Original Research Article

Mendelian randomisation for mediation analysis: current methods and challenges for implementation

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Data and code availability: All code for simulation analyses, applied analyses and example code is available on Github (simulations: https://github.com/eleanorsanderson/MediationMR, applied analyses and example code: https://github.com/alicerosecarter/MediationMR). The cleaned dataset for UK Biobank analyses will be archived with UK Biobank. Please contact access@ukbiobank.ac.ukfor further information

Abstract

Background

Mendelian randomisation uses genetic variants randomly allocated at conception as instrumental variables for a modifiable exposure of interest. Recent methodological advances allow for mediation analysis to be carried out using Mendelian randomisation. When genetic instruments are available for both an exposure and mediator, both multivariable and two-step Mendelian randomisation may be applied.

Methods

We use simulations and an applied example to demonstrate when multivariable Mendelian randomisation and two-step Mendelian randomisation methods are valid and how they relate to traditional phenotypic regression-based approaches to mediation. We demonstrate how Mendelian randomisation methods can relax assumptions required for causal inference in phenotypic mediation, as well as which Mendelian randomisation specific assumptions are required. We illustrate our methods in data from UK Biobank, estimating the role of body mass index mediating the association between education and cardiovascular outcomes.

Results

Both multivariable Mendelian randomization and two-step Mendelian randomization are unbiased when estimating the total effect, direct effect, indirect effect and proportion mediated when both confounding, and measurement error are present. Where both the exposure and mediator are continuous, in the presence of a rare or common binary outcome, we found little evidence of bias from non-collapsibility of the odds ratio.

Conclusion

Phenotypic mediation methods require strong, often untestable, assumptions. Mendelian randomisation provides an opportunity for improving causal inference in mediation analysis. Although Mendelian randomisation specific assumptions apply, such as no weak instrument bias and no pleiotropic pathways, strong assumptions of no confounding and no measurement error can be relaxed.

Key Words: Mendelian randomisation, mediation analysis, multivariable Mendelian randomisation, two-step Mendelian randomisation

Introduction

Mediation analysis aims to quantify the contribution of intermediate variables that lie on the causal pathway from an exposure to an outcome. However, in order to make causal inference, a number of strong assumptions are required. Mendelian randomisation (MR) is an instrumental variable method, where genetic variants randomly allocated at conception can be used as instruments for modifiable phenotypes, eliminating reverse causality and, if the assumptions hold confounding [1]. In this paper we compare phenotypic regression-based methods for mediation analysis with MR methods for mediation analysis, and illustrate the assumptions required for MR mediation methods to make valid causal inference.

Mediation Analysis

Mediation analysis has long been carried out in the health and social sciences estimating the role of an intermediate variable between an exposure and an outcome. It is often carried out with the motivation of either improving aetiological understanding or, when intervening on an exposure is not feasible, identifying intermediate variables that could make suitable intervention targets. Methods for mediation analysis have been in use since the early twentieth-century, although they were not described as such at the time [2]. Formal methods were developed by Baron and Kenny in the 1980s [3]. Following this, a large amount of research has built on and improved mediation methods to better answer causal questions. Mediation analysis has been used in an increasing number of disciplines, including in epidemiology and population health research.

Three parameters are typically estimated in mediation analysis i) the total effect (the effect of the exposure on the outcome through all potential pathways, adjusted for confounders of the exposure and outcome) ii) the direct effect (the remaining effect of the exposure on the outcome that acts through pathways other than the specified mediator or set of mediators) and iii) the indirect effect (the path from exposure to outcome that acts through the mediator(s)). In situations where the total effect, direct effect and indirect effect all act in the same direction, an estimate of the "proportion mediated" (i.e. proportion of the total effect mediated by the mediator) can be calculated by dividing the indirect effect by the total effect. Traditional methods for mediation analysis centre around the use of phenotypic regression models. The total effect is estimated from a regression of the outcome on the exposure, adjusting for all potential confounders of the exposure-outcome association, exposure-mediator association and mediator-outcome association [4]. The direct effect is estimated from a regression of the outcome on the exposure, adjusting for confounders and the mediator.

There are two approaches to estimating the indirect effect. The difference in coefficients method calculates the indirect effect by subtracting the direct effect from the total effect (C-C', Figure 1A) [4]. The product of coefficients method estimates the exposure-mediator association and mediator-outcome association separately then multiplies the two estimates (A*B, Figure 1A) to calculate the indirect effect.

Traditional Baron and Kenny methods were introduced to estimate mediation in the presence of three continuous variables, although they are now often applied more generally to binary variables, including binary outcomes and mediators. In the presence of a rare binary outcome the estimates from the difference in coefficients and the product of coefficients method should coincide [4,5]. Where effects are estimated on the odds ratio scale, the causal effects are only approximated due to non-collapsibility of odds ratios, where the association between an exposure and outcome would not be constant by strata of categorical covariate. This is a major limitation as binary disease status is often of interest as an outcome.

These approaches rely on several strong, untestable assumptions to provide unbiased, causal estimates. These include, among others i) a causal effect of the exposure on the outcome, exposure on the mediator and mediator on the outcome ii) no unmeasured confounding between the exposure and outcome, exposure and mediator or mediator and outcome iii) no unmeasured confounders of the mediator-outcome association that are descendants of the exposure (intermediate confounders), iv) no reverse causality from the outcome or the mediator to the exposure, and v) no exposure-mediator interaction [4,6]. Furthermore, measurement error can introduce bias. For example, non-differential measurement error in the mediator can lead to an underestimate of the indirect effect [7]. Bias from differential measurement error, or measurement error in the exposure could either over- or under-estimate the indirect effect.

Counterfactual reasoning has been used to develop methods that can relax some of these strong assumptions [8-12]. These methods can estimate mediation in the presence of exposure-mediator interactions and account for measured intermediate confounders (confounders of the mediator-outcome association that are descendants of the exposure). Additionally, these more flexible counterfactual methods can allow for binary mediators and rare binary outcomes. The estimated direct effect is described as being a "controlled direct effect" if the value of the mediator is fixed at a certain value for all individuals in the population, or a "natural direct effect", when the value of the mediator is allowed to take the value for each person that it would have taken had they been unexposed, in a counterfactual scenario. The "natural indirect effect" represents the average change in an outcome if the value of the exposure was fixed, but the value of the mediator is allowed to

vary for each individual. If there is no interaction between the exposure and mediator, the estimate of the natural direct and controlled direct effect will be the same [4,5,13]. However, methods nested in counterfactual theory remain biased in the presence of unmeasured confounding, measurement error in the exposure or mediator, or in a mis-specified model with reverse causality [4,14].

Mendelian randomisation

In Mendelian randomisation (MR) randomly allocated genetic variants are used as instrumental variables for a modifiable phenotype [1,15,16]. Given the random allocation of genetic variants at conception, in the absence of pleiotropic effects, MR estimates are robust to biases from confounding, reverse causation and measurement error [15]. Three core assumptions are required for a genetic variant to be a valid instrumental variable, these are i) the genetic variants are robustly associated with the exposure (the relevance assumption) ii) there are no confounders between the genetic variants and the outcome, including unmeasured confounders (the independence assumption) and iii) the genetic variants do not affect the outcome via any path other than the exposure (the exclusion restriction criteria) (Supplementary figure 1) [1] .

Rationale for using Mendelian randomisation to answer mediation questions

Mendelian randomisation can itself be thought of as a form of mediation analysis, where the entire effect of the genetic variant on the outcome acts via the modifiable exposure of interest [17]. However, in addition to this, recent methodological developments mean MR can be used to estimate the mediating role of an intermediate phenotypic risk factor between a phenotypic exposure and outcome. Mendelian randomisation can be used to overcome some of the previously described strong assumptions required for causal inference in mediation analysis. For example, using MR, all estimates (including the direct and indirect effect) are more robust to bias from unmeasured confounding, including that of intermediate confounding. Crucially, MR can be used to estimate a causal effect of the exposure on the outcome, exposure on the mediator and mediator on the outcome. Although, it is worth highlighting that a number of MR specific assumptions are still required, such as that the genetic variants do not have horizontally pleiotropic effects and strongly predict the exposure and mediator.

In mediation terms, a univariable MR estimates the total effect, where the instrument (either the genetic variants or polygenic risk score) is used to estimate the effect of a single exposure on the outcome. Two differing MR approaches can be used which broadly mirror traditional phenotypic regression-based approaches to mediation to decompose the direct and indirect effects: multivariable MR and two-step MR.

