & Burgess (93) have shown that, in the case of strong instruments, two-sample and subsample MR analyses have much the same power as a full-sample MR analysis. This has important implications for the design of MR studies because exposure and outcome data can be expensive to measure in large numbers of individuals. Importantly, the two-sample approach means that summary-level data from publicly available GWAS data sets could be used in the analysis. The results of many entire GWAS are now available freely online (83) or in public archives such as the Database of Genotypes and Phenotypes (dbGAP) (74) and the European Genome-Phenome Archive (47). **Table 4** lists several websites where the results of GWAS may be downloaded and used in two-sample MR. Summary association statistics have also been made publicly available for molecular phenotypes, including the results of expression quantitative trait locus (eQTL) (144) and metabolomic GWAS analyses (113). These studies could be combined efficiently and cost effectively using the two-sample framework and then easily harvested to examine possible causal associations of interest. An overall estimate of the causal effect could then be obtained by combining IV estimates from several variants using inverse variance meta-analysis (78) or simple likelihood or Bayesian procedures (19).

Given its potential, two-sample MR has, until recently, been underutilized in the literature to date, with only a handful of studies taking advantage of the approach (91). However, one of the problems of relying on summary statistics to perform MR is that it may be difficult to test the underlying IV assumptions (e.g., IV core assumption two—that the instrument is not related to variables confounding the exposure-outcome association) and the linearity of the genotype-exposure association or of the risk factor–outcome association (40). In the latter case, methods have been proposed for examining the linearity of the association between the potential causal factor of interest and the disease outcome within the MR framework (21, 115). Publication bias and the winner's curse (29)—i.e., the phenomenon that effect sizes in discovery GWAS are

Table 4 Publicly available genome-wide association study meta-analysis results

Phenotype	Study	Reference	Study size	Website
Alzheimer's disease	Lambert et al. (2013)	70	17,008 cases 37,154 controls	**http://www.pasteur-lille.fr/en/recherche/u744/igap/igap_download.php**
Anorexia	Boraska et al. (2014)	16	2,907 cases 14,860 controls	**http://www.med.unc.edu/pgc/downloads**
Bipolar disorder	Psychiatr. GWAS Consort. Bipolar Disord. Work. Group (2011)	99	7,481 cases 9,250 controls	**http://www.med.unc.edu/pgc/downloads**
BMI	Speliotes et al. (2010)	120	123,865 individuals	**http://www.broadinstitute.org/collaboration/giant**
Bone mineral density	Estrada et al. (2012)	42	32,961 individuals	**http://www.gefos.org/?q=content/data-release**
CHD	Schunkert et al. (2011)	111	22,233 cases 64,762 controls	**http://www.cardiogramplusc4d.org**
Crohn's disease	Barrett et al. (2008), Franke et al. (2010)	10, 51	6,333 cases 15,056 controls	**http://www.ibdgenetics.org**
Depression	Major Depress. Disord. Work. Group Psychiatr. GWAS Consort. (2013)	75	9,240 cases 9,519 controls	**http://www.med.unc.edu/pgc/downloads**
Educational attainment	Rietveld et al. (2013)	103	101,069 individuals	**http://ssgac.org/Data.php**
Height	Lango Allen et al. (2010)	71	133,653 individuals	**http://www.broadinstitute.org/collaboration/giant**
Rheumatoid arthritis	Stahl et al. (2010)	121	5,539 cases 20,169 controls	**http://www.broadinstitute.org/ftp/pub/rheumatoid_arthritis**
Schizophrenia	Schizophr. Work. Group. Psychiatr. Genomics Consort. (2014)	110	36,989 cases 113,075 controls	**http://www.med.unc.edu/pgc/downloads**
T2D	Morris et al. (2012)	80	12,171 cases 56,862 controls	**http://diagram-consortium.org/downloads.html**
Ulcerative colitis	Anderson et al. (2011)	3	6,687 cases 19,718 controls	**http://www.ibdgenetics.org**
Waist-hip ratio	Heid et al. (2010)	58	77,167 individuals	**http://www.broadinstitute.org/collaboration/giant**

Abbreviations: BMI, body mass index; CHD, coronary heart disease; T2D, type 2 diabetes.

