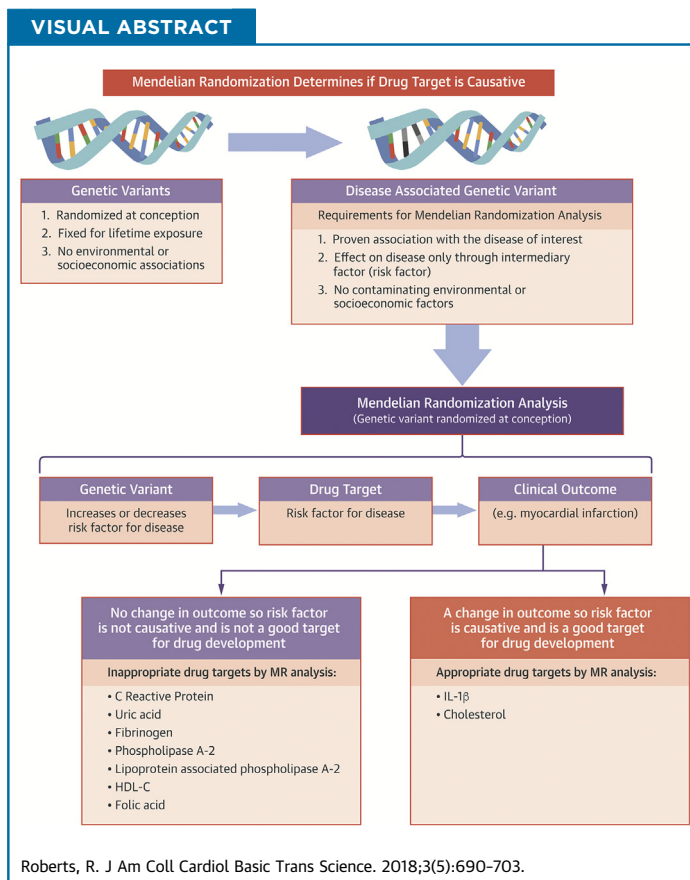


TRANSLATIONAL PERSPECTIVES

Mendelian Randomization Studies Promise to Shorten the Journey to FDA Approval



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SUMMARY

There has been a dearth of new drugs approved for cardiovascular disorders. The cost is prohibitive, averaging to \$2.5 billion, and requiring 12.5 years. This is in large part due to the high failure rate, with only 5% approval by the Food and Drug Administration. Despite preclinical studies showing potential safety and efficacy, most fail when they go to clinical trials phase I to III. One cause for failure is the drug target, often discovered to be a biomarker rather than causative for the disease. Mendelian randomization (MR) studies would determine whether the drug target is causative and could save millions of dollars and time, and prevent unnecessary exposure to adverse drug effects. This was demonstrated in 3 clinical trials that were negative with 2 drugs, veraspladib and darapladib. MR studies during the trials showed the targets of secretory and lipoprotein-associated phospholipids A2 are not causative for coronary artery disease and predicted negative results. The requirement for MR studies is a genetic risk variant with altered function, randomized at conception that remains fixed throughout one's lifetime. It is not confounded by dietary, lifestyle, or socioeconomic factors. It is more sensitive than randomized controlled trials because exposure to the risk factor is fixed for a lifetime. MR studies showed plasma high-density lipoprotein cholesterol is not a causative target of coronary artery disease, and neither is uric acid, C-reactive protein, and others. MR studies are highly sensitive in determining whether drug targets are causative, and are relatively easy, inexpensive, and not time consuming. It is recommended that drug targets undergo MR studies before proceeding to randomized controlled trials. (J Am Coll Cardiol Basic Trans Science 2018;3:690-703) © 2018 The Author. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

For more than a decade, there has been a dearth of new drugs approved for cardiovascular disorders. The process from conception to approval by the Food and Drug Administration (FDA) is a strenuous and perilous path. The cost has become prohibitive, which in the United States for a cardiovascular drug averages \$2.5 billion and requires an average of 12.5 years (1). The time and cost beckons that the drug has the potential to be a blockbuster or it will not be financially worthy. Although the need for improved patient care remains great, a potential therapeutic agent must be evaluated and proven to meet rigorous safety and efficacy standards established by the FDA before routine patient access.

This review will discuss how advances in genetics and the use of Mendelian randomization (MR) studies have the potential to significantly decrease the cost and time required for a drug to go from concept to FDA approval. The emphasis is on the application of MR studies to development of drugs for cardiovascular disorders. However, the principle involved is similar for development of drugs for any disorder.

MR STUDIES AND THEIR APPLICATION TO DRUG DISCOVERY

MR is the term given to studies that use genetic variants to determine whether there is a causal relationship between modifiable risk factors (nongenetic) for a disease and disease-related outcomes. MR

studies utilize mathematical models to analyze disease-associated genetic variants that have been randomly assigned at conception and sustained unchanged and unfounded throughout one's lifetime. Thus, it is possible to compare the effect of a lifetime exposure to a disease-related genetic variant (allele) with a loss or gain of function (analogous to a drug in a randomized clinical trial) to that of neutral genetic variant that serves as the reference (analogous to a placebo in a randomized clinical trial). If the altered genetic variant is modifying a risk factor that is causative of the disease, the MR study will detect a change in the clinical outcome. A risk factor that is proven by MR to be causative of the disease is a worthy target for drug development, whereas the reverse is true if not causative. For example, a genetic variant that increases plasma low-density lipoprotein cholesterol (LDL-C) will increase coronary atherosclerosis and the incidence of myocardial infarction (MI), a known clinical consequence of coronary artery disease (CAD). Thus, LDL-C is causative of coronary atherosclerosis and, as a worthy drug target, led to the development of statin drugs, the main therapy for prevention of CAD. If there are no effects on clinical outcome, time and money will be saved by not pursuing the development of a drug targeted for this risk factor.