Multivariable Mendelian randomisation (MVMR) is a method to estimate the direct effect of multiple exposures on an outcome. These exposures can be highly correlated or share some of the same genetic variants (pleiotropic variants). In MVMR, the genetic variant, or polygenic risk score, for both the primary exposure and the second exposure are included as instruments in the analysis (Figure 1B) [18,19]. Subject to power and instrument strength, MVMR can include more than two exposures. Using MVMR an estimate of the direct effect of each exposure on the outcome is obtained. This direct effect is equivalent to the natural direct effect estimated in mediation analysis. Similar to the difference method in traditional approaches, the indirect (mediated) effect can be calculated by subtracting the MVMR direct effect from the total effect of the exposure on the outcome estimated by univariate MR [20].

Two-step Mendelian randomisation (sometimes called network MR) is akin to the product of coefficient methods in traditional phenotypic regression-based mediation analysis [21,22]. Two MR estimates are calculated i) the causal effect of the exposure on the mediator and ii) the causal effect of the mediator on the outcome (Figure 1C) [21-23]. These two estimates can then be multiplied together to estimate the indirect effect.

These methods are increasingly being used in mediation analyses [20,24-27]. In this paper, we demonstrate how MVMR and two-step MR can estimate mediated effects (direct effects, indirect effects and the proportion mediated) and which assumptions are required for the resulting estimates to be unbiased [18,19,22]. We provide guidance about how to carry out each method, with code provided (available on github), and illustrate each method using both simulated and real data, applied to an individual level (one-sample) MR analysis. Additionally, we summarise scenarios where one method may be favourable compared to another and include a decision flow chart for current available methods.

Methods

Overall study design

This study uses simulations and real-data examples to demonstrate how MR can be applied to mediation analysis. In an applied example we use multivariable regression and MR to investigate the role of body mass index (BMI) in mediating the association between years of education and systolic blood pressure, cardiovascular disease (CVD) and hypertension (continuous, rare binary and common binary outcomes, respectively).

Simulations

We simulated data under the model illustrated in Supplementary figure 2 with continuous, rare binary (5% prevalence) and common binary (25% prevalence) outcomes. We varied the total effect of our exposure and proportion mediated and obtained results for the phenotypic estimates and MR methods for both the difference and product of coefficients methods. Additionally, we simulated results where the total effect of the exposure estimates a small amount of the variation in the outcome and where each of the exposure and mediator were subject to non-differential measurement error. The full range of scenarios simulated are presented in Supplementary table 1.

The simulations were interpreted using the size of bias between the true and estimated value for each parameter. The size of bias in simulation results was estimated in two ways. Firstly, absolute bias was ascertained by subtracting the estimated value from the true value, for each of the total effect, direct effect, indirect effect and proportion mediated. Secondly, for all parameters relative bias was ascertained by dividing the absolute size of bias by the true value of the parameter.

UK Biobank

UK Biobank is a population-based cohort study which recruited 503,317 UK adults between 2006 and 2010. At baseline assessment centres individuals reported their highest qualification completed, ranging from no qualifications (equivalent to leaving school after 7 years) up to degree level (equivalent to 20 years of schooling). Clinic nurses measured their systolic blood pressure (mmHg) and BMI, which was calculated from measured height and weight (kg/m^2). Hypertension was defined according to the World Health Organisation definition of a systolic blood pressure of greater than 140mm/Hg and a diastolic blood pressure of greater than 90 mm/Hg or were taking a prescription for antihypertensive medication. Individuals are linked with hospital episode statistics, where incident cases of cardiovascular disease are reported. Cardiovascular disease was defined as having a recorded ICD10 code of I or G45 or an ICD9 code of 390-459. Confounders considered were age, sex, place of birth (northing and easting co-ordinates), birth distance from London, and Townsend deprivation index at birth. See supplementary methods for full details.

Participants were restricted to White British participants, defined from self-report data and confirmed using genetic principal components (PCs) where individuals with PCs suggesting a broader ancestry were excluded. Participants were included if they had complete data on education, BMI, systolic blood pressure, hypertension, CVD diagnoses and all considered covariates (N = 216 359) (Supplementary Figure 3). In MR analysis, polygenic risk scores were created for education and BMI, using the most recent genome-wide association studies from European individuals, which did not include UK Biobank participants (see supplementary methods for full details) [28,29].

Statistical analysis

The following approaches were applied to both UK Biobank data and simulated data. UK Biobank analyses were performed using Stata version 15 (StataCorp LP, Texas) and R version 3.5.1 was used for simulation scenarios.

The effects on binary outcomes (hypertension and incident CVD) were estimated on risk difference, log odds ratio, and odds ratio scales.

Phenotypic based methods

The total effect

Multivariable linear and logistic regression were used to estimate the association of education with each outcome (equation 1, see Supplementary material). All phenotypic analyses were adjusted for potential confounders; age, sex, place of birth, birth distance from London, and Townsend deprivation index at birth.

The indirect effect: difference method

Using multivariable regression, each outcome was regressed on education adjusting for the mediator (BMI) to estimate the direct effect of education (equation 2). The direct effect was subtracted from the total effect model, to provide an estimate of the indirect effect (equation 3). Confounders were included as previously described. In the applied example, confidence intervals for the direct effect are provided by the regression output, whilst those for the indirect effect were estimated using bootstrapping with 1000 replications. In simulation studies the standard deviation of the regression coefficients was calculated across repeats to evaluate precision.

The indirect effect: product of coefficients method

Two regression models were estimated. Firstly, multivariable linear regression was carried out to estimate the effect of education on BMI (equation 4). Secondly, multivariable linear and logistic regression was carried out for the effect of BMI on each outcome also adjusting for education (which acts as a confounder of this association) (equation 5). These two estimates are then multiplied together to provide a single estimate of the indirect effect (equation 6). Confidence intervals were estimated by bootstrapping with 1000 replications, whilst precision was evaluated in simulation analyses by estimating the standard deviation across regression coefficients.

Mendelian randomisation methods

The total effect

The total effect of education on all outcomes was investigated using two-stage least squares regression (2SLS). In the first regression, the effect of the education weighted allele score on self-reported educational attainment was estimated (equation 7A). In the second stage, the outcome is regressed on the genetically predicted exposure (equation 7B). `ivreg2` in Stata was used for continuous outcomes. Where outcomes were binary, the predicted value of the genetically predicted exposure from the fitted model was stored and included in the second stage regression models. Second stage regressions were carried out on both the log odds and odds ratio scale. Robust standard errors were specified for the second stage [30]. In addition to previously listed phenotypic confounders, both regression stages were adjusted for the first ten genetic PCs to control for population structure.

Multivariable Mendelian randomisation

Multivariable MR estimates the direct effect of education and BMI individually on each outcome by including instruments for both education and BMI using 2SLS regression. The first stage regression for the effect of the genetic variants for education and BMI are used to predict each exposure (education and BMI) (equation 8A and equation 8B). In the second stage regression, the outcome was regressed on the predicted values of each exposure (equation 8C). Both regression stages were adjusted for confounders and genetic principal components as in the total effect models. The direct effect was then subtracted from the total effect to estimate the indirect effect (equation 9) and bootstrapping used to estimate the indirect effect confidence intervals. The standard deviation of the regression coefficients was calculated across repeats to evaluate precision of simulation results. The Stata package `ivreg2` was used to carry out analyses for continuous outcomes, whilst this was carried out in two separate stages as described previously for binary outcomes on the log odds or odds ratio scale.

Two-step Mendelian randomisation

Two-step MR involves first estimating the effect of the exposure on the mediator in a univariable MR model and then estimating the effect of the mediator on the outcome in a second univariable MR model.

The univariable MR model was carried out to estimate the effect of education on BMI, 2SLS regression with `ivreg2` in Stata (equation 10A and equation 10B). A second model estimating the effect of BMI on each outcome was carried out using MVMR. Both the BMI and education genetic

variants were included in the first and second stage regressions (equation 11A-C). For continuous outcomes, the Stata package *ivreg2* was used and was carried out in two stages as previously described for binary outcomes. Previous approaches in the literature have not used MVMR for this second step [21,22] and propose carrying out a univariable MR of the effect of the mediator on the outcome. However, using MVMR ensures any effect of the mediator on the outcome is independent of the exposure. Additionally, this method provides an estimate of the direct effect of the exposure on the outcome.

