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New proposal to address mediation analysis interrogations by using genetic variants as instrumental variables

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Abstract

The application of causal mediation analysis (CMA) considering the mediation effect of a third variable is increasing in epidemiological studies; however, this requires fitting strong assumptions on confounding bias. To address this limitation, we propose an extension of CMA combining it with Mendelian randomization (MRinCMA). We applied the new approach to analyse the causal effect of obesity and diabetes on pancreatic cancer, considering each factor as potential mediator. To check the performance of MRinCMA under several conditions/scenarios, we used it in different simulated data sets and compared it with structural equation models. For continuous variables, MRinCMA and structural equation models performed similarly, suggesting that both approaches are valid to obtain unbiased estimates. When noncontinuous variables were considered, MRinCMA presented, overall, lower bias than structural equation models. By applying MRinCMA, we did not find any evidence of causality of obesity or diabetes on pancreatic cancer. With this new methodology, researchers would be able to address CMA hypotheses by appropriately accounting for the confounding bias assumption regardless of the conditions used in their studies in different settings.

Núria Malats and Teresa Pérez contributed equally to this study and are joint senior authors.

KEYWORDS
causal inference, causal mediation analysis, Mendelian randomization, pancreatic cancer, type 2 diabetes mellitus

1 | INTRODUCTION

In epidemiological studies, an important goal is to analyse the causal relationship between an exposure and an outcome. In general, epidemiological analysis relies on observational data and may present bias generated by confounding factors that are associated with the outcome and the exposure and may distort the real effect of the exposure (Hernán & Robins, 2020; Imbens & Rubin, 2015; Pearl, 2010). According to the availability of these variables, they are defined as measured (VanderWeele, 2016) (i.e., all the confounders are collected and measured) or unmeasured confounders (Hernán & Robins, 2020; Imbens & Rubin, 2015; Pearl, 2010). Several statistical methods have been proposed according to the type of confounding variables: for instance, causal mediation analysis (CMA) assumes measured confounding, although Mendelian randomization (MR) can deal with unmeasured confounding.

CMA is, in general, used to estimate the causal effect of the exposure on the outcome considering the effect of a third variable, called mediator, which occurs in the pathway from the exposure to the outcome (Valeri & VanderWeele, 2013; VanderWeele, 2016; Vanderweele & Vansteelandt, 2009) (Figure 1a). In CMA there are three main estimates: (a) the direct effect: effect of the exposure on the outcome independent of the mediator; (b) the indirect effect: effect of the exposure on the outcome that occurs through the mediator; and (c) the total effect: effect of the exposure on the outcome considering all previous effects. These effects may be estimated by either the product or the different methods proposed by Baron and Kenny (BK) (De Stavola et al., 2015; Vanderweele & Vansteelandt, 2009). However, to interpret these estimates as causal, strong assumptions are required according to the confounding assumptions (Vanderweele & Vansteelandt, 2009). When applying CMA, all the confounding variables should be known and measured. These assumptions are difficult to meet (Carter et al., 2021) and may lead to biased and not representative results.

MR analysis is extensively used in epidemiology to analyse the effect of an exposure and an outcome (Burgess et al., 2013; Coscia et al., 2022; Davies et al., 2018) (Figure 1b). This procedure applies either genetic variants

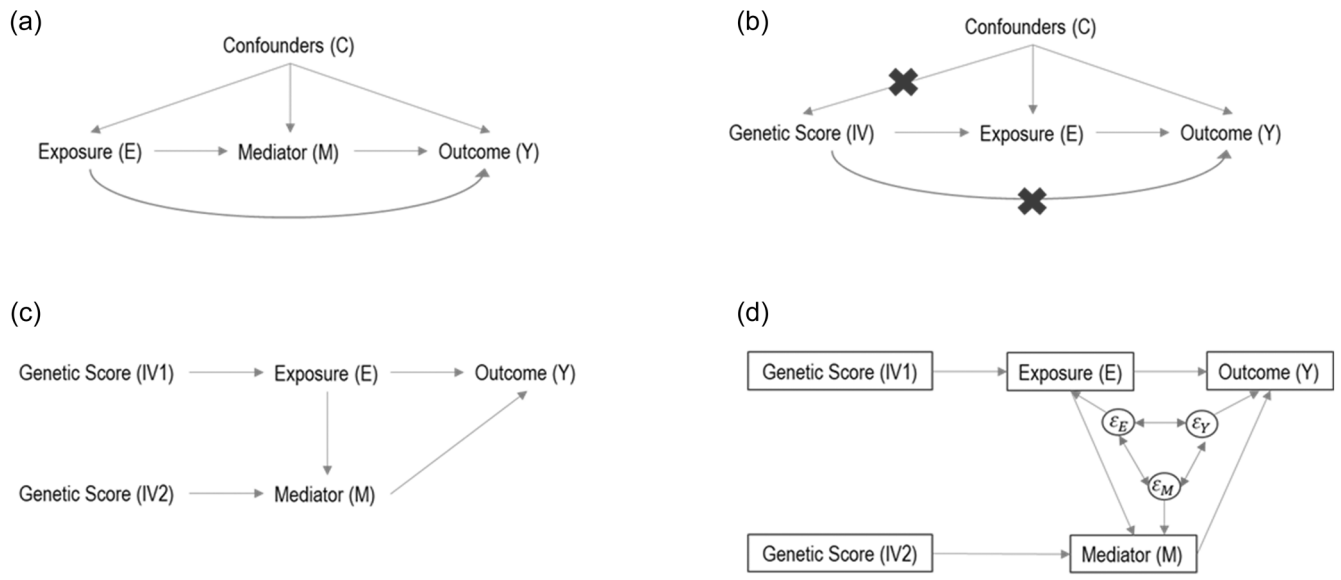

FIGURE 1 Causal diagrams. (a) Causal mediation analysis (CMA) graph. (b) Mendelian randomization graph. (c) Mediation analysis with instrumental variables (IVs) (MRinCMA as the specific case). Confounders are considered between E, M and Y, but not represented in the directed acyclic graph (DAG). (d) Structural equation model (SEM), where directed arrows represent causal effect and bidirectional arrows indicate a correlation between variables. Measurement variables are represented by squares and latent variables, that is, not observed are represented by circles. Confounders are considered between E, M and Y, but not represented in the DAG.

or a genetic score (Burgess & Thompson, 2013) as the instrumental variable (IV), leading to unbiased estimates of the causal effect, even when there is unmeasured confounding bias (Burgess & Thompson, 2013; Burgess et al., 2017, 2020; Coscia et al., 2022; Davies et al., 2018). Three core MR assumptions are needed for the estimation of the causal effect: (i) the IV is associated with the exposure, (ii) the IV is associated with the outcome only through the exposure and (iii) the instrument is not associated with any confounder (Burgess & Thompson, 2013; Coscia et al., 2022). Assuming individual level data and one-sample MR (Burgess et al., 2020), a common approach used in MR is the two-stage least squares (2SLS). The 2SLS is a regression-based method that uses the predicted values of the exposure to estimate the causal effect on the outcome (Burgess et al., 2017; Uddin et al., 2015). There are some extensions of the 2SLS depending on the type of the variables as the two-stage residual inclusion (2SRI), which is a good alternative for noncontinuous variables (Palmer et al., 2017; Terza et al., 2008).