Mathematical and statistical polygenic models employing large sample sizes have been developed for MR utilizing disease-related genetic variants to determine whether the risk factor contributes to the

cause of the disease by showing it alters the expected clinical outcome. These models are very sensitive in showing causation even with genetic variants that mediate minimal risk (e.g., a risk ratio of 1.10). The failure rate for drug development can be significantly decreased if the risk factor targeted is proven a priori to be causally related to the disease. MR is a rapid, inexpensive method with the power to determine, before initiating drug development, whether the targeted risk factor is causally related to the disease. It is not the purpose or the scope of this review to discuss the mathematical foundations used to analyze MR studies. Reviews of the statistical methods are available such as that by Lawlor et al. (2).

INNOVATION AND RISK NECESSARY FOR NEW DRUG DEVELOPMENT

There has been a decline in the number of new drugs approved per dollar spent (3,4). The funding by the pharmaceutical industry has increased from \$10 billion to \$60 billion per year (5). Despite the increased funding, the number of new drugs approved remains constant at about 20 per year. In 2007, for example, only 19 new molecules were approved by the FDA, the fewest since 1983 (6). In 2008, 21 new drugs were approved by the FDA, of which only 6 were developed by the large pharmaceutical companies and, perhaps more important, only 29% would be considered “first-in-class” medicines. In 2009, only 17% of the 24 new drugs approved were considered first-in-class (6). The trend is to develop drugs within a class previously approved by the FDA. Diseases with unknown targets represent a major challenge and are associated with a high failure rate, leading to decreased innovation and new therapies. The average lifespan of a female in Britain from 1900 to 2000 more than doubled from 40 years to 80 years (7). This increased longevity was in large part due to antibiotics and the treatment of post-partum infections. This, of course, will only continue if we are innovative in developing new therapies for old and new diseases.

HIGH RATE OF FAILURE FOR DEVELOPMENT OF DRUGS

It is estimated that only about 5% of drugs evaluated in phase I clinical trials are ultimately approved by the FDA (3,4). Most failures are said to occur in phase II clinical trials, with about a quarter of the failures due to toxic effects and about one-half due to lack of efficacy (3,8). It has long been recognized that the cost of drug development is dominated primarily by the high rate of failures. It is worth noting that the

failures in phase II clinical trials occur despite prior extensive evaluation in *in vitro* and animal studies. To be evaluated in phase I clinical studies, preclinical studies must show the drug to be safe and effective. The preclinical studies are claimed to account for 32% of the cost for developing a new drug, and the clinical studies (phase I to III) for approximately 63% (4).

It is evident from the preceding discussion that increased productivity could come from several areas, but low-lying fruit would be attacking the high failure rate. Increased efficiency and productivity should target reducing the failure rate in phase II and phase III clinical trials. In contrast to this, the current trend in the pharmaceutical industry suggests that both phase II and phase III failure rates are increasing (3,6,9,10). Reduction in either phase II or III by 50% from a baseline value of 2.5 years to 1.25 years would reduce the cost per new drug by \$200 million. Similar results have been reported by DiMasi et al. (11).

SINGLE-GENE DISORDERS HELP TO ELUCIDATE NOVEL DRUG TARGETS

Discovery of rare single genes that induce disorders is somewhat analogous to MR studies. Families were discovered with familial hypercholesterolemia, shown to be caused by mutations in the gene encoding for the LDL receptor (12,13). These families were associated with increased risk of CAD. Individuals heterozygous for the mutant gene exhibited plasma concentrations of LDL-C much less than observed in individuals homozygous for the gene. Individuals heterozygous for the mutation develop CAD in their 40s and 50s, whereas homozygotes develop CAD in their teenage years and often die before the age of 20 years (14). This, in effect, showed a dose-response relationship and further supported the causative role of cholesterol in CAD and the causative role of the mutation. Familial hypercholesterolemia is inherited as an autosomal dominant disease, which means the mutant gene will be transmitted randomly to 50% of the offspring. The 50% of offspring without the mutant gene exhibit normal plasma concentrations of LDL-C, serving as a control to further support the role of cholesterol in CAD. β -Hydroxy β -methylglutaryl-CoA (HMG-CoA) reductase is the rate-limiting step in the synthesis of cholesterol and thus an appropriate drug target for reducing plasma LDL-C. Inhibition of HMG-CoA reductase gave rise to lovastatin (15), the first of a family of drugs referred to as statins that have since become the main class of drugs for primary and secondary prevention of CAD (16).

Discovery of a recent single-gene disorder further supports the hypothesis of a causative role for LDL-C in CAD. In 2003, families were discovered with increased plasma LDL-C and an increased incidence of CAD. This was shown to be due to an autosomal dominant inherited disease caused by a gain-of-function mutation in the gene that encodes for PCSK9 (17). Subsequent families, discovered with PCSK9 loss-of-function mutations, were associated with decreased plasma LDL-C and a reduced incidence of CAD (18-20). Subsequent randomized controlled clinical trials (RCTs) demonstrated that monoclonal antibodies inhibiting PCSK9 reduced LDL-C and the incidence of cardiac events (21-24).

