Applied causal inference methods for sequential mediators

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Abstract

Background: Mediation analysis aims at estimating to what extent the effect of an exposure on an outcome is explained by a set of mediators on the causal pathway between the exposure and the outcome. The total effect of the exposure on the outcome can be decomposed into an indirect effect, i.e. the effect explained by the mediators jointly, and a direct effect, i.e. the effect unexplained by the mediators. However finer decompositions are possible in presence of independent or sequential mediators.

Methods: We review four statistical methods to analyse multiple sequential mediators, the inverse odds ratio weighting approach, the inverse probability weighting approach, the imputation approach and the extended imputation approach. These approaches are compared and implemented using a case-study with the aim to investigate the role of adverse reproductive outcomes and infant respiratory infections on infant wheezing in the Ninfea birth cohort.

Results: Using the inverse odds ratio weighting approach, the direct effect of maternal depression or anxiety in pregnancy is equal to a 59% (95% CI: 27%-94%) increased prevalence of infant wheezing and the mediated effect through adverse reproductive outcomes is equal to a 3% (95% CI: -6%- 12%) increased prevalence of infant wheezing. When including infant lower respiratory infections in the mediation pathway, the direct effect decreases to 57% (95% CI: 25%-92%) and the indirect effect increases to 5% (95% CI: -5%,15%). The estimates of the effects obtained using the weighting and the imputation approaches are similar. The extended imputation approach suggests that the small joint indirect effect through adverse reproductive outcomes and lower respiratory infections is due entirely to the contribution of infant lower respiratory infections, independently from the increased prevalence of adverse reproductive outcomes.

Conclusions: The use of these methods allows the study of multiple mechanisms underlying the association between an exposure and an outcome and provides a solution for the problem of intermediate confounding by considering the intermediate confounder as a sequential mediator. The choice of the method may depend on what is the effect of main interest, the nature of the variables involved in the analysis and the truthfulness of the underlying assumptions.

KEYWORDS:
Causal inference, Mediation analysis, Sequential mediators, Direct and Indirect effects, Weighting, Imputation.

1 | BACKGROUND

Mediation analysis aims at estimating to what extent the effect of an exposure on an outcome is explained by a given set of mediators on the causal pathway between the exposure and the outcome. This goal is achieved by decomposing the total effect of the exposure on the outcome into a natural indirect effect, i.e. the effect explained through the given mediators, and a natural direct effect, i.e. the effect unexplained by the mediators [1]. Researchers often deal with research questions that involve more than one

mediator at a time. In the analysis of multiple mediators, the total effect of the exposure on the outcome can be decomposed into the natural direct and indirect effects considering the mediators jointly. Finer decompositions are however possible; in a setting with two mediators, for example, there are four possible pathways from the exposure to the outcome: through the first mediator alone, through the second mediator alone, through both mediators, and through neither of them. Identifying and estimating each effect is not trivial and the difficulty increases as the number of mediators increases[2].

The counterfactual approach provides a set of tools to identify and estimate direct and indirect effects using both linear and nonlinear models, with both discrete and continuous variables, and allowing interactions between the exposure and the mediators[1,3,4,5,6]. Furthermore it clearly specifies the assumptions needed to identify the direct and indirect effects and to allow their causal interpretation[1,7,8,9,10].

A number of methods, which derive from different characterisation of the non-parametric mediation formula[3] have been developed to carry out mediation analysis involving multiple mediators. Among the best known, Imai et al (2010)[11] proposed a quasi-Bayesian Monte Carlo method, or alternatively, a nonparametric bootstrap procedure, to draw counterfactuals from the outcome and mediators models and hence calculate direct and indirect effects. Lange et al (2014)[12] showed how independent mediation pathways can be modeled in a single natural effect model, which directly parameterizes natural direct and indirect effects, using a weighting-based approach. In order to take into account the dependence among mediators, Vanderweele and Vansteenlandt[13] proposed a regression-based approach to analyse multiple sequential mediators using a combination of regression parameters obtained from models for the mediators and the outcome. This approach can be used only in the context of continuous outcomes and continuous or binary mediators, or rare and binary outcomes with continuous mediators. When the integration is too cumbersome to be done analytically, Monte-Carlo integration methods can be used. Daniel at al (2015)[2] extended the parametric G-computation[14,15] to the context of multiple mediators. Albert et al (2019)[16] showed a further development of the parametric mediation formula approach to accomodate repeatedly measured mediators and multiple mediators at each stage and allow for multiple types of outcomes following generalized linear models. Being based on parametric models, these approaches provide valid estimates when all models are correctly specified. Furthermore the modeling complexity depends on the functional form of the mediators, the number of mediators and their potential dependence.

In this paper, we provide a detailed overview and step-by-step implementation with the statistical software R[17] of four weight-based and/or imputation-based methods to analyse multiple sequential mediators in a causal inference framework. The paper is organised as follows: (i) we first introduce the background and notation, (ii) then we describe the selected approaches to the analysis of multiple mediators, (iii) and finally we analyse a case-study using the selected approaches and compare the results. The case-study uses data of the NINFEA birth cohort[18] to investigate the role of maternal mental health during pregnancy on infant wheezing between 6 and 18 months, considering two potential mediators: adverse reproductive outcome (low birth weight and/or preterm birth and/or delivery with caesarean section) and lower respiratory infections in the first 6 months of infant life.