Owing to the potential limitation of having an unmeasured confounding bias in CMA, some authors already suggested that using IVs in the CMA context could be a practical alternative to *relax* the CMA assumptions and to obtain unbiased results (Carter et al., 2021). For instance, Burgess et al. (2015). (Figure 1c) proposed an extension of the 2SLS regression-based method adding a third stage (i.e., 3SLS) when regressing continuous outcome variables, and they compared the performance of this method to structural equation models (SEM) (Figure 1d) when analysing the causality between body mass index (BMI), C-reactive protein and uric acid. Also, in the context of continuous outcomes, Frölich and Huber (2017) applied a four-stage regression-based extension (i.e., 4SLS and 4SRI) to analyse the effect of education and income on the social functioning using school leaving and windfall income as IVs. Although Burgess et al. (2015) considered the exposure, mediator and outcome as continuous variables, Frölich and Huber (2017) included the exposure as binary, maintaining the mediator and outcome as continuous. In the same context, North et al. (2019) used the same causal diagram as Burgess et al. (2015) to estimate the interaction terms between the exposure and the mediator considering two different instruments, one per each exposure and mediator. Sanderson (2021) proposed an approach based on multivariable MR to assess the directionality of the exposure when there is another variable related to the IV; Relton & Davey Smith (2012) considered a two-sample MR in an epigenetic context. Finally, Carter et al. (2021). made a comprehensive summary of the current methods to use MR in mediation analysis and they proposed an alternative combining both approaches.

Up to now, only Carter et al. (2021). suggested a methodological alternative for categorical outcomes, pointing to the lack of development of methods when modelling this type of variables, which are those commonly used in epidemiological studies. For that reason, in this paper, we proposed to extend Burgess et al. (2015) and Frölich and Huber (2017) approaches, offering a simple, valid and flexible methodology that would consider not only continuous and normally distributed variables but also categorical variables.

Therefore, the aim of this study was to propose four extensions of the CMA, which we named "Mendelian Randomization in Causal Mediation Analysis" (MRinC-MA), by considering two genetic instruments, for exposure and mediator each, to correct for potential confounding bias in the mediation analysis framework.

In this paper, we first demonstrate the capacity of MRinCMA to obtain unbiased direct, indirect and total effects using simulation studies, and compare their performances to SEM, as the reference method. Then, we report the result of applying MRinCMA, to assess the causality between obesity, type 2 diabetes mellitus (DM) and pancreatic cancer (PC) in the context of the PanGenEU case–control study (Molina-Montes et al., 2021).

2 | METHODS

This section is structured as follows: first, we presented the new proposal statistical method and the approach used as a reference; second, we described the simulation process and the performance indicators considered to assess the efficiency of the statistical methods; third, a brief description of a real data set used in the empirical example.

2.1 | MRinCMA approaches to estimate direct, indirect, and total effects

We defined four MRinCMA approaches as extensions of 2SLS: 4SLS, 3SLS, 4SRI and 3SRI. The causal diagram is presented in Figure 1c, where Y is the outcome; E, the exposure; M, the mediator; IV_1, the exposure-weighted genetic score; IV_2, the mediator-weighted genetic score; and C, the vector of confounders.

Following the 2SLS procedure, the 4SLS approach requires four regression models to estimate direct, indirect and total effects

$$E = \beta_0 + \beta_1 IV_1 + \beta_2 C + \varepsilon_1, \quad (1)$$

$$M = \mu_0 + \mu_1 \hat{E} + \mu_2 IV_2 + \mu_3 C + \varepsilon_2, \quad (2)$$

$$Y = \alpha_0 + \alpha_1 \hat{E} + \alpha_2 \hat{M} + \alpha_3 C + \varepsilon_3, \quad (3)$$

$$M = \gamma_0 + \gamma_1 \hat{E} + \gamma_2 C + \varepsilon_4, \quad (4)$$

where $\hat{E}$ and $\hat{M}$ are the predictor vectors obtained from Equations (1) and (2), respectively.

Based on BK methodology (De Stavola et al., 2015; Vanderweele & Vansteelandt, 2009), the direct effect is the estimated effect of $\hat{E}$ over Y, $\hat{\alpha}_1$, derived from Equation (3); the indirect effect is the product $(\hat{\gamma}_1 \times \hat{\alpha}_2)$, where $\hat{\gamma}_1$ is the estimated effect of $\hat{E}$ over M, obtained from Equation (4); and $\hat{\alpha}_2$ is the estimated effect of $\hat{M}$ over Y, derived from Equation (3). The total effect is the sum of both effects, $\hat{\alpha}_1 + (\hat{\gamma}_1 \times \hat{\alpha}_2)$ for continuous outcomes and the product $\hat{\alpha}_1 \times (\hat{\gamma}_1 \times \hat{\alpha}_2)$ for binary outcomes (Vanderweele & Vansteelandt, 2010). The 3SLS approach is similar to 4SLS, but only needs three regression models, Equations (1)–(3). The direct effect is $\hat{\alpha}_1$, as for 4SLS, although the indirect effect is $(\hat{\mu}_1 \times \hat{\alpha}_2)$, and the total effect is the sum, or the product when appropriate, of both effects. The 4SRI approach is similar to the 4SLS, but now instead of using the fitted values of E and M, $(\hat{E}, \hat{M})$, the regression models included the observed values (E, M) and the residuals $(\hat{\varepsilon}_1, \hat{\varepsilon}_2)$ obtained in the previous steps. The models are as follows:

$$E = \tau_0 + \tau_1 IV_1 + \tau_2 C + \varepsilon_1, \quad (5)$$

$$M = \eta_0 + \eta_1 E + \eta_2 \hat{\varepsilon}_1 + \eta_3 IV_2 + \eta_4 C + \varepsilon_2, \quad (6)$$

$$Y = \nu_0 + \nu_1 E + \nu_2 \hat{\varepsilon}_1 + \nu_3 M + \nu_4 \hat{\varepsilon}_2 + \nu_5 C + \varepsilon_3, \quad (7)$$

$$M = \lambda_0 + \lambda_1 E + \lambda_2 \hat{\varepsilon}_1 + \lambda_3 C + \varepsilon_4, \quad (8)$$

where $\hat{\varepsilon}_1$ and $\hat{\varepsilon}_2$ are the residual vectors obtained from Equations (5) and (6), respectively.