POLYGENIC DISORDERS—THE ERA OF GENOME-WIDE ASSOCIATION STUDIES

Rare single-gene disorders inherited by Mendelian dominant or recessive patterns have made great contributions to the understanding of human disease, but are limited or less appropriate paradigms for understanding common diseases. Common diseases such as CAD are polygenic disorders. In CAD, about 50% of its predisposition is due to genetic inheritance transmitted through many genes (25-27). The remaining predisposition is from interaction with environmental or acquired factors. Each single genetic variant predisposing to CAD exhibits only minimal increased risk, and thus, no one gene is sufficient or necessary for induction of CAD. Genetic linkage analysis of pedigrees affected by the disease are appropriate to map the chromosomal location of genes responsible for single-gene disorders but less appropriate for polygenic disorders (28,29). The case-control association study is the more appropriate approach for polygenic disorders. This requires a large number of cases and controls, and to be unbiased, would require hundreds of thousands of DNA markers evenly distributed throughout the human genome. The technology and required DNA markers did not become available until 2005 (30,31). The Human Genome Project, completed in 2000 (32-34), provided a DNA sequence reference for the whole genome of 3.2 billion nucleotides. The HapMap Project annotated over 3 million single nucleotide polymorphisms (SNPs) (31,35), which provided the appropriate DNA markers to perform genome-wide association studies (GWAS).

Investigators quickly pursued GWAS to discover genetic variants predisposing to a variety of diseases, particularly true for cardiovascular disorders. In 2007, the first genetic risk variant, 9p21, for CAD was discovered in GWAS simultaneously by our

group (36) and the Icelandic group (37). This led to an international pursuit for genetic variants predisposing to CAD by the formation of the international consortium of CARDIoGRAM (Coronary Artery Disease Genome-Wide Replication And Meta Analysis) (38,39), followed by CARDIoGRAMplusC4D (Coronary Artery Disease Genome Wide Replication and Meta-Analysis plus the Coronary Artery Disease (CAD) Genetics) (40). These consortia brought together several groups performing GWAS, enabling them to perform a meta-analysis with a large sample size, currently over 300,000 cases with CAD and control subjects (40-44). These studies from CARDIoGRAMplusC4D in combination with recent availability of subjects from the U.K. biobank have led to the discovery of over 160 genetic risk variants for CAD (41,45,46). All of these genetic risk variants predisposing to CAD have been shown to be genome-wide significant ($p = 10^{-8}$) and replicated in an appropriate independent population. Similarly, in conjunction with the Lipid Consortium, more than 150 genetic variants were discovered that regulate plasma lipid levels (41,43,44). The discovery of multiple genetic variants associated with polygenic diseases with robust associations to their disease and confirmation in independent populations are desirable features for utilization in MR studies. More than 50% of the genetic risk variants predisposing to CAD mediate their effect through mechanisms that remain unknown (45). These CAD-associated risk variants provide a rich source of future drug targets to treat CAD and can be used in MR studies to determine their causality of CAD. MR studies utilizing genetic variants that regulate plasma high-density lipoprotein cholesterol (HDL-C) showed plasma HDL-C is not protective of CAD, which will be described later. The use of GWAS in pursuing genetic risk variants predisposing to CAD has been equally successfully in discovering genetic risk variants predisposing to many other polygenic diseases. The GWAS NHGRI (National Human Genome Research Institute) repository reports over 3,000 genetic risk variants predisposing to more than 300 complex polygenic human traits (47).

Throughout this review, the terms *genetic variant* and *allele* are considered interchangeably. The preferred term is *genetic variant*. An allele is defined as a form of a gene that usually differs by only 1 nucleotide, referred to as a SNP, which is also correct for a genetic variant. However, a gene includes a DNA sequence that codes for a protein, whereas most genetic variants predisposing to common diseases (over 80%) are located in non-protein-coding regions and manifest their effect through regulation of a downstream sequence (45).

MR DETERMINES CAUSATION OF RISK FACTOR

Embarking on a project to develop a new drug to treat or prevent a disease requires years and millions, if not hundreds of millions, of dollars. It would be crucially important to know before such commitment whether the target selected for the drug is causative of the disease of interest. If the target (usually a risk factor) is causative, one would be encouraged to pursue drug development. If the target is a risk biomarker without causative effects, the drug may very well decrease the concentration of the biomarker, but would not be expected to affect the disease process or reduce the deleterious clinical effects. This was, in fact, the case shown by MR studies for C-reactive protein (CRP), a well-known risk factor for CAD. MR is the term given to studies that use genetic variants to determine whether there is a causal relationship between modifiable risk factors (nongenetic) for a disease and disease-related outcomes. The concept of MR was outlined some time ago (48), but its application was limited until the beginning of this century (49,50). In the past decade, its application increased and most recently has experienced an exponential growth. This is in part due to the availability of genetic risk variants for multiple common diseases discovered by the many GWAS.

MR utilizes mathematical formulations designed to analyze whether disease-related genetic variants that alter the risk for the disease of interest influence the clinical outcome of that disease. The use of a genetic variant (genotype) to determine whether there is a causal relationship between the effect of a risk factor and outcome of disease in statistics is referred to as an application of the general theory of instrumental variable (IV) analysis. The IV variable in MR studies is the genetic variant that must affect the outcome (clinical consequence of the disease) only through an intermediary modifiable variable (risk factor for the disease). Its formal use in MR studies was introduced relatively recently (51). If the MR analysis concludes the selected genetic variant is associated with a change in the expected clinical outcome, it is proof that the risk factor is causative of the disease. Thus, determining by MR studies whether the drug target is causative is crucially important to the development of new drugs. The conclusion of causation by MR is fulfilled if and only if the genetic variants selected for an IV analysis satisfy the following assumptions (Table 1):

1. The genetic variant is robustly associated with the modifiable (nongenetic) risk factor of interest for that disease.

TABLE 1 Requirements of a Genetic Variant to Qualify for MR

1. The genetic variant is associated with the modifiable (nongenetic) risk factor of interest for that disease.
2. The genetic variant is not associated with confounding factors that bias associations between the modifiable risk factor and clinical outcomes.
3. The genetic variant is related to the clinical outcome only via its association with the modifiable risk factor.

MR = Mendelian randomization.