The two regression estimates from the second stage regression are multiplied together to estimate the indirect effect (equation 12), with bootstrapping used to estimate the confidence intervals. In simulation studies the standard deviation of the regression coefficients was calculated across repeats to evaluate precision.

Estimating the proportion mediated

The proportion mediated is calculated by dividing the indirect effect (either estimated using the difference method/MVMR or the product of coefficients method/two-step MR) by the total effect. Confidence intervals were calculated via bootstrapping.

Sensitivity analyses

In the applied phenotypic analysis, sensitivity analyses were carried out dichotomising education and/or BMI to a binary variable to further test non-collapsibility, where analyses were carried out on the log odds ratio scale. See supplementary methods for details.

Instrument strength was assessed by calculating F-statistics for univariable MR and conditional F-statistics for MVMR [31]. Sensitivity analyses for MR methods included using MR-Egger and MVMR-Egger to test for pleiotropy in the applied example [32,33].

Results

Simulations

Continuous outcomes

Phenotypic methods

In all simulated scenarios, both the difference method and product of coefficients method estimate the same value for the indirect effect and proportion mediated (Figures 2 and 3 and supplementary tables 2-4). In the presence of residual covariance to reflect confounding, estimates of the total

effect, direct effect and indirect effect using the difference method and product of coefficients were equally biased.

For example, on the linear risk difference scale with a continuous outcome, for a true total effect size of 0.5 and an indirect effect of 0.125, equating to a proportion mediated of 0.25, the absolute size of bias for the indirect effect, from both the difference method and product of coefficients method, was 0.3 and the absolute size of bias for the proportion mediated was 0.5, equating to a relative bias of 3.8 and 1.2 respectively (Supplementary tables 2-4).

Where no confounding is simulated in the case of no true total effect, phenotypic methods estimated the total effect, direct effect and indirect effect with no bias (Supplementary table 5).

Where measurement error was simulated, measurement error in the exposure led to an overestimate in the mediated effect, whilst measurement error in the mediator led to an underestimate in the mediated effect (Supplementary table 6).

Mendelian randomisation methods

With the exception of some simulations where the true total effect was equal to zero, in the presence of residual covariance to reflect confounding, MVMR and two-step MR both estimated the total effect, the indirect effect and the proportion mediated with no absolute bias (Figures 2 and 3 and supplementary tables 7 and 8).

Where there was no true total effect (i.e. total = 0) or if the total effect was estimated with poor precision, the proportion mediated estimated by MR methods was out of the bounds of a proportion, with very wide confidence intervals. For a sample size of 1000 with a true total effect size of 0.2 and a proportion mediated of 0.25, both MVMR and two-step MR estimated the indirect effect (size of absolute bias = 0.0, size of relative bias = 0.02) and proportion mediated (size of absolute bias = -0.02, size of relative bias = -0.09) with little bias. However, the standard deviation of the proportion was large (proportion = 0.23, SD = 3.53) (supplementary tables 9 and 10). Phenotypic methods had much smaller standard deviations, but larger bias values (Supplementary tables 3 and 4)

Where measurement error was simulated either in the exposure or the mediator, both MVMR and two-step MR estimated the mediated effects with little bias (Supplementary table 11).

Binary outcomes on the risk difference scale

In both phenotypic methods, with binary outcomes on a linear relative scale, bias was present. Considering a rare binary outcome with a true total effect size of 0.5 and an indirect effect of 0.125, equating to a proportion mediated of 0.25 both the difference method and product of coefficients method the size of absolute bias for the indirect effect and proportion mediated was 0.03 and 0.29 respectively (Figures 2 and 3 and supplementary tables 12-15). In the same scenario but considering a common binary outcome the size of absolute bias from both methods estimating the indirect effect was 0.08, whilst the bias for the proportion mediated was 0.29 (Figures 2 and 3 and Supplementary tables 12-15).

In the MR scenarios, estimated effects were concordant between MVMR and two-step MR, with little to no bias. For example, in the previously described example with a rare binary outcome, the size of absolute bias for the indirect effect and proportion mediated was zero. With a common binary outcome, the size of absolute bias for the indirect effect was -0.01, whilst the size of bias for the proportion mediated was zero (Figures 2 and 3 and Supplementary tables 16-19).

Binary outcomes on the log odds ratio scale

In the scenarios simulated, there was some evidence of bias when analysing binary outcomes on the log odds ratio scale. In the example of a rare binary outcome, where the true total effect is set to 0.5 and the proportion mediated is 0.25, both MR methods estimated the indirect effect with a bias of 0.03 and the proportion mediated with zero bias. In the same scenario for a common binary outcome, both methods estimated the indirect effect with no bias. Using MVMR the proportion mediated was estimated with minimal bias (-0.01) and zero bias using two-step MR (Supplementary tables 20 and 21).

As the true value of the proportion mediated increased, typically the absolute size of bias also increased. For example, where the true total effect size is set to 0.5 and the proportion mediated to 0.75, in the case if a rare binary outcome the size of bias for the indirect effect was 0.09 and the proportion mediated 0.01, estimated using both MVMR and two-step MR. For a common binary outcome, the size of bias for the indirect effect was -0.01 and the proportion mediated -0.01, estimated using MVMR. However, there was zero bias for both the indirect effect and proportion mediated using two-step MR (Supplementary tables 20 and 21).

Binary outcomes on the odds ratio scale

In simulation scenarios explored in this analysis, neither MVMR nor two-step MR were able to estimate the mediated effects without bias when using the odds ratio scale (Supplementary 22 and 23).

Inconsistent mediation

Inconsistent mediation exists when the indirect effect and total effect are in opposing directions, leading to a negative proportion mediated [34]. In this case, a proportion mediated is not meaningful and only the direct and indirect effects are interpreted. Both the difference method and product of coefficients methods were able to accurately estimate the direction of the indirect effect (in this case a positive total effect and negative indirect effect) but the indirect effect estimate was biased compared with the true values. Both MVMR and two-step MR were able to estimate the size of the indirect effect without bias (Supplementary table 7).

Real data example results

Descriptive characteristics of UK Biobank participants included in the real data example are shown in Supplementary table 24.

Effect of education on systolic blood pressure, CVD and hypertension

Both multivariable regression and univariable MR provided evidence to support a causal effect of education on systolic blood pressure, as well as for a role of BMI mediating this effect on the risk difference scale. Phenotypically, the difference method estimated the indirect effect for a one standard deviation increase in education on systolic blood pressure mediated via a one standard deviation increase in BMI to be -0.28 (-0.29 to -0.26) and the proportion mediated to be 24.0% (95% CI: 21.2% to 26.9%) (Figure 4 and Supplementary table 25). Using MVMR the indirect effect estimated was -0.52 (95% CI: -0.74 to -0.29). Despite the MVMR indirect effect and total effect being larger than the phenotypic difference estimate, this corresponded to a smaller proportion mediated of 13.5% (95% CI: 6.9% to 20.2%).

Using the product of coefficients method, the indirect effect of a one standard deviation increase in education via a one standard deviation increase in BMI on systolic blood pressure was -0.28 (-0.29, -0.27) with a proportion mediated of 24.0% (95% CI: 22.8% to 25.2%) (Figure 4 and Supplementary table 25). Comparatively, using two-step MR, the indirect effect was estimated to be -0.50 (95% CI: -0.83 to -0.17) corresponding to a proportion mediated of 13.0% (95% CI: 4.8% to 21.2%).

Both multivariable regression and univariable MR provided evidence to support a causal effect of education on CVD, including for a mediating role of BMI. Considering analyses on the risk difference scale, estimates from MVMR were larger than those from two-step MR. For example, the indirect effect via a one standard deviation increase in BMI on the effect of a standard deviation increase in education on incident CVD, was estimated to be -0.01 (95% CI: -0.01 to 0.00) using MVMR and -0.003 (95% CI: -0.005 to -0.001) using two-step MR. The estimated proportion mediated was 14.2% (95% CI 16.4% to 44.8%) using MVMR compared with 9.05% (-3.4% to 21.5%) using two-step MR. The estimates of the decomposed mediated effects were similar when analysed using the log odds ratio scale, however estimates had wider confidence intervals (Figure 4 and Supplementary table 25).