The direct effect is now $\hat{\nu}_1$, the indirect effect is $(\hat{\lambda}_1 \times \hat{\nu}_3)$ and the total effect is the sum, $\hat{\nu}_1 + (\hat{\lambda}_1 \times \hat{\nu}_3)$, or the product, $\hat{\nu}_1 \times (\hat{\lambda}_1 \times \hat{\nu}_3)$, of both continuous and categorical outcomes, respectively.

Analogously to 3SLS, the 3SRI method only requires models Equations (5)–(7). The direct effect is $\hat{\nu}_1$, the indirect effect is $(\hat{\eta}_1 \times \hat{\nu}_3)$ and the total effect is the sum, or the product, as appropriate, of both effects.

2.2 | SEM as a reference method

We also estimated the three effects using the SEM as was previously done by Burgess et al. (2015) to compare our results. It is a multivariate technique widely used in sociological and econometrical fields (Belope, 2020; Pearl, 2012), which specifies measurement errors of the variables and permits to incorporate both observed and unobserved variables (Muthén, 1984, 2011; Pearl, 2010, 2012).

We defined the equations as shown in Figure 1d. Coefficient estimates of E and M in SEM represented direct and indirect effects, and the total effect is the sum of both assuming no interaction. The maximum likelihood approximation is used for continuous and normally distributed variables, although the weighted least squares (WLS) was considered for categorical variables (Li, 2016; Muthén, 1984; Muthén & Asparouhov, 2015; Olsson et al., 2000).

2.3 | Simulation study design

Simulation studies are a common tool for assessing the performance of statistical methods (Morris et al., 2019). For that reason, we evaluated the efficiency of the five abovementioned methods (i.e., 4SLS, 3SLS, 4SRI, 3SRI and SEM) in estimating the parameters of interest: direct, indirect and total effects, under different scenarios. The summary and workflow are shown in Figure 2.

An observational study with an outcome Y, an exposure E and a mediator M was simulated, under different settings, 2000 times.

Each simulated data set was generated based on the PanGenEU study data frequency, a European case–control study including 2500 PC patients and 1500 controls (López de Maturana et al., 2021; Molina-Montes et al., 2021) (Supporting Information: Appendix A).

2.3.1 | Independent variables and outcomes of interest

The outcomes, exposures and mediators have been defined as either continuous or binary variables. We considered two outcomes, a binary trait reflecting PC status and a continuous one corresponding to the PC risk score. BMI (continuous) and obesity (categorized as 1 if BMI > 30 and 0 otherwise; Kent et al., 2017; Molina-Montes et al., 2021) were the exposures (E). Long-standing DM (LSDM) (i.e., diabetes diagnosed >2 years before the study recruitment [Molina-Montes et al., 2021] and categorized as yes/no) and glycated haemoglobin (continuous) were considered as mediators (M). We included two continuous genetic scores (IV_1 and IV_2) weighting by the effect of each genetic