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In addition to the assumptions outlined in the preceding text, in MR studies to determine causality, one must assume all associations are linear, which is not always the case, but there are statistical corrections available to express nonlinear parametrics, such as odds ratios or risk ratios (2).

If we assume the aforementioned assumptions are met with a continuous clinical outcome, variable (y), the IV calculation of the regression coefficient for the effect of risk factor exposure (x) on clinical outcome (y), is as follows:

$$\beta_{IV} = \beta_{zy} / \beta_{zx}$$

where β_{zy} is the coefficient for the regression of clinical outcome (y) on the IV (z), and β_{zx} is the coefficient for the regression of exposure to the risk factor (x) on the IV. The β_{IV} provides an estimate of the causal effect of the risk factor on clinical outcome even in the presence of unmeasured cofounders of the exposures–outcome association (2).

The principle of MR is based on Mendel's second law that inheritance of any one trait is independent of the inheritance of all other traits. A functional genetic variant known to affect the risk of disease is close to ideal in satisfying the 3 assumptions outlined in the preceding text for the following reasons (Table 2):

1. The genetic variants inherited from each parent are randomly assigned at conception.
2. The association between the genetic variant and the disease remains unchanged because germline DNA does not change during one's lifetime.
3. The genetic variant transmitted by the parents as a DNA sequence, is not confounded by environmental factors such as dietary, lifestyle, environmental, or socioeconomic factors.

TABLE 2 The Properties of a Genetic Variant Are Close to Optimal for its Use in MR Studies

1. The genetic variants inherited from each parent are randomly assigned at conception.
2. The association between the genetic variant and the disease remains unchanged because germline DNA does not change during one's lifetime.
3. The genetic variant, transmitted by the parents as a DNA sequence, is not confounded by environmental factors such as dietary, lifestyle, environmental, or socioeconomic factors.
4. The disease process does not alter the germline genotype, therefore the association between genetic variant and disease is not influenced by reversed causality.
5. The genetic variant remains fixed throughout life, providing for the assessment of a lifetime exposure.
6. There is no problem with compliance because the genetic variant is fixed for life.
7. Because germline DNA remains unchanged throughout life, there is no regression dilution effect.

MR = Mendelian randomization.

4. The disease process does not alter the germline genotype, therefore, the association between genetic variant and disease is not influenced by reversed causality.
5. Because germline DNA remains unchanged throughout life, there is no regression dilution effect; and lastly,
6. There is no problem with compliance because the variable being assessed is an inherited genetic variant that remains fixed throughout life.

LIMITATIONS TO MR

AVAILABILITY OF A GENETIC VARIANT FOR MR.

The most common problem is the lack of functional genetic variants appropriate for use in MR studies. A requisite requirement to perform a MR study is having available a functional genetic variant associated with either loss or gain of function that relates to the disease of interest. The usual genetic variant is loss of function, but it could be gain of function. The initial discovery of PSCK9 as a risk factor for CAD was due to a mutation that was associated with gain of function. Secondly, the genetic variant must strongly influence the risk of the disease of interest, but not be associated with the clinical outcome other than through its effect on the risk factor. The data we have today on the human genome are very encouraging and would indicate almost all DNA sequences, including genes, have multiple forms (alleles, genetic variants) with some alteration in function. The alteration in function is due to the presence or absence of a SNP. The human genome with 3.2 billion nucleotides shows a fairly constant number of about 3.5 million SNPs per

genome (52-55). It is estimated that these 3.5 million SNPs account for over 80% of the variation observed in human phenotypes and are also responsible for most of the predisposition for disease (56). Although each human genome has only 3.5 million SNPs, they have been selected by their parents from over 30 million SNPs known to circulate in the general population. Thus, most DNA sequences exhibit different forms (alleles) due to SNPs, making available 1 of the necessary requirements for MR studies.

UNRELIABLE GENETIC VARIANTS. In the 1990s, many genotypes were claimed to be associated with risk for disease. These claims were based on the candidate gene approach (57,58) and were subsequently discredited. A fundamental component of the MR study is a genetic variant with a well-characterized function that relates to the modifiable risk factor for the disease. The association between the genetic variant and the disease should be confirmed in several datasets, preferably within the MR study population. Today, this is much more likely because we have learned a considerable amount from the pursuit of genetic risk variants predisposing to polygenic diseases. The SNPs used as markers are distributed throughout the genome, which made it possible to pursue GWAS, an unbiased approach to discover disease-related genetic variants. Genetic variants worthy of an association as a predisposing risk factor must have a p value of 10⁻⁸ (referred to as genome-wide significant) and be replicated in an appropriate independent population. The candidate approach has been discarded, and GWAS is the recommended approach for genetic risk variants. Over 3,000 genetic variants have been confirmed to be associated with multiple polygenic disorders. Of the estimated 7,000 rare, single-gene disorders, several mutations have been discovered for over 4,000 diseases (56). There is also the concern for genotyping errors due to poor quality DNA, artifacts, faulty equipment, and human errors.

PLEIOTROPIC GENETIC VARIANTS. This refers to a genetic variant with multiple functions. It is desirable the genetic variant has a single function that relates to the risk of the disease of interest. However, if the pleiotropic effects of the genetic variant do not affect the clinical outcome, it does not violate the core assumption. Pleiotropic effects that do affect clinical outcomes could invalidate the MR approach. Sometimes the pleiotropic effect can be very confusing if it exhibits opposing effects. In the case of using a genetic variant that inhibits interleukin (IL)-1a and also IL-1b, it was expected that the risk for CAD would be decreased

on the assumption that inhibiting the actions of these 2 interleukins would decrease inflammation and be beneficial. However, an increased incidence of MI was observed in part due to increased plasma levels of LDL-C. A subsequent monoclonal antibody specific for IL-1b was associated with decreased CAD and had no effect on lipids or MI. The implication is that IL-1a may have opposing effects such as those mediated through increased plasma lipids (59).