Mendelian randomisation suggested more education reduced risk of hypertension; however, estimates were imprecise and confidence intervals were consistent with an increased risk. This led to very large confidence intervals around the estimate of the proportion mediated by BMI. On the risk difference scale, the proportion mediated that was estimated by MVMR was 56.5% (95% CI: 7.9% to 105.1%), whilst the proportion estimated using two-step MR was 60.9% (95% CI: -275.8% to 397.5%). Similar values were obtained using the log odds ratio scales (Figure 4 and Supplementary table 25).

For both CVD and hypertension, the decomposed mediated effects estimated on the odds ratio scale were discordant compared with those on either the risk difference or log odds ratio scale.

Sensitivity analyses

Applied examples using phenotypic mediation methods were extended to examine the role of binary exposures or mediators on non-collapsibility. In both rare and common binary outcomes, where the education exposure was dichotomized to low (10 years of education or less) compared with high education (greater than 10 years of education) the difference in coefficients method and product of coefficients method estimated similar mediating roles by a continuous standard deviation increase in BMI. For example, the proportion mediated by BMI on the association between education (high vs low) and CVD was 15.8% (13.5% to 18.2%) for the difference method and 15.6% (15.1% to 16.2%) for the product of coefficients method. Where the mediator was binary (normal and underweight vs overweight and obese) the two methods diverged. For example, the proportion mediated by high versus low BMI on the association between a standard deviation increase in education and incident CVD was 8.6% (7.3% to 10.2%) for the difference in coefficients method and 45.3% (38.8% to 51.9%) for the product of coefficients method. This was similar when both the exposure and outcome were

considered as binary. Similar results were also seen when considering common hypertension as the outcome (Supplementary table 26).

All instruments had strong F statistics and conditional F statistics, where the minimum value was 935 (conditional F-statistic of education) (Supplementary table 27).

Both MR-Egger and MVMR-Egger provide little evidence to support pleiotropic effects of the instruments biasing results (Supplementary table 28).

Discussion

In this analysis we have demonstrated four methods for mediation analysis; the difference method and the product of coefficients method using phenotypic data, and MVMR and two-step MR using genetic data. We have demonstrated using a range of scenarios in simulation studies when the estimates of an indirect effect or proportion mediated using MR methods are more robust to bias, compared with phenotypic regression methods.

Sources of bias in phenotypic analyses

Measurement error

Our results show that in phenotypic approaches, with a continuous exposure and mediator, non-differential measurement error in the mediator leads to an underestimate of the mediated effect, where MR methods show no bias. This is consistent with previous methodological and applied work [7]. In previous MR mediation analyses looking at the role of BMI and blood pressure as mediators of education and cardiovascular disease, we identified larger differences between the results of phenotypic and genetic analyses when the mediator was more likely to be measured imprecisely or with error, such as systolic blood pressure, compared with mediators less likely to be measured with error, such as BMI [27].

Confounding and the cross-world assumption

Four key causal assumptions in phenotypic mediation relate to unmeasured confounding. Specifically these are of i) no unmeasured cofounding between the exposure and outcome ii) no unmeasured confounding between the exposure and mediator iii) no unmeasured confounding between the mediator and outcome and iv) no unmeasured confounding between the mediator and outcome that is itself a descendant of the exposure (independent confounder known as the cross world assumption). These assumptions remain present even in causal inference methods such as those based on counterfactual theory.

Phenotypic analyses typically control for all possible confounders available, but it is generally impossible to measure sufficient confounders, and frequently those that are measured are measured with error. Additionally, collider bias can be introduced by adjusting for the mediator in the presence of unmeasured mediator-outcome confounders [35].

Our simulation results show that whilst phenotypic mediation methods are prone to bias by unmeasured confounding, MR methods are robust to this [1,16]. Additionally, mediation estimates from MR are robust to unmeasured intermediate confounders.

Providing the instrumental variable assumptions hold, including assuming linearity (i.e. no exposure-mediator interactions), there can be no path from the exposure to unmeasured intermediate confounders as depicted in the directed acyclic graph in Figure 6. Nor is there a path from the unmeasured intermediate confounder to the instrument for the mediator. Where the linearity assumption cannot be made, adjusting for all phenotypic confounders of the mediator and outcome association will better justify the assumption of no intermediate confounding.

Sources of bias in MR analyses

In order to obtain valid causal inference for mediation, all standard MR assumptions must be met. In particular, having strong instruments, no evidence of pleiotropy and no associations between the genetic instruments and confounders are important.

Overlapping instruments

MVMR can include overlapping instruments for multiple exposures but is biased if instruments for any of the exposures are also independently instruments for the outcome i.e. the genetic variants predict the outcome of interest not via the exposure [19]. In a two-step MR mediation analysis, the mediator is considered as both an exposure (of the outcome) and as an outcome (of the exposure) and therefore any instruments for the exposure that are also instruments for the mediator should be excluded from the analysis as they are pleiotropic in the estimation of the effects of the exposure on the mediator. Additionally, any instruments for the exposure that are also (direct) instruments for the mediator will be pleiotropic in the estimation of the total effect of the exposure on the outcome through a univariable MR. Although these overlapping instruments can be included in the MVMR, if the association between the exposure and mediator or the total effect of the exposure on the outcome is to be estimated as part of the analysis, the overlapping instruments should be excluded and would likely want to be excluded from all analyses for consistency.

Small total effects

In simulation studies with no true total effect the MR estimate of the proportion mediated is implausible. A lack of evidence of a total effect could be because in truth there is no causal effect or may be due to a lack of power. Where there is no evidence of a total effect, mediation analyses should not be carried out.

Where the total effect is weak or estimated imprecisely (with confidence intervals crossing the null) simulations show the indirect effect and the proportion mediated using MR can be estimated but have large standard deviations. In this case, results should be interpreted with caution, especially considering the bounds of error.

Power

Mendelian randomisation studies require very large sample sizes to achieve adequate statistical power. Although power calculators are available for univariable MR analyses, methods are not currently available to estimate power in more complex scenarios, such as when using multivariable MR or two-step MR [36]. Conditional F-statistics in MVMR are typically weaker than standard F-statistics, further decreasing the power of analyses. Indeed, although some methods exist, power calculators for observational mediation methods are also limited [37]. In the absence of formal power calculations, the power of these analyses can be considered by evaluating the precision of the confidence intervals for all of the total, direct and indirect effects, as well as assessing the conditional instrument strength.

Interactive effects

Currently, MR methods are not available for combining both mediation with interactions between the exposure and mediator. Separately, methods are available for estimating interactions in an MR framework with individual level data, provided large enough sample sizes are available , but methods developed for phenotypic data that decompose a total effect into mediation and interaction terms are not yet developed for MR [11,38,39]. In our applied example we have assumed no interaction between education and BMI based on previous MR literature [38].

Analysis using binary outcomes

Odds ratios are non-collapsible. This means the association between an exposure and outcome would not be constant on the odds-ratio scale by strata of categorical covariate. The stratum specific values of the covariates differ from the marginal value not accounting for covariates [40,41]. Whilst typically discussed in the context of confounding, including a mediator as a covariate, as is done when estimating direct effects, can lead to issues with non-collapsibility because the two models

(the first for the total effect and the second for the direct effect) are no longer comparable [4]. The mediation literature indicates that to estimate the direct and indirect effects of a binary outcome, the outcome must be rare (less than 10% prevalence), so the odds ratio approximates the risk ratio, and the product of coefficients method should be used for phenotypic data [5]. In the presence of a common binary outcome, estimates from the product of coefficients method and difference method are unlikely to align (and indeed the literature suggests both are likely biased).

In our simulation scenarios, we found the estimated mediated effects for both rare binary outcomes and common binary outcomes were biased compared with the true values, using both MVMR and two-step MR. In our simulations, this bias was small and typically would not alter conclusions made. However, the exact bias from non-collapsibility will be unique to each scenario, including depending on the number of and strength of confounding variables. Additionally, our phenotypic applied examples indicate that the difference in coefficients method and product of coefficients method are more likely to diverge, and for bias by non-collapsibility to be present, when the mediator is binary in addition to the outcome. Alternatively, analyses can be conducted on the risk difference scale, which reduces the risk of bias due to the non-collapsibility.

Both our simulation results and applied example show estimating effects on the odds ratio scale, as opposed to the log odds ratio scale are heavily biased. Where odds ratios are required, analyses should be carried out on the log odds ratio scale and exponentiated. However, researchers should be aware of the potential bias from non-collapsibility.