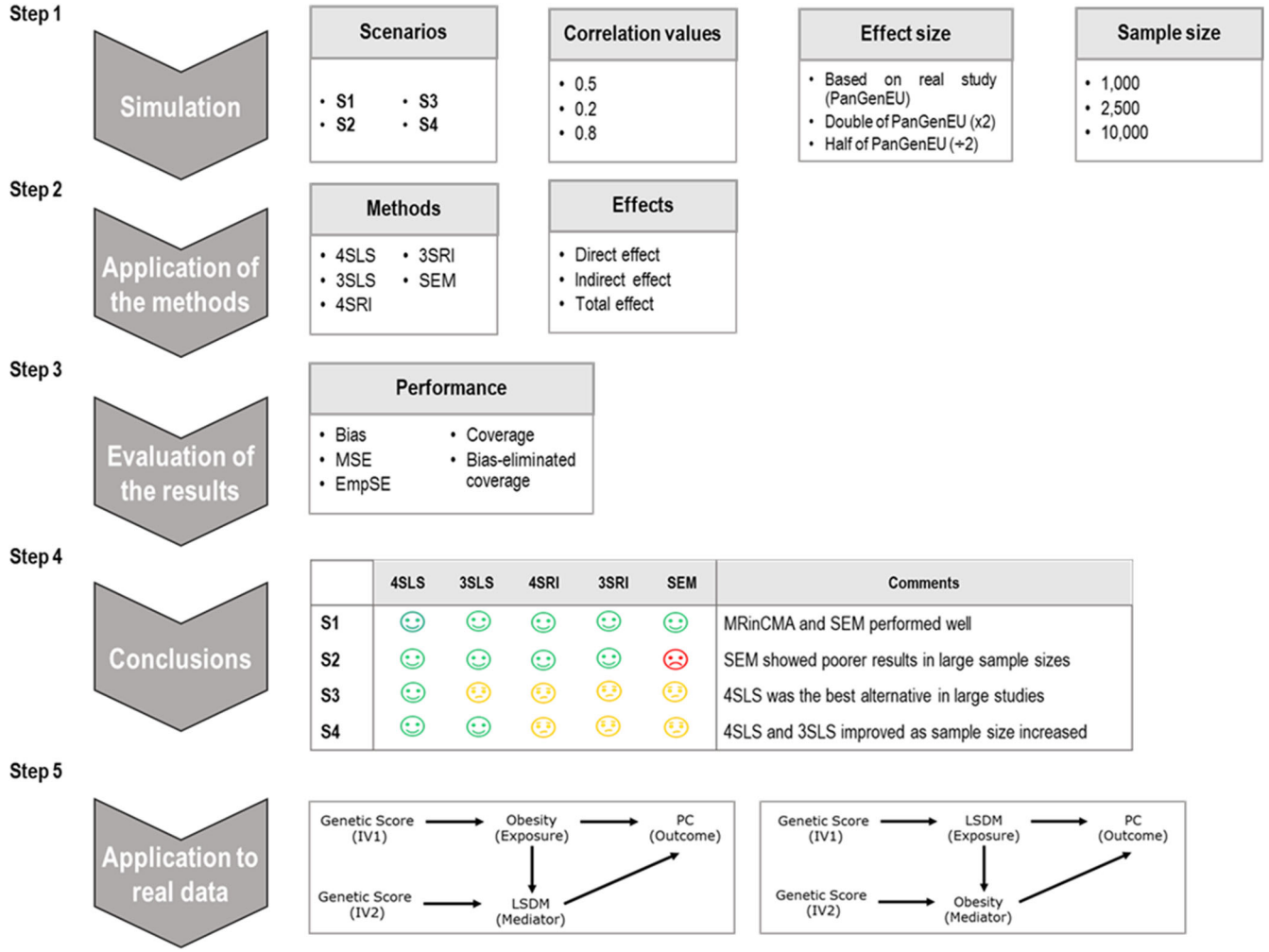

FIGURE 2 Simulations steps and workflow: S1: *Y*, *E* and *M* continuous; S2: *Y* binary and *E* and *M* continuous; S3: *Y* continuous and *E* and *M* binary; S4: *Y*, *E* and *M* binary. EmpSE, empirical standard error; IV, instrumental variable; LSDM, long-standing diabetes mellitus; MSE, mean-squared error; PC, pancreatic cancer; 3SLS, three-stage least squares; 4SLS, four-stage least squares; 4SRI, four-stage residual inclusion; 3SRI, three-stage residual inclusion; SEM, structural equation models.

variant on the exposure and the mediator, respectively. We incorporated sex (categorical: man/woman), age (continuous) and smoking status (categorical: nonsmoker/occasional/former/current smoker) as confounding variables (*C*).

2.3.2 | Data-generating mechanisms

According to the definition of the variables *Y*, *E* and *M*, the following four scenarios were studied:

S1: *Y*, *E* and *M* as continuous.
S2: *Y* as binary and both *E* and *M* as continuous.
S3: *Y* as continuous and both *E* and *M* as binary.
S4: *Y*, *E* and *M* as binary.

Variables *Y*, *E* and *M* were simulated as continuous in all cases by using their probit or logit transformation (*Y**, *E** and *M**), when appropriate; therefore, linear regression models were assumed regardless of the character of these variables. The original *Y*, *E* and *M* were recovered subsequently by inverting this transformation. Note that *Y* equalled *Y**, *E* equalled *E** and *M** equalled *M* when they were continuous (Supporting Information: Appendix B).

We simulated *Y**, *E** and *M** using a multivariate normal model, assuming random errors with vectors of means $\mu = (0,0,0)$ and variance–covariance matrix:

$$\Sigma = \begin{pmatrix} \sigma_E^2 & \rho\sigma_E\sigma_M & \rho\sigma_E\sigma_Y \\ \rho\sigma_E\sigma_M & \sigma_M^2 & \rho\sigma_M\sigma_Y \\ \rho\sigma_E\sigma_Y & \rho\sigma_M\sigma_Y & \sigma_Y^2 \end{pmatrix}.$$

Standard deviations ($\sigma_E, \sigma_M, \sigma_Y$) were considered, based on PanGenEU results. Correlation, ρ, was moved from weak (0.2), and moderate (0.5) to strong (0.8).

We considered different simulation settings according to: (i) the nature of the variables (S1–S4), (ii) the correlation between variables $\rho = (0.2, 0.5, 0.8)$, (iii) the effect sizes (based on PanGenEU estimates, half and double of PanGenEU estimates) and (iv) the study sample size (1000, 2500 and 10,000). Additional details about the simulation procedure are available in Supporting Information: Appendix B and values of the prefixed coefficients considered are shown in Supporting Information: Appendix D, Table ST1. When dealing with categorical variables, the probit transformation was always considered for SEM, while for MRinCMA, we considered the probit transformation for scenario S3 (continuous outcome) and the logit transformation in other cases, as better performance results were observed.

2.4 | Applied example: Effect of LSDM and BMI on PC

We applied the MRinCMA approaches and SEM to investigate the effect of LSDM on PC considering the mediation effect of BMI, and the effect of BMI on PC with LSDM as a mediator. To this goal, we used the resources of the PanGenEU study, a European multicentre hospital-based case–control study designed to assess environmental and genetic factors associated with PC risk (López de Maturana et al., 2021; Molina-Montes et al., 2021). PC is the seventh most common death by cancer worldwide, and LSDM and obesity are the two main complex risk factors due to the coexistence of both. For that reason, it is important to assess the causal effect of obesity and the mediation role of LSDM to better understand their role in PC risk. We included those participants with available genetic information, (1040 cases and 738 controls). More details are provided in Supporting Information: Appendix A and in Molina-Montes et al. (2021) and López de Maturana et al. (2021).

2.5 | Performance indicators

We evaluated the five approaches using the following metrics: bias, mean-squared error (MSE), empirical standard error (EmpSE) 95% confidence interval coverage rate (CI-C) and bias-eliminated coverage rate (BE-C). A detailed definition of these performance measures is presented in Supporting Information: Appendix C and in Morris et al. (2019).

All analyses were performed using R software version 4.0.1. SEMs were fitted using package *lavaan* (Rosseel, 2012), which implements WLS for categorical data fitting a probit regression model.

3 | RESULTS

3.1 | Results from the simulation study

A summary of the performance of the five methods, also described in Figure 1, is as follows:

S1: Y, E and M as continuous: All methods yielded a null or negligible bias in the estimation of the three effects, Table 1. In Table 2 and in Supporting Information: Appendix D, Table ST2, we presented the estimated 95% CI-C and BE-C. For the direct effect, SEM outperformed the MRinCMA methods with results around the nominal 95% CI cut-off point. On the other hand, for the indirect and total effects, we noted that CI-C and BE-C were reasonably close to the cut-off point for the five methods.

S2: Y as binary and both E and M as continuous: The results obtained for MRinCMA methods were similar to those from *S1*, with good performance results overall, Tables 2 and 3 and Supporting Information: Appendix D, Table ST2. On the contrary, SEM showed higher bias and presented poor results with CI-C deteriorated to a clearly unacceptable coverage for indirect and total effects when the sample size increased, Table 2 and Supporting Information: Appendix D, Table ST2.

S3: Y as continuous, and E and M as binary: Biased estimates and high MSE values were obtained in both MRinCMA and SEM (Table 4). SEM showed poorer coverage rates than MRinCMA (Table 2). 4SLS was the best alternative with a large sample size (Supporting Information: Appendix D, Tables ST2 and ST3).