POPULATION STRATIFICATION. This occurs when population subgroups exist that experience both different disease rates and have different frequencies of the genetic variant of interest. The example often given is that of a study involving 5,000 Native Americans of the Pima and Papago Indians (60). A strong inverse relationship between the HLA haplotype and type 2 diabetes was shown. This was interpreted as the absence of the haplotype being causally related to diabetes. However, further analysis was performed that showed those who were of full Native American heritage had a haplotype frequency of 1% and diabetes prevalence of 40%. In the Caucasian population, the haplotype frequency is 66% and the prevalence of diabetes is 15%. Appropriate analysis showed no relationship between haplotype and diabetes (61). This could have caused significant confusion in MR studies. Suppose the haplotype had been used in an IV analysis as a modifying risk of MI. It would have shown the false conclusion of no relationship between diabetes and MI. Nevertheless, population stratification when present is almost always recognized today and seldom represents a problem for MR studies.

LINKAGE DISEQUILIBRIUM. The principal of MR as stated previously is based on Mendel's second law that each genetic trait is independently inherited. However, if a genetic variant A with a defined function qualifying it for use in a MR study is in close physical proximity to variant B that has a different function, both are coinherited and thus not independent. This in genetics is referred to as linkage disequilibrium. If genetic variant B affects the clinical outcome of the disease, it could falsely be ascribed to genetic variant A, and it would invalidate the core assumption of a MR study. If genetic variant B does not affect the outcome of the disease, it does not invalidate the MR approach.

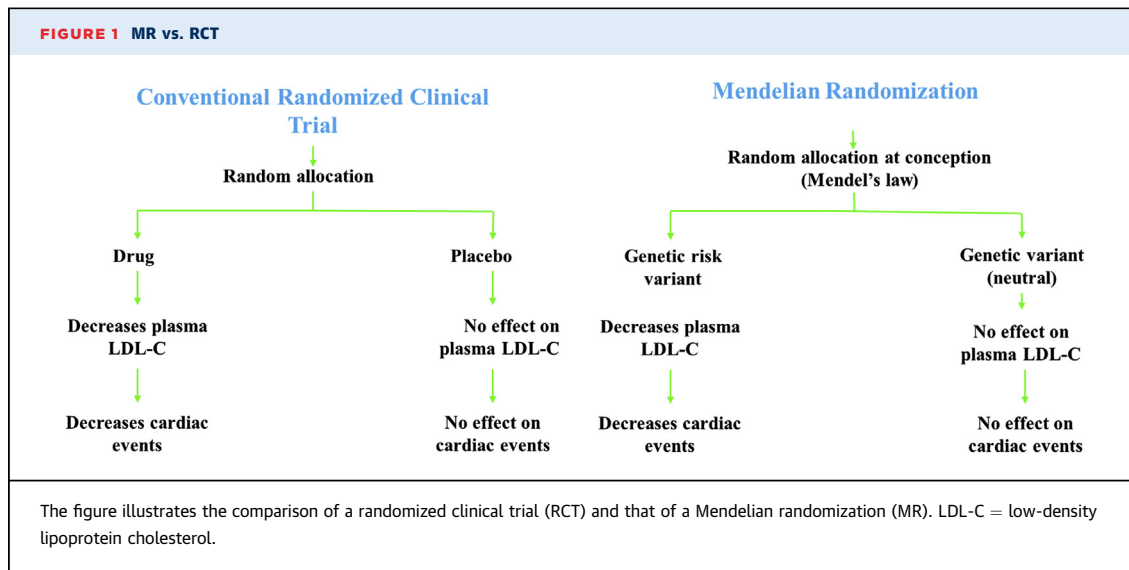
CANALIZATION. Canalization refers to the buffering effects of either environmental or genetic factors that may compensate for the modifying effect of the selected genetic variant on outcome of the disease in a MR study. This comes from knockout studies in

animal models where the gene is knocked out and does not show the expected effect due to redundant genetic variants that compensate for the effects of the eliminated gene. There is no easy solution to this problem, and the results could lead to invalid conclusions. This must always be considered when the MR study shows lack of effect. It is a rare problem for MR studies.

SAMPLE SIZE. Sample size is a concern because most common genetic variants exhibit minimal effect on risk. It requires a large sample size to perform an MR study, usually consisting of thousands despite the lifelong exposure. Nevertheless, the lifelong exposure compensates in large part for the minimal effect, giving most MR studies exquisite sensitivity compared with that of clinical trials. The establishment of biorepository and Internet posting of data from GWAS provides a rich source for large populations that have often already been genotyped for the genetic variant of interest. However, the problem of a weak effect of the genetic variant on outcome of the disease is a concern (62). A weak effect could lead to no information or imprecise estimates of the causal effect. It has been stated that before performing the MR, if a weak effect is expected, one should attempt to estimate if the sample size is adequate. F-statistics from the first-stage regressions should give values >10 if the sample size is adequate (63).

MR STUDY COMPARED WITH A RCT

MR has been compared with the randomized controlled clinical trial. MR is often referred to as a naturalized randomized trial. The analogy follows from the fact that at conception, the parents transmit randomly the genetic variants of maternal and paternal genotypes, which remain fixed throughout one's lifetime. Comparing the design of a MR study to that of the RCT may elucidate the power and simplicity of MR, both in concept and in operation (Figure 1). The RCT example compares a drug that is known to lower plasma LDL-C with that of a placebo, which has no effect on LDL-C. The result is a decrease in cardiac events in the arm receiving a statin with no change in cardiac events in the arm receiving a placebo. Increased plasma LDL-C has long been recognized as a risk factor for CAD. LDL-C has also been shown to be a major culprit in the pathogenesis of coronary atherosclerosis leading to such consequential cardiac events as MI and sudden death. Evidence in support of LDL-C as an etiological factor for coronary atherosclerosis and its clinical sequelae have been obtained from multiple sources, ranging from in vitro data and animal studies to single-gene



disorders associated with hypercholesterolemia. To our knowledge, the primary mechanism whereby cholesterol can influence the clinical cardiac events of CAD is through its effect on coronary atherosclerosis. The development of a statin drug was based on the evidence that increased plasma LDL-C is not only a biomarker indicating increased risk of CAD, but also causative of CAD.