Which method and when

Our results suggest that both MR mediation methods can be used for both continuous and binary outcomes, but caution is required in some instances, for example where total effects are weak. However, the most appropriate method to use, including whether to carry out an MR analysis rather than phenotypic analysis, will primarily depend on i) the research question including the main effects of interest and ii) the data (including genetic) available. The flow chart in Figure 5 aims to help with the decision-making process, based on practical limitations of MR. Mendelian Randomisation has specific advantages compared with phenotypic methods where causal assumptions are required. The causal effect of the exposure on the outcome, the exposure on the mediator and the mediator on the outcome can all be tested. Additionally, bi-directional MR could be used to determine which of two variables is the causal exposure and causal mediator, where this may not be known.

Although MR is robust to many of the untestable causal assumptions in phenotypic mediation analysis, these are replaced with a set of MR specific causal assumptions (Figure 6), and careful

consideration should be given to which assumptions are most plausible. Best practice would always be to triangulate across phenotypic and genetic approaches, and across multiple data sources wherever possible [42].

Additional considerations

Multiple mediators can be assessed using MVMR, where non-overlapping SNPs for all exposures and mediators are included in one instrument. Under this approach, all mediators will be considered simultaneously, and the effect of each mediator individually is not estimated. This may mean the causal question of interest cannot be answered. A key consideration in carrying out these analyses would be having sufficient power for analyses; particularly as conditional instrument strength is likely to reduce with each additional mediator. Given the current available sample sizes, investigators may wish to focus on a small number of exposures and mediators, for example one exposure and one mediator in each analysis.

Methods applied in this paper can be used with summary data MR [43]. Much of the same considerations will apply for both individual level MR, as presented here, and summary data MR. Importantly, all sources of summary statistics for the exposure, mediator and outcome will need to be non-overlapping. As the mediator is considered an outcome in the exposure-mediator model, sample overlap can introduce bias [44]. As individual level data is not available in summary data MR, bootstrapping cannot be sued to estimate the confidence intervals for the indirect effect or proportion mediated, and the delta method can be used to approximate these confidence intervals [27]. Analyses will also be restricted to the scale reported by the GWAS used.

In addition to genetic instrumental variables (IVs), as is the case with MR, non-genetic IVs can be used to estimate mediation in a similar manner. Indeed, using a variety of IVs to answer the same question can improve causal inference. For example, the raising of the school leaving age can be applied as an IV for education [45]. For a number of exposures, or mediators of interest, genetic IVs are not available. Applying IV approaches equivalent to MVMR and two-step MR for use with non-genetic IVs can be used to minimise bias by reverse causality and confounding, providing all IV assumptions are met.

Applied results in context

Our applied example demonstrates a causal total effect of education on systolic blood pressure, supporting results in the wider literature [46-48] and shows that BMI is a mediator of the association between education and systolic blood pressure. Given our previous analyses showing that systolic blood pressure is itself a mediator of the association between education and CVD, this work suggests

systolic blood pressure is downstream of BMI on the causal pathway, although we have not explored bi-directional association in this analysis.

Limitations of our approach

Although we have included a range of simulation scenarios, including both continuous and binary outcomes, this is not an exhaustive range of scenarios and it is plausible that there are a number of further simulations when MR methods, or indeed phenotypic methods, may provide biased answers.

Whilst MR has advantages when applied to mediation analysis, it has limitations. The exclusion restriction criteria assuming no pleiotropic pathway is an important assumption which applies equally when MR is used for mediation analysis. Some methods are available to assess pleiotropy including for the use of MVMR [32,33,49].

Very few binary exposures will be truly binary and are likely a dichotomization of an underlying continuous variable (the liability scale), changing the interpretation of an MR analysis [50]. For example, smoking is often defined as ever versus never smokers, when really the causal exposure is a continuous reflection of smoking heaviness and duration. As a result, the exclusion restriction criteria are violated, where the genetic variant can influence the outcome via the unmeasured continuous exposure, even if the binary exposure does not change [50]. In a mediation setting, the same would apply to a binary mediator. In these scenarios, two-step MR could be used to test whether there is evidence of a causal pathway between the binary exposure and/or mediator. However, the estimates of mediation would likely be biased.

Although MR is more robust to confounding than traditional analyses, confounding can be introduced through population stratification, assortative mating, and dynastic effects [51]. Adjusting for genetic principal components which capture population structure and other explanatory variables can minimise bias.

Future research

Methods for estimating and decomposing a total effect into mediation and interaction parameters within an MR framework would be useful. Similarly, methods to estimate the effects of multiple mediators will be important, as more complex causal questions begin to be explored using these methods. Additionally, developing methods to estimate power in MR and mediation will be important.

Conclusions

Mendelian randomisation can be extended to estimate direct effects, indirect effects and proportions mediated. MR estimates are robust to violations of the often untestable assumptions of phenotypic mediation analysis, including unmeasured confounding, reverse causality and measurement error. MR analysis makes its own strong, but distinct assumptions, especially relating to instrument validity. To estimate mediation using MR, we require large sample sizes and strong instruments for both the exposure and mediator.

Box 1: Definitions of the mediated effects

The Total effect: The effect of the exposure on the outcome

The Direct effect: The effect of the exposure on the outcome, not explained by the mediator(s) under consideration. In addition to controlling for confounders (of all paths, as described for the total effect), the mediator of interest should be controlled for.

The Indirect effect: The effect of the exposure on the outcome acting through the mediator, or mediators, of interest.

The proportion mediated: Proportion of the total effect explained by the mediator

Box 2: Summary of Mendelian randomisation

Individual level data Mendelian Randomisation
Individual SNPs or polygenic risk scores are created for each individual in a study, where all study information and genetic information is provided for each individual.
Both the gene-exposure and gene-outcome estimates are calculated in the same sample
Analyses can be carried out on either a binary (log odds ratio) or continuous scale
The F-statistic and Sanderson-Windmeijer F-statistic can be used to assess instrument strength in univariable and multivariable MR respectively.
Summary data MR
Summary estimates of the gene-exposure and gene-outcome association are estimated in separate samples
Analyses must be carried out on the scale reported by the outcome genome wide association study
Provides an opportunity to maximise statistical power by using multiple data sources
MR-Egger can be extended to investigate pleiotropy in MVMR [33]

Box 3: Key recommendations when using Mendelian randomisation for mediation analysis

- Ensure strong instruments are available for exposures and mediators and test instrument strength using the F-statistic. Test the conditional instrument strength for multivariable MR using the Sanderson-Windmeijer F-statistic [31]
- Instruments for the exposure and mediator must be independent for both multivariable MR and two-step MR
- The instruments must not have a pleiotropic effect on the mediator or outcome
- Current MR methods are optimised for use with continuous exposures and mediators, where binary exposures or mediators which are a reflection of a true underlying continuous measure can lead to violation of the exclusion restriction criteria
- Use univariable MR to test for evidence of causal association in each step of the mediation path, from the exposure to the outcome, exposure to the mediator and mediator to the outcome
- Ensure that there is a strong total effect of the exposure on the outcome, with confidence intervals not spanning the null
- Where individual-level data are being used and outcomes are binary estimate effects on a linear scale to alleviate potential bias from non-collapsibility of odds ratios
- If using summary level data with a binary outcome, estimate effects on the log odds ratio scale and transform after analysis if odds ratios are required

Main Paper Figures

Figure 1: Directed acyclic graphs demonstrating A) the decomposed effects in phenotypic regression-based mediation analysis where C represents the total effect, C' represents the direct effect and the indirect effect can be calculated by subtracting C' from C (difference method) or multiplying A times B (product of coefficients method) B) multivariable MR, using a combined genetic instrument for both the exposure and mediator of interest, to estimate the direct effect (C') of the exposure and C) two-step Mendelian randomisation, where the effect of the exposure on the mediator and mediator on the outcome are estimated separately, using separate genetic instrumental variables for both the exposure and mediator. These estimates are then multiplied together to estimate the indirect effect of the media tor