S4: Y, E and M as binary: Similar conclusions as those obtained in scenario S3 could be derived here, 4SLS was, in general, the best option (Tables 2 and 4). Its performance improved as sample size increased (Supporting Information: Appendix D, Tables ST2 and ST3). A sensitivity analysis has been done considering higher and lower effect sizes and stronger or weaker correlations between Y, E and M, and we obtained similar performance results (data not shown).

3.2 | Application to real data: The PanGenEU case–control study

Considering the performance of the five approaches, we applied the appropriate methodology to the PanGenEU study, to investigate the effect of LSDM and BMI on PC risk.

TABLE 1 Bias, MSE and EmpSE in scenario S1, according to different sample sizes, based on coefficients shown in Appendix D, Table ST1.

	Direct			Indirect			Total		
	Bias Estimate (SE)	**MSE Estimate (SE)**	**EmpSE Estimate (SE)**	**Bias Estimate (SE)**	**MSE Estimate (SE)**	**EmpSE Estimate (SE)**	**Bias Estimate (SE)**	**MSE Estimate (SE)**	**EmpSE Estimate (SE)**
$n = 1000$									
4SLS	−0.001 (0.001)	0.003 (0.000)	0.055 (0.001)	0.002 (0.001)	0.001 (0.000)	0.030 (0.000)	0.001 (0.001)	0.004 (0.000)	0.066 (0.001)
3SLS	−0.001 (0.000)	0.003 (0.000)	0.055 (0.001)	−0.004 (0.001)	0.001 (0.000)	0.029 (0.000)	−0.004 (0.001)	0.004 (0.000)	0.066 (0.001)
4SRI	−0.001 (0.001)	0.003 (0.000)	0.053 (0.001)	0.002 (0.001)	0.001 (0.000)	0.028 (0.000)	0.001 (0.001)	0.004 (0.000)	0.066 (0.001)
3SRI	−0.001 (0.001)	0.003 (0.000)	0.053 (0.001)	−0.003 (0.001)	0.001 (0.000)	0.027 (0.000)	−0.004 (0.001)	0.004 (0.000)	0.066 (0.001)
SEM	−0.001 (0.001)	0.003 (0.000)	0.053 (0.001)	−0.003 (0.001)	0.001 (0.000)	0.027 (0.000)	−0.004 (0.001)	0.004 (0.000)	0.066 (0.001)
$n = 2500$									
4SLS	0.000 (0.001)	0.001 (0.000)	0.034 (0.000)	0.004 (0.000)	0.000 (0.000)	0.018 (0.000)	0.004 (0.001)	0.002 (0.000)	0.040 (0.001)
3SLS	0.000 (0.001)	0.001 (0.000)	0.034 (0.000)	−0.001 (0.000)	0.000 (0.000)	0.017 (0.000)	−0.002 (0.001)	0.002 (0.000)	0.040 (0.001)
4SRI	−0.001 (0.001)	0.001 (0.000)	0.033 (0.000)	0.004 (0.000)	0.000 (0.000)	0.017 (0.000)	0.004 (0.001)	0.002 (0.000)	0.040 (0.001)
3SRI	−0.001 (0.000)	0.001 (0.000)	0.033 (0.000)	−0.001 (0.000)	0.000 (0.000)	0.016 (0.000)	−0.002 (0.001)	0.002 (0.000)	0.040 (0.001)
SEM	−0.001 (0.001)	0.001 (0.000)	0.033 (0.000)	−0.001 (0.000)	0.000 (0.000)	0.016 (0.000)	−0.002 (0.001)	0.002 (0.000)	0.040 (0.001)
$n = 10{,}000$									
4SLS	0.000 (0.000)	0.000 (0.000)	0.016 (0.000)	0.005 (0.000)	0.000 (0.000)	0.009 (0.000)	0.005 (0.000)	0.000 (0.000)	0.019 (0.000)
3SLS	0.000 (0.000)	0.000 (0.000)	0.016 (0.000)	0.000 (0.000)	0.000 (0.000)	0.008 (0.000)	0.000 (0.000)	0.000 (0.000)	0.019 (0.000)
4SRI	0.000 (0.000)	0.000 (0.000)	0.016 (0.000)	0.005 (0.000)	0.000 (0.000)	0.008 (0.000)	0.005 (0.000)	0.000 (0.000)	0.019 (0.000)
3SRI	0.000 (0.000)	0.000 (0.000)	0.016 (0.000)	0.000 (0.000)	0.000 (0.000)	0.008 (0.000)	0.000 (0.000)	0.000 (0.000)	0.019 (0.000)
SEM	0.000 (0.000)	0.000 (0.000)	0.016 (0.000)	0.000 (0.000)	0.000 (0.000)	0.008 (0.000)	0.000 (0.000)	0.000 (0.000)	0.019 (0.000)

Abbreviations: EmpSE, empirical standard error; MSE, mean-squared error; S1, Y, E and M continuous; SE, standard error; SEM, structural equation models; 3SLS, three-stage least squares; 4SLS, four-stage least squares; 3SRI, three-stage residual inclusion; 4SRI, four-stage residual inclusion.

TABLE 2 95% Confidence interval coverage rate and bias-eliminated coverage for the direct, indirect and total effect estimation in scenarios S1–S4 and according to different sample sizes, based on PanGenEU coefficients shown in Supporting Information: Appendix D, Table ST1.