The analogy of MR to the RCT requires substitution of the drug by a genetic variant. The genetic variant is a known polymorphism that has been well characterized to be associated with a decrease in plasma LDL-C. This genetic variant was transmitted randomly by the parents at the time of conception and remains fixed throughout life. To perform the MR study, one would genotype a large population for the genetic variant, and results would be compared with that cohort of the population without genetic variant A, having what is referred to as the wild-type genetic variant. The population would be carefully phenotyped for the disease of interest, which in this example would be CAD and its sequelae, such as MI and death. If the MR study shows the genetic variant modifies clinical outcome, it is proof of causation and makes the risk factor a worthy target for drug development.

The degree of risk mediated by a genetic variant for any polygenic disease, including CAD, is usually minimal (45). However, the effect of a minimal genetic risk variant can be detected by MR in part because of lifetime exposure. Thus, genotyping a

population with a mean age of 55 years will have on average 55 years of exposure to these alleles with 100% compliance. Nevertheless, even with lifetime exposure, the minimal risk imparted by a single genetic variant still requires a large sample size of several thousand to provide a robust definitive conclusion. MR analysis with large sample sizes provide for much greater sensitivity than can be expected from a RCT (Table 3). MR studies are inexpensive, relatively easy to perform, and can be completed within months. MR study requires phenotyping and genotyping of the population. Usually, though, there are available populations already phenotyped and often also genotyped. It is also worthy of emphasis that in addition to proving causation

TABLE 3 MR vs. RCT

Mendelian Randomization	Randomized Clinical Trial
• Genetic risk variants randomized at conception	• Therapy or placebo randomized upon initiation of trial
• No confounding factors	• Could be multiple confounding factors
• Fixed for life	• Not fixed even during the trial
• Design enables one to determine whether the risk factor is causative	• Design does not necessarily enable one to determine causation
• Safety and efficacy assessed from birth (lifetime of exposure)	• Safety and efficacy assessed for duration of trial (3 to 5 yrs)
• Relatively inexpensive	• Invariably costs millions
• Markedly less time-consuming	• Invariably 3 to 5 yrs

MR = Mendelian randomization; RCT = randomized clinical trial.

through its effect on clinical outcome, it also provides an important screen for safety and untoward side effects.

The greater sensitivity of MR over RCT is evident from a recent study by Ference et al. (64) involving a meta-analysis of multiple studies involving over 300,000 individuals. MR analysis showed a great reduction in cardiac morbidity and mortality in individuals inheriting genetic variants with decreased plasma LDL-C. These investigators estimated that a decrease of 1 U of LDL-C (38.7 mg/dl) since birth was associated with a 54.5% in cardiac events. This is a 3-fold greater reduction in cardiac morbidity and mortality per unit of decreased plasma LDL-C than observed in clinical trials utilizing statin therapy.

Risk factors that contribute to disease are often detected by epidemiological observations and validated by RCT. There have been many risk factors based on epidemiological observations such as the relationship between smoking and lung cancer that have been confirmed by RCT and embraced by clinical practice. There have also been many epidemiological observations proven to be incorrect by RCT. It is recommended that observations from epidemiological studies be confirmed when possible by MR studies. Frequently, there are no genetic variants available to test many epidemiological observations such as hydrocarbon pollution. RCTs are very expensive and time consuming, involving thousands of patients, some of which may be exposed to unwanted drug side effects. MR has the advantage over RCT of determining whether the risk factor being assessed is causative of the disease.

Although MR is primarily used to determine causation between the risk factor and the outcome of the disease, it also provides important information on safety and efficacy.

MR INDICATED THE PHOSPHOLIPASE A2 ENZYMES ARE NOT APPROPRIATE DRUG TARGETS FOR PREVENTING CAD

In the search for new drugs to treat CAD, the observations relating to the pathogenesis of coronary atherosclerosis and etiology provide fodder for new drug targets. Our current drug armamentarium to prevent CAD is essentially limited to inhibition of cholesterol synthesis via statin therapy. The current high cost and time-consuming development of new drugs has in part prohibited pursuing high-risk novel targets. The current high cost and time-consuming development of new drugs has in part prohibited pursuing high-risk novel targets, despite recent evidence from the discovery of multiple genetic risk

variants, strongly indicating there are other factors contributing to CAD besides cholesterol and conventional risk factors. It is estimated that only about one-third of the genetic risk variants predisposing to CAD mediate their risk through known risk factors for CAD.

Over the past decade, observations strongly indicate that proinflammatory secretory phospholipase A2 (PLA2) and lipoprotein-associated phospholipase A2 (Lp-PLA2) are risk factors for CAD. These 2 enzymes specifically hydrolyze the glycerol backbone, releasing a fatty acid and a phospholipid that can act as a messenger signaling molecules for inflammation. The early evidence suggesting PLA2 was involved with CAD was a significant increase of these phospholipids in the arterial intima of atherosclerotic plaques (65,66). It is believed that these lipoproteins are prone to oxidation and uptake by macrophages that generate foam cells (67). Studies in knockout and transgenic animal models expressing sPLA2s reinforced the concept that sPLA2 is involved in the development of atherosclerosis. Observational studies also indicated that higher plasma levels of sPLA2-IIA and sPLA2 are associated with increased risk of CAD, MI, and stroke (68-75). Multiple other studies were evaluated in a meta-analysis of multiple clinical studies assessing PLA2s and concluded that these phospholipase A2 enzymes are associated with increased risk of CAD (76).