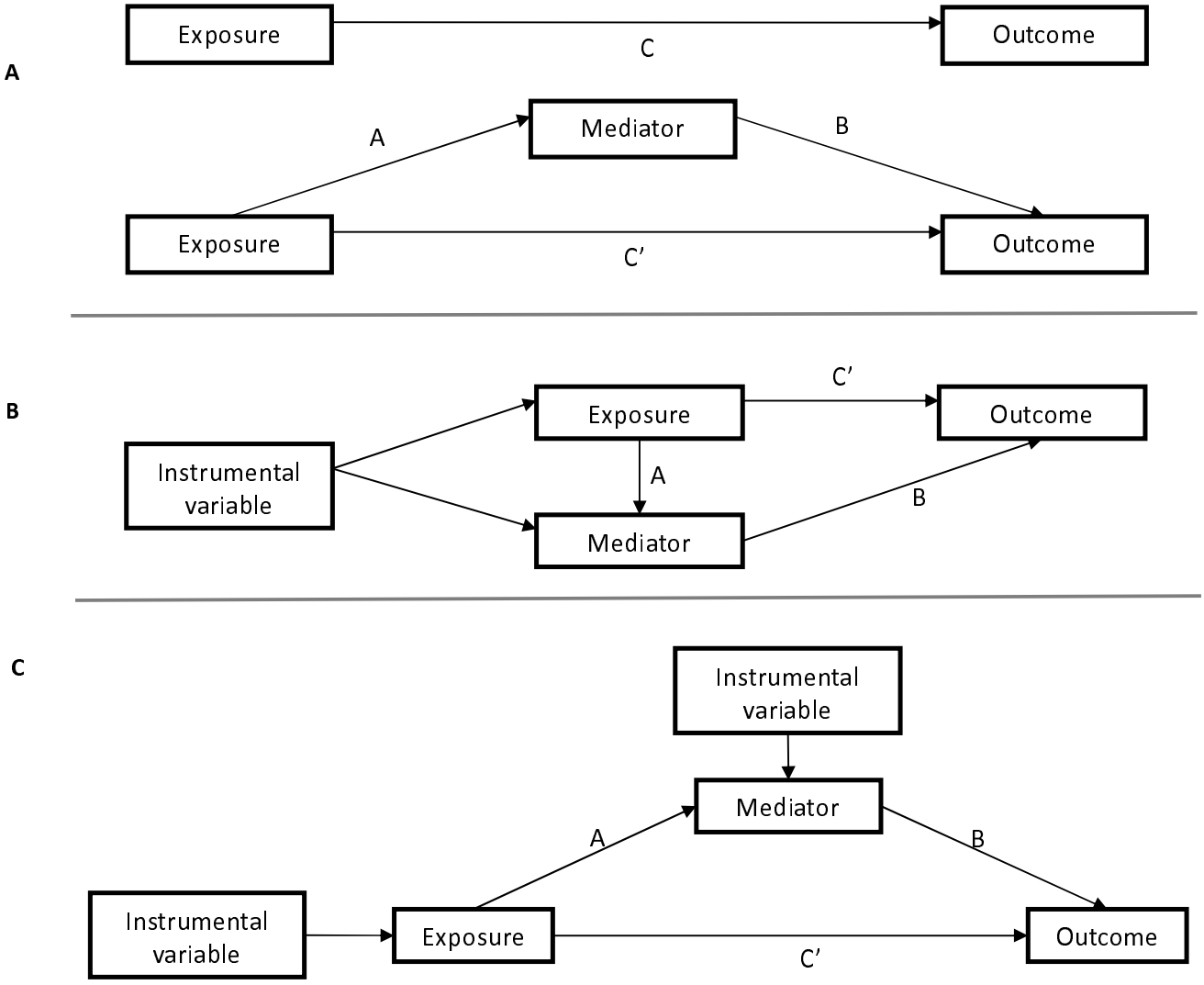
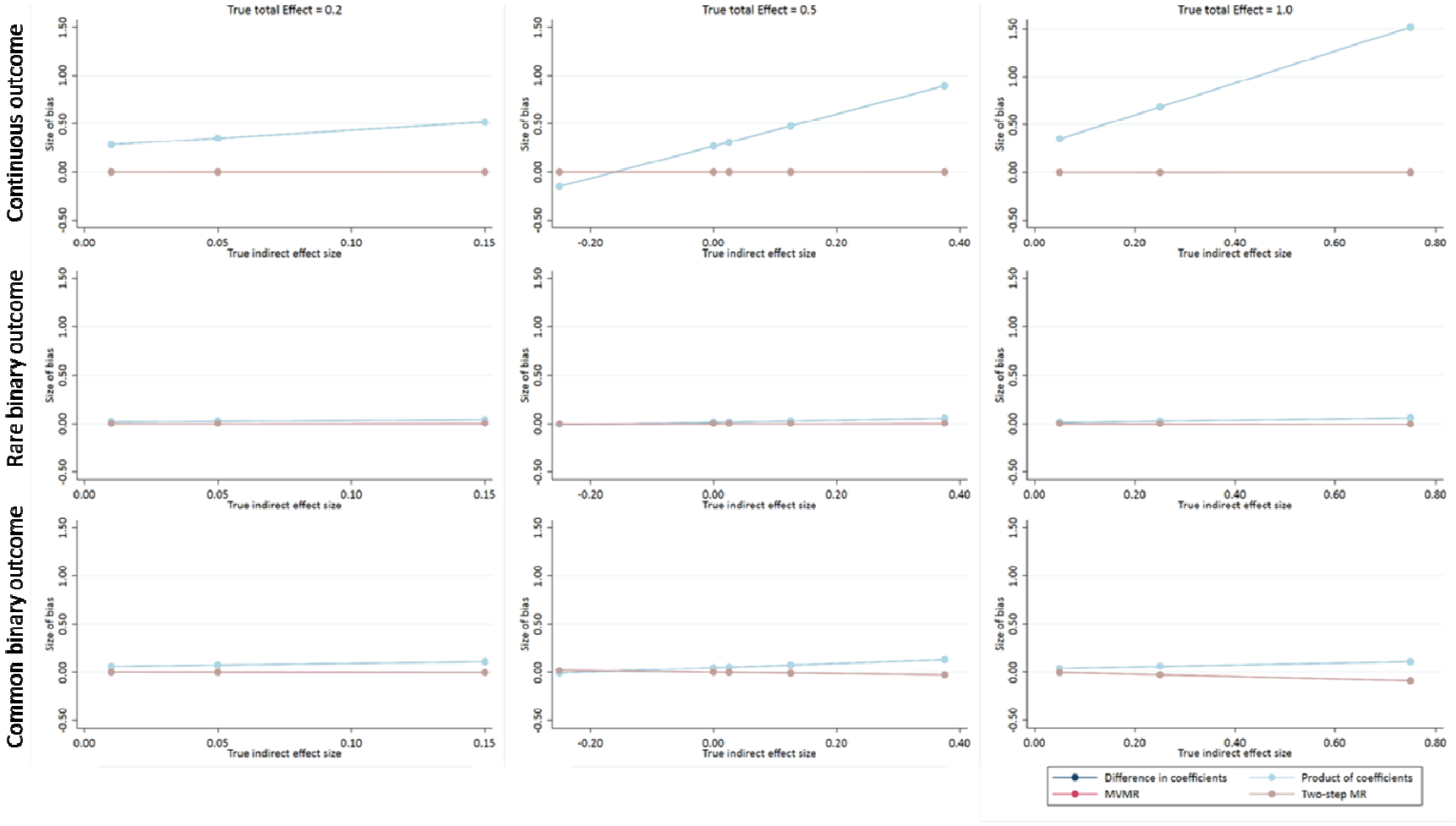
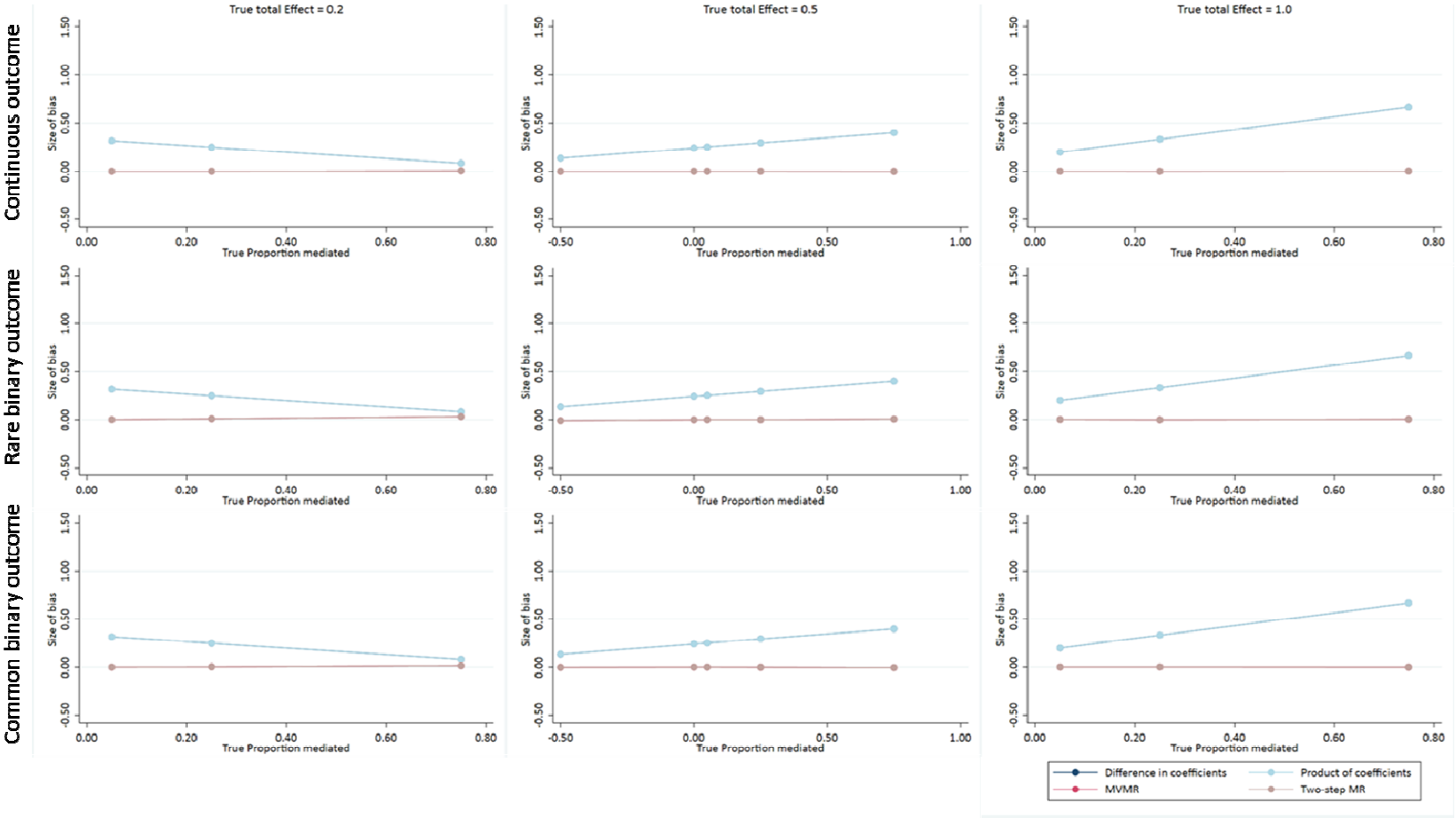