	$n = 1000$						$n = 2500$					
	Direct		Indirect		Total		Direct		Indirect		Total	
	CI-C	BE-C	CI-C	BE-C	CI-C	BE-C	CI-C	BE-C	CI-C	BE-C	CI-C	BE-C
S1												
4SLS	99.40%	99.40%	93.70%	93.80%	92.00%	92.10%	99.60%	99.60%	93.80%	93.00%	91.90%	92.55%
3SLS	99.40%	99.40%	92.20%	93.60%	91.50%	91.75%	99.60%	99.60%	92.70%	93.25%	92.90%	92.70%
4SRI	88.30%	88.65%	93.30%	92.70%	92.60%	92.30%	88.30%	87.95%	93.00%	93.20%	91.90%	92.40%
3SRI	88.30%	88.65%	91.40%	92.65%	92.50%	92.50%	88.30%	87.95%	92.60%	92.85%	92.50%	92.45%
SEM	94.70%	94.78%	91.40%	92.00%	92.10%	91.95%	94.70%	94.40%	93.10%	93.45%	93.10%	93.10%
S2												
4SLS[a]	99.00%	99.00%	93.00%	93.00%	92.00%	92.00%	99.00%	99.00%	94.00%	93.00%	91.00%	92.00%
3SLS[a]	99.00%	99.00%	92.00%	93.00%	92.00%	92.00%	99.00%	99.00%	94.00%	93.00%	93.00%	93.00%
4SRI[a]	88.00%	89.00%	94.00%	94.00%	92.00%	92.00%	88.00%	88.00%	93.00%	93.00%	93.00%	92.00%
3SRI[a]	88.00%	89.00%	92.00%	93.00%	92.00%	92.00%	88.00%	88.00%	93.00%	93.00%	93.00%	92.00%
SEM[b]	96.00%	97.00%	78.00%	94.00%	96.00%	99.00%	94.00%	95.00%	67.00%	92.00%	78.00%	97.00%
S3												
4SLS[b]	97.20%	96.90%	99.50%	99.50%	98.70%	98.30%	97.10%	96.20%	98.80%	98.80%	95.90%	96.30%
3SLS[b]	97.20%	96.90%	99.50%	99.50%	98.10%	98.00%	97.10%	96.20%	98.80%	98.70%	95.90%	95.60%
4SRI[b]	94.40%	94.10%	99.60%	99.60%	98.80%	98.70%	93.10%	94.00%	99.40%	99.50%	94.80%	96.60%
3SRI[b]	94.40%	94.10%	99.70%	99.80%	98.40%	99.00%	93.10%	94.00%	99.60%	99.60%	95.00%	96.60%
SEM[b]	91.90%	91.60%	73.70%	98.60%	93.60%	95.20%	93.60%	94.40%	39.30%	96.00%	81.80%	93.40%
S4												
4SLS[a]	95.00%	95.00%	99.00%	99.00%	98.00%	97.00%	95.00%	95.00%	99.00%	99.00%	95.00%	96.00%
3SLS[a]	95.00%	95.00%	99.00%	99.00%	98.00%	98.00%	95.00%	95.00%	99.00%	99.00%	97.00%	97.00%
4SRI[a]	95.00%	95.00%	99.00%	99.00%	99.00%	99.00%	96.00%	96.00%	99.00%	99.00%	97.00%	98.00%
3SRI[a]	95.00%	95.00%	99.00%	99.00%	99.00%	99.00%	96.00%	96.00%	99.00%	99.00%	97.00%	98.00%
SEM[b]	99.00%	99.00%	89.00%	99.00%	97.00%	100.00%	96.00%	98.00%	57.00%	99.00%	75.00%	99.00%

Abbreviations: BE-C, bias-eliminated coverage; CI-C, 95% confidence interval coverage rate; S1, Y, E and M continuous; S2, Y binary and E and M continuous; S3, Y continuous and E and M binary; S4, Y, E and M binary; SEM, structural equation models; 3SLS, three-stage least squares; 4SLS, four-stage least squares; 3SRI, three-stage residual inclusion; 4SRI, four-stage residual inclusion.

[a]Logit model was used.

[b]Probit model was used, when appropriate.

We defined Biological Model A to study the causal effect of obesity (binary) on PC (binary, case/control) risk considering LSDM (binary) as a mediator, and Biological Model B to analyse the causality of LSDM on PC considering obesity as a mediator, both corresponding to scenario S4.

The estimates and 95% CI for each effect were obtained based on the results shown in Tables 2 and 4, and the 4SLS approach is applied for both Biological Models A and B. Based on the results shown in Table 5, it can be concluded that there was no direct effect of LSDM and obesity on PC and that no factor mediated the relationship of the other.

4 | DISCUSSION

In this work, we proposed an extension of CMA and MR approaches (i.e., MRinCMA) to solve the potential confounding bias present in CMA when confounders

TABLE 3 Bias, MSE andEmpSE in scenario S2 according to different sample sizes, based on coefficients shown in Appendix D, Table ST1.

	Direct			Indirect			Total		
	Bias Estimate (SE)	**MSE Estimate (SE)**	**EmpSE Estimate (SE)**	**Bias Estimate (SE)**	**MSE Estimate (SE**	**EmpSE Estimate (SE)**	**Bias Estimate (SE)**	**MSE Estimate (SE)**	**EmpSE Estimate (SE)**
$n = 1000$									
4SLS[a]	−0.001 (0.001)	0.003 (0.000)	0.098 (0.001)	0.002 (0.000)	0.000 (0.000)	0.044 (0.001)	0.001 (0.001)	0.004 (0.000)	0.038 (0.001)
3SLS[a]	−0.001 (0.001)	0.003 (0.000)	0.098 (0.001)	−0.004 (0.001)	0.001 (0.000)	0.040 (0.001)	−0.004 (0.001)	0.004 (0.000)	0.034 (0.001)
4SRI[a]	−0.001 (0.001)	0.003 (0.000)	0.131 (0.001)	0.002 (0.000)	0.000 (0.000)	0.058 (0.001)	0.001 (0.001)	0.004 (0.000)	0.047 (0.001)
3SRI[a]	−0.001 (0.001)	0.003 (0.000)	0.131 (0.001)	−0.003 (0.000)	0.000 (0.000)	0.053 (0.001)	−0.004 (0.001)	0.004 (0.000)	0.043 (0.001)
SEM[b]	−0.012 (0.002)	0.004 (0.000)	0.060 (0.007)	−0.021 (0.001)	0.001 (0.000)	0.024 (0.007)	−0.001 (0.000)	0.000 (0.000)	0.027 (0.001)
$n = 2500$									
4SLS[a]	0.000 (0.000)	−0.001 (0.000)	0.062 (0.000)	0.004 (0.000)	0.000 (0.000)	0.026 (0.001)	0.004 (0.000)	0.002 (0.000)	0.022 (0.001)
3SLS[a]	0.000 (0.000)	−0.001 (0.000)	0.062 (0.000)	−0.001 (0.000)	0.000 (0.000)	0.024 (0.001)	−0.002 (0.000)	0.002 (0.000)	0.020 (0.001)
4SRI[a]	0.000 (0.000)	−0.001 (0.000)	0.082 (0.000)	0.004 (0.000)	0.000 (0.000)	0.033 (0.000)	0.004 (0.000)	0.002 (0.000)	0.028 (0.001)
3SRI[a]	0.000 (0.000)	−0.001 (0.000)	0.082 (0.000)	−0.001 (0.000)	0.000 (0.000)	0.031 (0.000)	−0.002 (0.000)	0.002 (0.000)	0.026 (0.001)
SEM[b]	−0.012 (0.001)	0.002 (0.000)	0.038 (0.000)	−0.020 (0.000)	0.001 (0.000)	0.014 (0.000)	−0.001 (0.000)	0.000 (0.000)	0.014 (0.001)
$n = 10{,}000$									
4SLS[a]	0.000 (0.000)	0.000 (0.000)	0.029 (0.000)	0.005 (0.000)	0.000 (0.000)	0.013 (0.000)	0.005 (0.000)	0.000 (0.000)	0.012 (0.000)
3SLS[a]	0.000 (0.000)	0.000 (0.000)	0.029 (0.000)	0.000 (0.000)	0.000 (0.000)	0.011 (0.000)	0.000 (0.000)	0.000 (0.000)	0.011 (0.000)
4SRI[a]	0.000 (0.000)	0.000 (0.000)	0.039 (0.000)	0.005 (0.000)	0.000 (0.000)	0.017 (0.000)	0.005 (0.000)	0.000 (0.000)	0.016 (0.000)
3SRI[a]	0.000 (0.000)	0.000 (0.000)	0.039 (0.000)	0.000 (0.000)	0.000 (0.000)	0.016 (0.000)	0.000 (0.000)	0.000 (0.000)	0.015 (0.000)
SEM[b]	−0.011 (0.000)	0.000 (0.000)	0.019 (0.000)	−0.020 (0.000)	0.000 (0.000)	0.007 (0.000)	−0.001 (0.000)	0.000 (0.000)	0.006 (0.000)