The accumulating evidence from experimental and clinical studies strongly indicated phospholipases, both the secretory and the lipoprotein-associated forms, were associated with increased risk for CAD. This led to the evaluation of varespladib, a known inhibitor of secretory phospholipase A2, and darapladib, which selectively inhibits lipoprotein-associated phospholipase A2. In the clinical trial VISTA-16 (Evaluation of Safety and Efficacy of Short-term A-002 Treatment in Subjects With Acute Coronary Syndrome), 5,189 patients were enrolled with acute coronary syndrome and received varespladib (5 mg/day) or placebo for a follow-up of 16 weeks (77). Analysis showed no effect on outcome, and the trial was discontinued.

Darapladib, an inhibitor of lipoprotein-associated PLA2, was evaluated in 2 clinical trials. The phase III randomized clinical trial, SOLID-TIMI 52 (The Stabilization of Plaques Using Darapladib-Thrombolysis In Myocardial Infarction 52), randomized 13,026 patients with acute coronary syndrome receiving darapladib (160 mg/day) or placebo with a follow-up of 2.5 years (78). The RCT STABILITY (Stabilization of Atherosclerotic Plaque by Initiation of Darapladib Therapy) randomized 15,828 with stable CAD receiving

darapladib (160 mg/day) or placebo with a follow-up of 3.7 years (79). There was no effect on death, MI, or stroke in either of these studies. Meta-analysis of both studies showed no statistically significant decreased risk of CAD (78,79).

Although clinical trials were being performed to assess the safety and efficacy of veraspladib and darapladib, MR studies were being performed. One MR study used a functional genetic variant of sPLA2 that was associated with 38% lower plasma levels with 1 copy and 60% lower levels with 2 copies. These reductions in sPLA2 were greater than observed with veraspladib or darapladib. In a sample size of 93,000, the MR study showed no evidence of a causal effect of sPLA2 for risk of CAD (80). Other MR studies in a pooled analysis of 27,230 events and 70,500 controls confirmed no causal effect of veraspladib for increased risk of CAD (81,82).

To further assess the causality of lipoprotein-associated PLA2 with CAD, another MR study used 7 genetic variants of Lp-PLA2 in a sample size of 10,494 CAD cases and 15,624 control subjects. Results indicated Lp-PLA2 is not associated with causality of CAD (83). A MR study using a loss-of-function variant for Lp-PLA2 was associated with a 3-fold lower level of Lp-PLA2, but exhibited no effect on the risk for CAD (84). It is evident from these MR studies that neither sPLA2 nor Lp-PLA2 is involved with the etiology of coronary atherosclerosis or CAD. The results of the MR studies indicate they are not appropriate targets for drug therapy if the design is to decrease CAD. Had MR studies been performed before these RCTs, millions of dollars would have been saved together with a decade of investigation.

MR STUDIES AND THE ROLE OF INFLAMMATION IN CAD

MR studies have already shown many targets traditionally associated with CAD such as folic acid (85,86), uric acid (87), and fibrinogen are not causative and should not be used as targets for development of new drugs. MR studies serve a very important function in distinguishing between a biomarker associated with risk of a disease as opposed to a biomarker that is causative of the disease. CRP is synthesized in the liver and as part of the immediate response of the innate immune system has long been recognized as a risk factor for CAD. Statin therapy, the primary preventive therapy for CAD, inhibits cholesterol synthesis and reduces cardiac events. Statin therapy also reduces plasma levels of CRP. This led to the suspicion that CRP, a part of the inflammatory pathway, may contribute directly to coronary

atherosclerosis. To test this hypothesis, the JUPITER (Crestor 20mg Versus Placebo in Prevention of Cardiovascular [CV] Events) trial (88) was performed, which enrolled people with low plasma LDL-C and increased plasma CRP levels. Statin therapy in this trial was associated with decreased plasma CRP levels and decreased cardiac events (88). This led to the possibility that CRP might be a drug target for the prevention of CAD. Several genetic variants of the gene encoding for CRP became available for use in MR studies. MR studies (83-86) using these genetic variants showed CRP had no effect on cardiac events, indicating that plasma CRP is not causative of CAD. Thus, it is not an appropriate target for drug development. Plasma CRP levels in CAD still represent a biomarker for inflammation but CRP is not causative of the inflammation that contributes to the pathogenesis of CAD. To decrease the CAD resulting possibly from inflammation, one must target the source of the inflammation that induces coronary atherosclerosis. CRP is a secondary inflammatory marker induced by other markers further upstream in the inflammatory pathway such as IL-6 and IL-1. IL-6 is the most potent stimulant of CRP synthesis and is consistent with the concept that downstream CRP is a secondary messenger.

IL-6 is also increased with inflammation and is a risk factor for CAD. IL-6 has been shown to induce endothelial dysfunction and plaque formation (89,90), and thus is potentially more causative than CRP. An MR study utilizing 2 polymorphisms in the IL-6 pathway were associated with lifetime low levels of plasma CRP and decreased vascular events (91,92). This would indicate a causal relationship between IL-6 and vascular events. However, these results were tempered by increased levels of IL-8, TNF, MMP-9, and Lp-PLA2. Secondly, it is known that further upstream in the inflammatory pathway is IL-1, which is primarily responsible for the synthesis of IL-6, thus IL-1 would be even more likely to be causative and a more appropriate drug target to decrease inflammation and cardiac events. An MR study (93) was performed using 2 genetic variants that encode the IL-1 receptor antagonist, both of which are endogenous inhibitors of both IL-1 α and IL-1 β . It is recognized that IL-1 α and IL-1 β have different actions, and thus, it may be confusing to interpret the results when both are simultaneously inhibited. Plasma levels of IL-1 α and IL-1 β are increased during inflammation and represent risk factors of CAD. The results of the MR study showed the genetic variants were associated with lower plasma levels of IL-6 and CRP, and reduced rates of rheumatoid arthritis, but with an increased incidence of MI and abdominal aortic

aneurisms (59). Interpretation of the results is difficult because one cannot distinguish between IL-1 α and IL-1 β . Part of the reason for the increased MI may be associated with increased plasma LDL-C levels. This MR study illustrates the preference to use a genetic variant with a single known function, otherwise the results can be uninterpretable. A major concern in MR studies is pleiotropic effects, resulting in a beneficial effect counteracted by an adverse effect.