Figure 2: Size of absolute bias for the indirect effect of an exposure on range of outcomes through a continuous mediator, for a range of fixed true effect sizes (0.2, 0.5 and 1.0) and range of true indirect effect sizes using phenotypic regression or Mendelian randomisation, on



the risk difference (relative) scale (simulated N = 5000)

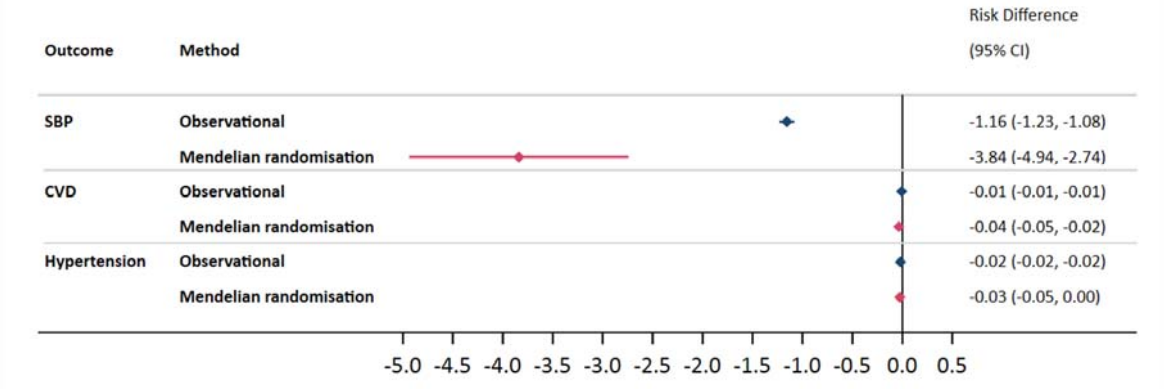
Figure 3: Size of absolute bias for the proportion mediated by a mediator between an associated between an exposure and outcome, for a range of fixed true total effect sizes (0.5), via a range of true proportions mediated, using multivariable regression or univariate Mendelian



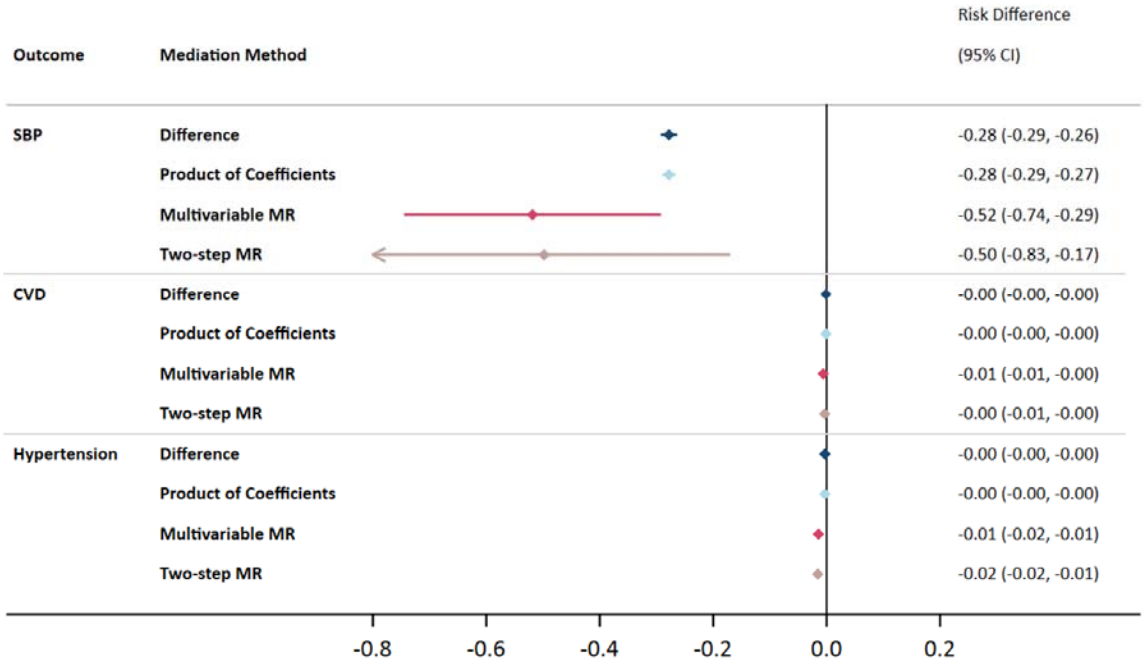
randomisation, on the risk difference (relative) scale (simulated N = 5000)

Figure 4: Total (A), indirect effects (B) and proportion mediated (C) estimating the mediating role of BMI between education and systolic blood pressure, cardiovascular disease (all subtypes combined) and hypertension in UK Biobank (N=216 359), using multivariable regression methods and Mendelian randomisation