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likely to be overestimated—may also inflate the strength of association between genotype and intermediate and hence bias the results of the MR analysis. If original data (i.e., data not based on summary statistics from meta-analysis) are available, then it may be possible to mitigate this problem by using half of the sample as the discovery set and the other half to estimate the causal effect (102). Interestingly, in two-sample MR, weak IVs lead to estimates of the causal effect that are biased toward the null hypothesis (4, 63), which is opposite to the situation in traditional MR analysis, where IV estimates are biased toward the observational association in the presence of weak instruments. Given that the two estimates are generally biased in opposite directions, the two forms of analysis can provide boundaries on the likely size of the causal effect.

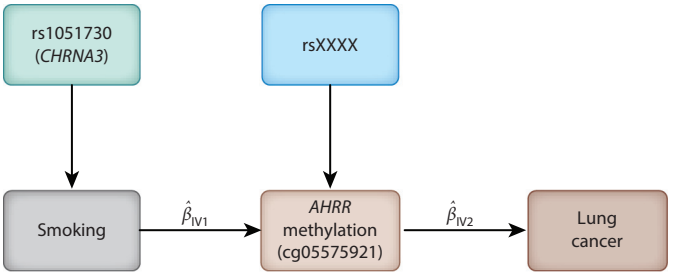

Figure 3

Example of two-step Mendelian randomization, which aims to quantify the extent to which a causal relationship between an exposure and outcome is mediated by a particular variable. In this hypothetical application from epigenetic epidemiology—where there is often considerable uncertainty about the extent to which observational associations reflect causality, as opposed to latent confounding or reverse causation (101)—the aim is to investigate the observational relationships between smoking (the exposure), methylation [a potential mediator, as measured on an Illumina 450K array (15)], and lung cancer (the outcome). Methylation is measured in peripheral blood at CpG site cg05575921 in the *AHRR* gene, which shows a strong observational relationship with smoking (151). The rs1051730 variant in the *CHRNA3* gene, which is known to be associated with the number of cigarettes smoked per day (133), is used as a genetic instrument for smoking in order to estimate the causal effect of smoking on methylation. Likewise, rsXXXX, a (fictitious) genetic variant putatively related to methylation at the cg05575921 *AHRR* site, is used as an instrumental variable to assess the causal relationship between the methylation site and lung cancer. In the first step of the process, instrumental variable analysis is performed between the exposure (smoking) and the putative intermediate variable (*AHRR* methylation) using the genetic instrument for the exposure (rs1051730). In the second step, instrumental variable analysis is performed between the outcome (lung cancer) and the intermediate (*AHRR* methylation) using the genetic instrument for the intermediate (rsXXXX). Under certain assumptions, including linearity among the different variables, the effect of the exposure (smoking) on the outcome (lung cancer) mediated by the intermediate is equal to the product of the regression coefficients ($\hat{\beta}_{IV1}$ multiplied by $\hat{\beta}_{IV2}$).

Two-Step Mendelian Randomization and Mediation Analysis

MR can be extended to examine the extent to which the causal relationship between an exposure and outcome is mediated by an intermediate variable of interest. The two-step approach (**Figure 3**) was originally developed within the framework of epigenetic epidemiology to examine the degree to which methylation mediates the relationship between an exposure and medically relevant outcomes (100, 101). However, it can be used to examine the degree to which any variable (i.e., not just methylation) mediates a causal relationship of interest. In the first step, a genetic instrument that is related to the exposure variable of interest is used to estimate the causal influence of the exposure on an intermediate variable. In the second step, a genetic instrument related to the intermediate variable is used to estimate the effect of the mediator on the outcome of interest. Under certain assumptions, including the linearity of relationships between variables and no effect modification (i.e., no statistical interactions between the variables), it is possible to estimate both the direct effect of the exposure on the outcome (i.e., independent of the mediator) and the effect of exposure on the outcome that is mediated through the intermediate variable (20, 36, 134).

Obviously, for two-step MR to be possible, genetic instruments must exist for both the exposure and intermediate of interest. However, the exposure, mediator, and outcome need not be measured in the same individuals (36). In fact, two-step MR could be combined with the two-sample MR approach described above to powerfully and efficiently examine the extent of mediation in causal networks. This gives two-step MR a distinct advantage over traditional mediation analysis and structural equation modeling approaches, which require the exposure, mediator, and outcome to

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be measured in at least a subset of all individuals (20). This advantage is particularly relevant for many molecular intermediates, which may be expensive to measure or difficult to collect (e.g., from tissues that are difficult to access), providing an opportunity to undertake MR analyses in far larger samples than would otherwise be possible (36).