Abbreviations: EmpSE, empirical standard error; MSE, mean-squared error; S2, Y binary and E and M continuous; SEM, structural equation models; 3SLS, three-stage least squares; 4SLS, four-stage least squares; 3SRI, three-stage residual inclusion; 4SRI, four-stage residual inclusion.

[a]Logit model was used.

[b]Probit model was used, when appropriate.

TABLE 4 Bias, MSE and EmpSE in scenarios S3 and S4 according to different sample sizes, based on coefficients shown in Appendix D, Table ST1.

	Direct			Indirect			Total		
	Bias Estimate (SE)	**MSE Estimate (SE)**	**EmpSE Estimate (SE)**	**Bias Estimate (SE)**	**MSE Estimate (SE)**	**EmpSE Estimate (SE)**	**Bias Estimate (SE)**	**MSE Estimate (SE)**	**EmpSE Estimate (SE)**
S3									
$n = 1000$									
4SLS[a]	−0.027 (0.068)	4.647 (0.329)	2.156 (0.001)	0.113 (0.1000)	10.005 (0.827)	3.162 (0.001)	0.904 (0.108)	12.532 (0.825)	10.720 (0.001)
3SLS[a]	−0.027 (0.068)	4.647 (0.329)	2.156 (0.001)	−0.027 (0.097)	9.493 (0.784)	3.082 (0.001)	0.764 (0.106)	11.889 (0.783)	10.185 (0.001)
4SRI	0.026 (0.082)	6.750 (0.461)	2.599 (0.001)	0.204 (0.152)	23.180 (2.237)	4.813 (0.001)	1.049 (0.157)	25.876 (2.058)	11.450 (0.001)
3SRI[a]	0.026 (0.082)	6.750 (0.461)	2.599 (0.001)	0.025 (0.149)	22.194 (2.114)	4.712 (0.001)	0.87 (0.155)	24.685 (1.923)	12.575 (0.001)
SEM[a]	−0.110 (0.014)	0.211 (0.010)	0.447 (0.002)	−0.128 (0.005)	0.040 (0.003)	0.255 (0.002)	-0.033 (0.003)	0.011 (0.002)	0.119 (0.001)
$n = 2500$									
4SLS[a]	−0.085 (0.037)	1.376 (0.066)	1.170 (0.000)	−0.189 (0.044)	1.996 (0.145)	1.401 (0.001)	0.544 (0.054)	3.184 (0.185)	2.130 (0.001)
3SLS[a]	−0.085 (0.037)	1.376 (0.066)	1.170 (0.000)	−0.314 (0.042)	1.840 (0.132)	1.320 (0.001)	0.419 (0.052)	2.893 (0.165)	2.212 (0.001)
4SRI[a]	−0.080 (0.046)	2.124 (0.116)	1.456 (0.000)	−0.208 (0.063)	3.993 (0.293)	1.988 (0.000)	0.531 (0.074)	5.739 (0.327)	3.347 (0.001)
3SRI[a]	−0.080 (0.046)	2.124 (0.116)	1.456 (0.000)	−0.389 (0.060)	3.697 (0.268)	1.884 (0.000)	0.350 (0.071)	5.222 (0.294)	3.284 (0.001)
SEM[a]	−0.110 (0.008)	0.077 (0.004)	0.154 (0.001)	−0.126 (0.002)	0.019 (0.000)	0.061 (0.001)	-0.020 (0.001)	0.001 (0.000)	0.018 (0.001)
S4									
$n = 1000$									
4SLS[b]	−0.107 (0.032)	2.026 (0.331)	1.420 (0.022)	0.023 (0.033)	2.161 (0.373)	1.470 (0.023)	−0.084 (0.039)	2.998 (0.353)	1.730 (0.027)
3SLS[b]	−0.107 (0.032)	2.026 (0.331)	1.420 (0.022)	−0.014 (0.032)	2.098 (0.362)	1.448 (0.023)	−0.121 (0.039)	3.052 (0.365)	1.743 (0.027)
4SRI[b]	−0.268 (0.032)	2.972 (0.124)	1.415 (0.022)	−0.119 (0.036)	2.666 (0.282)	1.629 (0.026)	−0.388 (0.039)	3.292 (0.189)	1.773 (0.028)
3SRI[b]	−0.268 (0.032)	2.972 (0.124)	1.415 (0.022)	−0.130 (0.036)	2.598 (0.027)	1.607 (0.025)	−0.398 (0.039)	3.260 (0.184)	1.764 (0.028)
SEM[a]	0.004 (0.005)	0.049 (0.002)	0.223 (0.003)	-0.119 (0.002)	0.026 (0.001)	0.109 (0.002)	−0.116 (0.006)	0.081 (0.003)	0.260 (0.004)
$n = 2500$									
4SLS[b]	−0.046 (0.013)	0.366 (0.020)	0.603 (0.009)	−0.004 (0.011)	0.225 (0.015)	0.475 (0.007)	−0.050 (0.016)	0.533 (0.039)	0.729 (0.011)
3SLS[b]	−0.046 (0.013)	0.366 (0.020)	0.603 (0.009)	−0.038 (0.010)	0.211 (0.015)	0.458 (0.007)	−0.084 (0.016)	0.536 (0.044)	0.727 (0.011)
4SRI[b]	−0.251 (0.016)	0.601 (0.027)	0.738 (0.012)	−0.108 (0.012)	0.335 (0.021)	0.569 (0.009)	-0.358 (0.019)	0.875 (0.043)	0.866 (0.013)