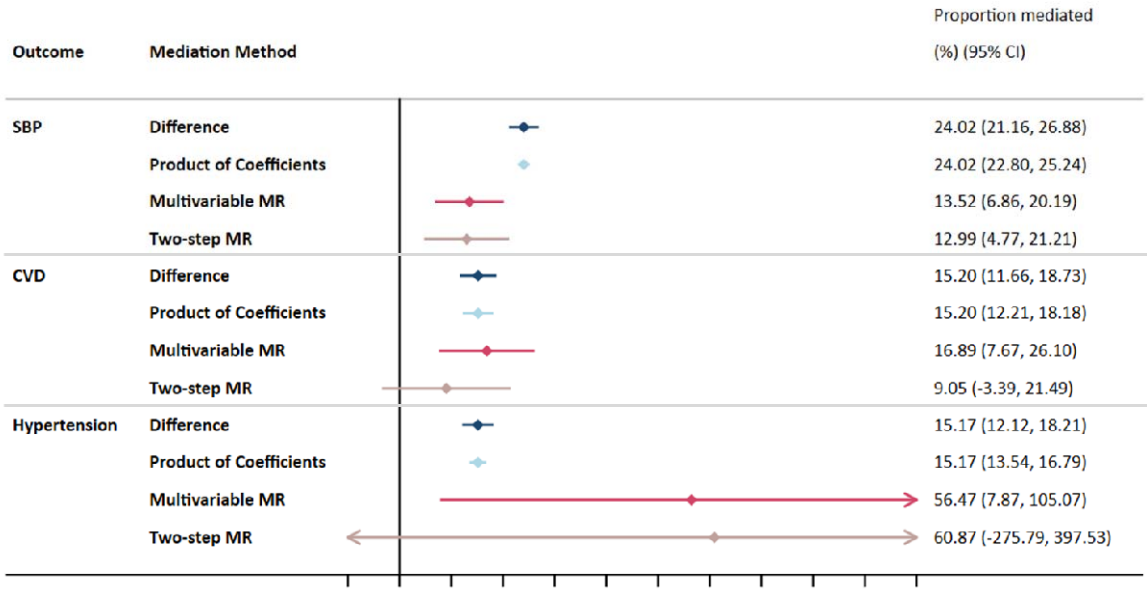
Total effect of education on CVD



Indirect effect via BMI of education on CVD



Proportion mediated by BMI on the effect of education on CVD



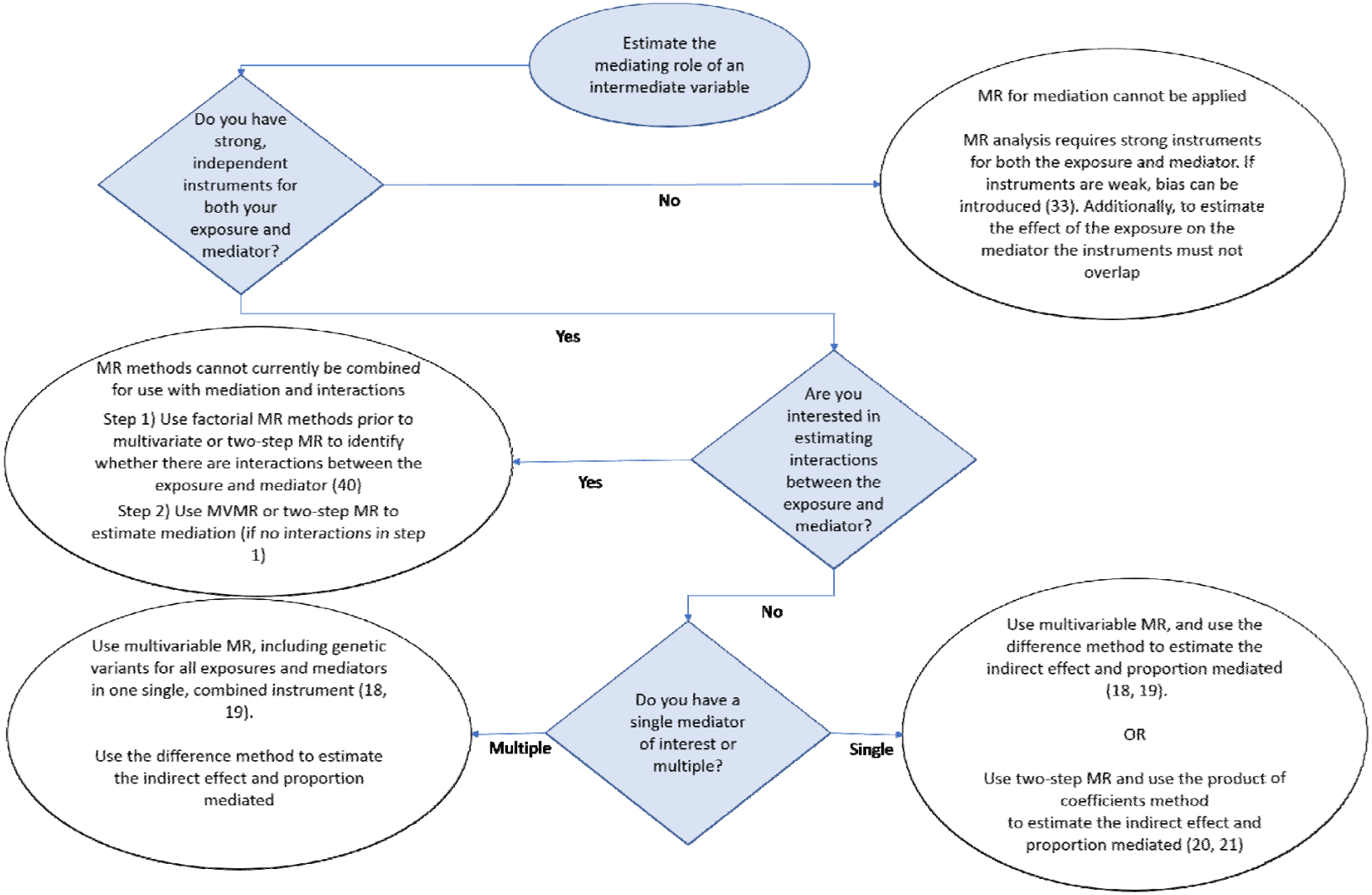
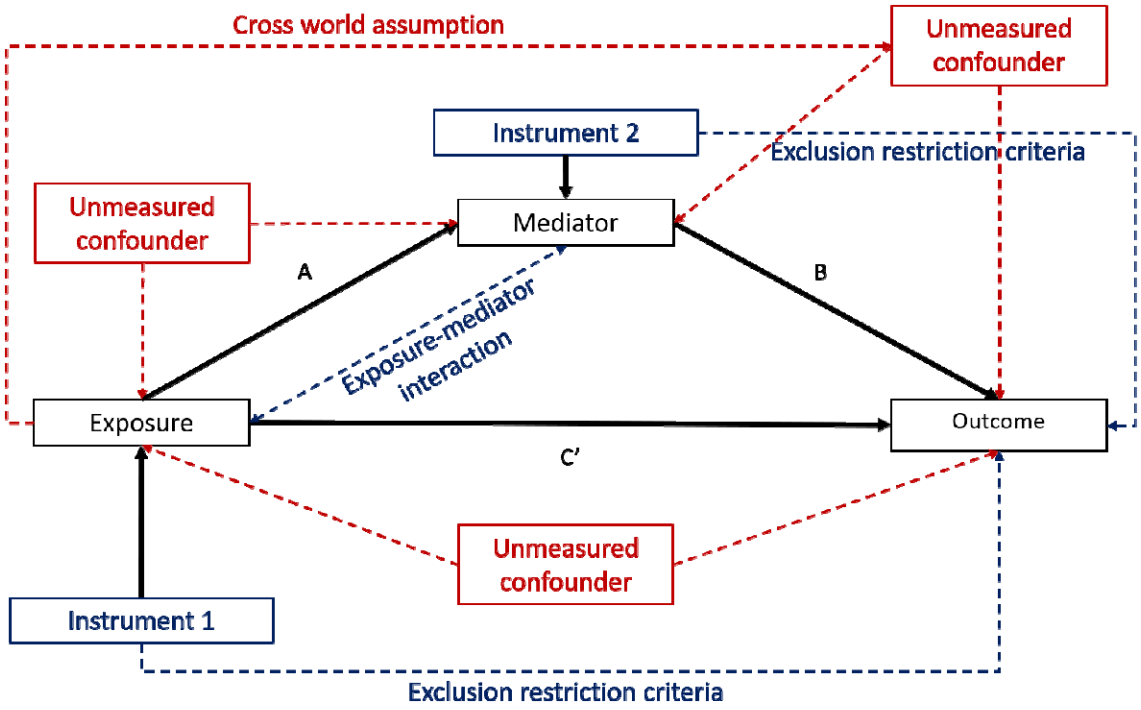


Figure 5: Decision flow chart to determine most appropriate mediation method

Figure 6: Schematic diagram illustrating the causal assumptions (dashed lines) in phenotypic regression-based mediation methods (red) and Mendelian randomisation mediation analysis (navy) with the measured associations in solid black lines



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