We expect application of the two-step MR approach to increase substantially as more genetic influences on intermediate traits are reported. Plentiful numbers of genetic variants related to molecular phenotypes such as gene methylation (13) and metabolomics have been reported (113), and large meta-analyses for many of these traits are already under way. Pleasingly, GWAS of methylation sites (e.g., as measured on microarray chips) have shown that methylation at many CpG sites is ubiquitously influenced by *cis*-genetic variants, implying that many instruments will be available for these types of analyses in the future (13).

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Mining the Phenome Using Mendelian Randomization

Whereas in the early part of the twenty-first century, use of MR was extremely limited by the paucity of genetic variants reliably associated with modifiable exposures and biological intermediates, the unbridled success of GWAS has meant that thousands of SNPs can now be used as IVs in MR analysis. These include the multitude of genetic variants that have been reliably associated with gene expression (144), methylation (13), the metabolome (113), and other molecular intermediates. The deposition of a substantial proportion of these data in public databases has created an unprecedented opportunity to data mine large-scale resources such as the UK Biobank (30) and other publicly available GWAS data collections for causal associations of interest (43).

In a proof-of-principle study, Evans et al. (43) constructed allelic scores consisting of known genetic variants that proxied body mass index (BMI) (120), CRP (39), and LDL cholesterol (126) in data from the first Wellcome Trust Case Control Study (142). The authors examined the association between these instruments and seven different diseases (bipolar disorder, coronary heart disease, hypertension, inflammatory bowel disease, rheumatoid arthritis, type 1 diabetes, and type 2 diabetes). They found that the method successfully identified strong known causal relationships (i.e., LDL allelic scores were associated with coronary heart disease, and BMI scores were related to type 2 diabetes) and was relatively robust in terms of not generating spurious relationships between allelic scores related to exposures not causally related to disease [e.g., CRP allelic scores were not related to coronary heart disease (25) or type 2 diabetes (131)].

As the authors note, there is no reason why this basic strategy could not be scaled up to examine tens of thousands of molecular phenotypes that may causally affect risk of disease in the many GWAS that are publicly available from the different data repositories. Although Evans et al. (43) constructed allelic scores using raw genotypes, two-sample and meta-analytic approaches could also be used if only summary results data are available. Indeed, there now exists software that can efficiently create genetic instruments from publicly available data and provide a preliminary IV analysis of GWAS data for potential causal associations (150). The likely success or failure of this approach will depend on several factors, including the degree to which the biological intermediates of interest are polygenic [otherwise, allelic scores cannot be formed, and one might as well just look at, e.g., the concordance between single eQTLs or metabolic QTLs (mQTLs) and disease-related SNPs] and the extent to which associations are tissue specific (i.e., if an allelic score proxies a relevant intermediate but not in the correct tissue, then it will not show association with the disease). In the future, to maximize application of the method, much could be gained from funding large-scale public resources of data from GWAS of gene expression and methylation in many different tissues and cell types, including cells in stimulated and resting states. Indeed, a "mining the phenome"–type approach could be carried out using a recently developed methodology to

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screen large publicly available disease and multi-omic GWAS summary results for evidence of genetic correlation (18). Those disease-omic pairs showing evidence of genetic correlation could then be followed up using bidirectional MR to investigate the possibility of a causal relationship (see below).

Another interesting possibility concerns the extent to which genome-wide allelic scores could be used in MR analysis. In other words, rather than just using known variants (or combinations of known variants) as genetic instruments, one could in theory use hundreds (or even thousands) of genetic variants scattered across the genome that GWAS indicate are nominally associated with the exposure of interest (i.e., variants that are not robustly associated with disease). Most exposures and intermediates are at least partially heritable. Several studies have shown that genome-wide allelic scores explain significant proportions of the variance in intermediates of interest and in some cases explain more variance than scores constructed only of known variants (43, 44). This idea has appeal in that not only could explaining more variance in the exposure of interest increase the strength of instruments and the power of studies, but it might even be possible to use genome-wide allelic scores to proxy traits with no known associated variants. However, the obvious problem is that genome-wide allelic scores will almost certainly violate at least one of the core IV assumptions (core assumptions two and/or three), reintroducing confounding into the analysis via genetic pleiotropy. A preliminary analysis showed that genome-wide scores lacked specificity and showed unexpected associations with diseases (43), and the authors concluded that there is currently little to recommend the use of genome-wide allelic scores, although they may be useful as a screening tool for generating hypotheses where known variants do not exist. The use of genome-wide information in MR continues to be an active area of research (146).