Recently, a monoclonal antibody, canakinumab, was developed which selectively inhibits IL-1 β . Preliminary studies showed reduced plasma levels of IL-1 β , IL-6, and CRP but no effect on IL-1 α and no effect on plasma levels of HDL-C or LDL-C. This led to the design of a clinical trial referred to as the CANTOS (Canakinumab Antiinflammatory Thrombosis Outcome) trial (94). Patients enrolled in this study were required to have increased plasma levels of CRP reflecting increased ongoing inflammation. This study evaluated 3 doses of the monoclonal antibody (canakinumab) that inhibit IL-1 β . The 2 higher doses were both associated with a reduction in IL-1 β , IL-6, and CRP with no change in IL-1 α . There were no change in plasma LDL-C or HDL-C. The monoclonal antibody was associated with a 15% reduction in major cardiovascular events. These results suggest the results of the MR study referred to previously may be due to opposing actions of IL-1 α and IL-1 β . This is the first major trial to demonstrate a reduction in inflammation was associated with a reduction in cardiac events (94).

It would be of significant importance to the pathogenesis of coronary atherosclerosis if one could perform MR study utilizing genetic variants that altered either IL-1 α solely or IL-1 β solely. This may also give us the mechanism as to why IL-1 α is associated with increased MI and plasma lipids. Such studies may be used to assess the etiological factors associated with on-target side effects and off-target beneficial and side effects. In addition to determining causality, it could be very helpful in assessing risk/benefits of drugs. Such studies are likely to increase as more experience is obtained with MR studies.

MR STUDIES STRONGLY SUGGEST PLASMA HDL-C IS NOT AN APPROPRIATE DRUG TARGET TO DECREASE CAD

The cardiovascular field has been indoctrinated to accept as dogma that increased plasma concentrations of HDL-C are protective of CAD. This stems from

a fundamental investigation performed by Gofman et al. (95) published in 1966. This has been further enhanced by the results of epidemiological studies and a variety of interventions, namely, niacin, exercise, fibrates, and red wine. Unfortunately, the support afforded by these interventions is highly confounded because they all simultaneously decrease LDL-C (96-98). Recent studies have made available more than 160 genetic variants that regulate plasma lipid levels (99,100). Utilizing a variant in LIPG p.Asn396Ser and 14 common SNPs associated solely with increased plasma concentrations of HDL-C (101), we performed a MR study. The control was 13 genetic variants associated solely with increased concentrations of plasma LDL-C. The MR study was designed with a sample size of 50,763 and 4,228 MIs having 90% power to detect a 13% reduction in risk of MI. Results were replicated in an independent population of 16,685 cases of MI and 48,872 controls followed by a meta-analysis of both populations. These results showed no association between plasma HDL-C concentrations and protection from MI. By contrast, the control study with 13 SNPs associated solely with increased plasma concentrations of LDL-C showed a 2-fold increased risk of MI. Several studies have since confirmed that plasma HDL-C offers no protection from CAD (102-107). A recent meta-analysis randomized 39 trials involving 117,411 patients to assess the effects of niacin, fibrates, and cholesteryl ester transfer protein (CETP) inhibitors on cardiovascular events (108). All interventions increased plasma LDL-C, but neither niacin, fibrates, nor the CETP inhibitors exhibited any effect on cardiac events. The group receiving niacin showed a reduction in the incidence of MI and was associated with increased HDL-C and decreased LDL-C. In the group receiving statin therapy, the addition of niacin offered no benefit. Similar results were observed for fibrates. This meta-analysis strongly suggests the benefit of niacin and fibrates is due to its minimal effect of decreasing plasma LDL-C concentrations. These results would suggest plasma LDL-C is not protective and is not a target for drug therapy designed to prevent CAD. Results of other MR studies showed increased plasma HDL-C is not associated with protection of CAD (101). These findings have major implications for future research involving prevention of CAD. In the past decade, HDL-C has been the target of several clinical trials despite increasing plasma levels of HDL having had a negative effect on cardiac events (102,105,106). It is of note that MR studies all consistently show plasma LDL-C and triglycerides are risk factors for CAD (98,109).

MR STUDIES SHOULD BE CONSIDERED BEFORE PERFORMING A CLINICAL DRUG TRIAL

In this review, we have shown the power of MR to determine whether a particular drug target is a causative risk as opposed to being simply a biomarker for the disease. In planning future drug therapies, given the high failure rate of about 95% and the limitation in choosing preclinical testing models, it would be highly recommended that the drug target be confirmed as causative for the disease before pursuing phase I, II, and III clinical trials. It behooves the medical community and the pharmaceutical industry to be certain the drug target is causative before exposing a large patient population inappropriately to a large clinical trial. This was

amply proven in the trials evaluating the secretory and lipoprotein-associated phospholipase A2 enzymes (previously discussed). The purpose of this review is to strongly enforce that MR studies can reduce the time, cost, and failures associated with clinical trials despite positive results obtained in the preclinical studies.

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