TABLE 4 (Continued)

	Direct			Indirect			Total		
	Bias Estimate (SE)	MSE Estimate (SE)	EmpSE Estimate (SE)	Bias Estimate (SE)	MSE Estimate (SE)	EmpSE Estimate (SE)	Bias Estimate (SE)	MSE Estimate (SE)	EmpSE Estimate (SE)
3SRI[b]	−0.251 (0.016)	0.601 (0.027)	0.738 (0.012)	−0.118 (0.012)	0.318 (0.021)	0.551 (0.009)	−0.369 (0.019)	0.875 (0.047)	0.860 (0.014)
SEM[a]	−0.009 (0.003)	0.017 (0.001)	0.129 (0.002)	−0.120 (0.001)	0.017 (0.000)	0.054 (0.001)	−0.130 (0.003)	0.037 (0.001)	0.144 (0.002)

Abbreviations: EmpSE, empirical standard error; MSE, mean-squared error; S3, Y continuous and E and M binary; S4, Y, E and M binary; SE, standard error; 3SLS, three-stage least squares; 4SLS, four-stage least squares; 3SRI, three-stage residual inclusion; 4SRI, four-stage residual inclusion; SEM, structural equation models.

[a]Probit model were used, when appropriate.

[b]Logit model was used.

are not measured by incorporating two IVs, which can deal with both continuous and categorical variables. Despite that the 4SLS, 4SRI and 3SLS approaches were already developed for continuous and normally distributed variables, here we assessed their performance also for noncontinuous variables. Moreover, we proposed the 3SRI approach using three stages considering both observed and residual values rather than the predicted values. In summary, we showed that MRinCMA provided unbiased estimates of the direct, indirect and total effects regardless the type of variables considered.

More specific, in scenario S1, where *E*, *M* and *Y* were defined as continuous variables, we concluded that MRinCMA worked properly, obtaining unbiased results equivalent or better than SEM, in all simulation settings. Our results, in terms of bias, were similar as those obtained by Burgess et al. (2015) when they considered 3SLS and SEM. Frölich et al. (2017) also confirmed that 4SLS worked properly in several simulation conditions.

For the rest of scenarios, where we had at least one categorical variable, as it was already described by Burgess (2013) and by Carter et al. (2021), these approaches can lead to less precise estimations due to the noncollapsibility of the odds ratio, ending in higher bias. Even so, in these cases, MRinCMA always outperformed SEM, where biased estimates and therefore worst coverage rates were obtained. Up to now, only Carter et al. (2021). and our proposal provided unbiased estimates with noncontinuous variables, although our approaches incorporate the residual values instead of predicted values, which have been described to be more adequate for categorical variables (Burgess et al., 2015; Palmer et al., 2017). Despite the good performance measures obtained overall for all MRinCMA approaches, some issues regarding the estimation of the SEs should be mentioned. As it was already described (Palmer et al., 2011, 2017; Terza et al., 2008), approaches that involve several stages tend to underestimate the SE and consequently this effect would impact on the performance of the MRinCMA. However, to avoid this limitation, the SE and the 95% CI showed in this work were derived using bootstrapping.

This work represented an exhaustive analysis where different MRinCMA approaches were tested in several settings. MRinCMA approach is an extension of the CMA method, and in this manuscript, we showed that unbiased direct, indirect, and total effects can be estimated when considering two IVs. We proved that MRinCMA can also be used when the variables of interest are noncontinuous, as commonly used in epidemiological studies. Considering the wide simulation frameworks raised, we also believe that this

TABLE 5 Direct, indirect and total effects and 95% CI from the PanGenEU case–control study.

Biological Model A			Direct effect		Indirect effect		Total effect	
Exposure	**Mediator**	**Method**	**OR**	**95% CI (OR)**	**OR**	**95% CI (OR)**	**OR**	**95% CI (OR)**
Obesity	LSDM	4SLS	0.97	[0.62;1.53]	1.13	[0.89;1.88]	1.12	[0.72;1.65]
Biological Model B			**Direct effect**		**Indirect effect**		**Total effect**	
Exposure	**Mediator**	**Method**	**OR**	**95% CI (OR)**	**OR**	**95% CI(OR)**	**OR**	**95% CI (OR)**
LSDM	Obesity	4SLS	1.20	[0.87;1.64]	1.00	[0.87; 1.12]	1.19	[0.83;1.73]

Note: CI was calculated using bootstrap, considering 2.5% and 97.5% percentiles. Logit models were considered.

Abbreviations: CI, confidence interval; LSDM, long-standing diabetes mellitus; OR, odds ratio; 4SLS, four-stage least squares; 3SRI, three-stage residual inclusion.

project could be considered as a guideline for investigators that are interested in applying CMA, whereas the confounding assumptions needed cannot be held. The advantage of incorporating two IVs would allow researchers to easily run mediation analysis while obtaining unbiased estimates. Further studies are needed to assess how MRinCMA performs with weak instrument bias, or potential horizontal pleiotropy (Bowden et al., 2015; Burgess & Thompson, 2017; Carter et al., 2021; Rees et al., 2017; Sanderson, 2021). Moreover, some possible extensions of these methods could be explored in further studies because the interactions between E and M were not considered (Burgess et al., 2015; North et al., 2019) and potential differences in the estimation of the indirect effect can be observed using MRinCMA.

In this work we also proposed an empirical example to study the direct and indirect effect of LSDM and obesity, respectively, on PC risk. The results were similar from those obtained in previous studies (Molina-Montes et al., 2021). The nonsignificance levels are plausible, due to the limited sample size used, in contrast what both MR and MRinCMA analyses require. As observed in the simulation studies, a large sample size is needed to obtain unbiased estimates. However, these results also showed the complex relationship between LSDM, obesity and PC, and further studies are required to proper identify the causes and effects of these risk factors on PC risk.

In conclusion, this study proposes and supports the use of a new approach to address CMA interrogations by incorporating two genetic instruments related to exposure and mediator that may correct potential confounding bias. MRinCMA can be easily applied in a wide range of epidemiological and clinical scenarios, regardless the nature of the variables and it could be considered as a solution in those studies where the main objective is to apply CMA, where the confounding assumptions needed cannot be guaranteed.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data produced for the simulation task and the corresponding R scripts are available from the first author upon request. The authors are not allowed to release or distribute the PanGenEU database.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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