Bidirectional Mendelian Randomization, Network Mendelian Randomization, and the Coming Age of Hypothesis-Free Causality

If the precise biological function of the genetic variant that one is using in MR is unknown (which is not uncommon when using genetic instruments derived from GWAS), it can sometimes be difficult to know a priori whether the genetic instrument exhibits a primary effect on the exposure of interest or the effect on the exposure is secondary to the outcome. In these ambiguous situations, traditional MR analysis has the potential to produce erroneous conclusions regarding the direction of causation. Bidirectional MR (also known as reciprocal MR) can quantify the causal effect of each variable on the other, provided that genetic instruments are available to proxy both variables.

In bidirectional MR, separate MR analyses are performed in both directions to ascertain the direction of any causal relationship. For example, if the observational relationship of interest is an association between BMI and CRP, then one would instrument BMI to estimate the causal effect of BMI on CRP and then instrument CRP to assess the causal effect of CRP on BMI. Bidirectional MR has been used in several recent applications to study the relationship between BMI and CRP (132, 143), BMI and fetuin A (128), BMI and vitamin D (135), and serum uric acid and adiposity (73). The bidirectional framework can be combined with the two-sample approach, yielding an efficient method for establishing causal relationships.

An obvious precondition for using the bidirectional approach is that appropriate independent genetic instruments for both variables are known in advance. Caution must be exercised when selecting genetic instruments from GWAS so that the variants chosen have primary rather than secondary effects on the variable of interest. For example, variants within the *fat mass and obesity associated* (*FTO*) gene could erroneously be selected as instruments for CRP based on their low p values in a CRP GWAS meta-analysis (39). However, these low p values arise because *FTO* variants are strongly related to BMI, which in turn causally affects CRP. Another limitation is

that bidirectional MR will have difficulty in the presence of reciprocal feedback loops where both variables influence each other. In these situations, a structural equation modeling framework might be a useful alternative for estimating reciprocal relationships.

Considerable potential exists for extending the basic MR framework to more complicated situations involving networks of variables. The central idea is that if there are several risk factors in a data set that each have instruments to proxy them, then in principle it should be possible to estimate the causal effect of each of these risk factors on each outcome of interest. Similarly, each of the risk factors could be examined for causal relationships with each other using bidirectional MR (20). In this way, genotypes can be used as IVs at various points to anchor the networks, and an overall picture of the causal relationships between the different variables can be constructed (20). This approach assumes that the genetic instruments are known a priori and that, in situations where an instrument correlates with multiple variables [as is common in, e.g., metabolomic networks (147)], it is known which of these associations represents a primary as opposed to secondary effect (i.e., an effect mediated through another variable in the network). When this information is not known, it may still be possible to orient the network by using other statistical techniques, such as structural equation modeling (8, 72, 105), partial correlation, or likelihood-based techniques (77, 109), to investigate which configuration of variables is most likely, and then use MR to estimate the size of causal effects between the different network nodes (114). These methods hold great promise for investigating and integrating multiple tiers of "-omics" data and for gaining insight into causal relationships between the different layers. Indeed, in the near future, MR methods could conceivably be used regularly to investigate all pairwise relationships within large multidimensional data sets in a hypothesis-free manner, letting the data speak for itself (33). The associations for which there is good evidence of causality would then become targets for formal and exhaustive hypothesis-testing studies.

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CONCLUSION

MR is a flexible and robust method that can be used to test whether an observational association between a modifiable exposure and a health-related outcome represents a causal relationship. The method has steadily grown in importance, utility, and scope as the number of genetic variants reliably associated with modifiable exposures, biological intermediates, and medically related outcomes has increased over the last decade. In this review, we have endeavored to show that MR not only has provided valuable insights into many areas of traditional epidemiology, but also is playing an increasingly important role in drug discovery and has the potential to assist in the dissection of high-dimensional complex networks consisting of thousands of molecular variables. Indeed, we envisage a time in the not too distant future when MR methods are regularly applied to all pairwise relationships within large multidimensional data sets in a hypothesis-free manner, producing evidence that can then be followed up in a hypothesis-testing manner with a high probability of success.

DISCLOSURE STATEMENT

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