2 | METHODS

2.1 | Marginal and conditional effects

We consider a setting with two sequential mediators. Let A denote the exposure, Y denote the outcome, and M_1 and M_2 denote two potential mediators on the pathway from the exposure to the outcome (with A affecting both M_1 and M_2, and M_1 affecting M_2). Let C denote the set of confounders that may affect the exposure, the mediators and/or the outcome. The relationships between A, M_1, M_2, Y and C are represented in the Directed Acyclic Graph (DAG) shown in Figure 1. Let $Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))$ be the individual counterfactual outcome that would have been observed had the exposure A been set to a and had M_1 and M_2 been set to the natural value they would have taken if A had been a^*, where a and a^* denote two possible exposure levels (e.g. $a = 1$ and $a^* = 0$).

Under the composition assumption, which postulates that $Y(a) = Y(a, M_1(a), M_2(a, M_1(a)))$[19] (that is, the potential outcome $Y(a)$, when A is set to a, is equal to the potential outcome $Y(a, M_1(a), M_2(a, M_1(a)))$ when A is set to a and M_1 and M_2 are set to the values they would have taken if $A = a$), at the population level the marginal total effect of A on Y can be decomposed with respect to the joint mediator $\{M_1, M_2\}$ as follows:

$$\overbrace{g\{E[Y(a, M_1(a), M_2(a, M_1(a)))]\} - g\{E[Y(a^*, M_1(a^*), M_2(a^*, M_1(a^*)))]\}}^{\text{Marginal total effect}} = \tag{1}$$

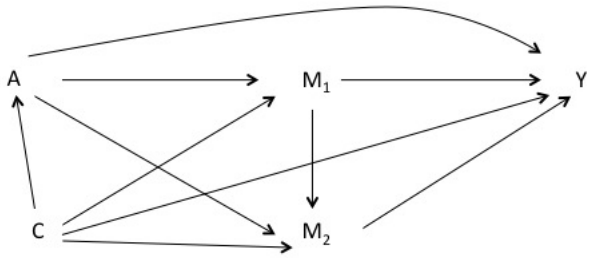

FIGURE 1 DAG representing the hypothesized causal structure. A: exposure, M1: first mediator, M2: second mediator, Y: outcome, C: confounders of A-Y, A-M1, A-M2, M1-Y, M2-Y, M1-M2 associations.

$$\overbrace{g\{E[Y(a, M_1(a), M_2(a, M_1(a)))]\} - g\{E[Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))]\}}^{\text{Marginal total indirect effect}} + \tag{2}$$

$$\overbrace{g\{E[Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))]\} - g\{E[Y(a^*, M_1(a^*), M_2(a^*, M_1(a^*)))]\}}^{\text{Marginal pure direct effect}} \tag{3}$$

where g is a link function[1]. If the g-scale is additive the total effect equals the sum of the pure direct and total indirect effects, while, if the g-scale is multiplicative, the total effect equals the product of those two effects. The formula above states that the marginal total effect (1) can be decomposed into the marginal total indirect effect that acts through at least one of the mediators (2) and the marginal pure direct effect that does not involve any of the mediators (3). In this decomposition the indirect effect captures the potential interaction effect between the exposure and the mediators on the outcome, hence the distinction between total and pure effects, as an effect is named pure when it does not involve the interaction with the exposure[20].

The marginal total effect expresses how much the outcome would change (on the scale defined by g) if the exposure were set from level a^* to level a uniformly in the population. The marginal pure direct effect expresses how much the outcome would change if the exposure were set at $A = a$ versus $A = a^*$ but both mediators were kept at the level they would have naturally taken had the exposure been set at $A = a^*$. Thus this effect captures the remaining effect of the exposure on the outcome if we were able to disable the pathways from the exposure to the mediators. The marginal total indirect effect expresses how much the outcome would change if the exposure were fixed at the level $A = a$ but both mediators were changed from the level they would have taken if $A = a^*$ to the level they would have taken if $A = a$. Thus this estimand captures the effect of the exposure on the outcome that operates through the mediators jointly.

Alternatively the total, pure direct, and total indirect effects can be defined conditionally on a set of baseline confounders $\boldsymbol{C}$ as follows:

$$\overbrace{g\{E[Y(a, M_1(a), M_2(a, M_1(a)))|C = c]\} - g\{E[Y(a^*, M_1(a^*), M_2(a^*, M_1(a^*)))|C = c]\}}^{\text{Conditional total effect}} = \tag{4}$$

$$\overbrace{g\{E[Y(a, M_1(a), M_2(a, M_1(a)))|C = c]\} - g\{E[Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))|C = c]\}}^{\text{Conditional total indirect effect}} + \tag{5}$$

$$\overbrace{g\{E[Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))|C = c]\} - g\{E[Y(a^*, M_1(a^*), M_2(a^*, M_1(a^*)))|C = c]\}}^{\text{Conditional pure direct effect}} \tag{6}$$

The interpretation of the conditional effects is similar to that of the marginal effects, with the difference that the former maximize the precision of the causal effect estimate in the study sample, while the latter is the average effect in the study sample and subsequently in the population conditional on the sample being representative of the population.

2.2 | Assumption

The identifying and estimating assumptions for the effects reported above include the following, which are defined in terms of counterfactuals in the Supplemental Material:

- consistency: the counterfactuals $M_1(a)$, $M_2(a, m_1)$ and $Y(a, m_1, m_2)$ are equal to the observed M_1, M_2 and Y when $A = a$, $M_1 = m_1$ and $M_2 = m_2$
- positivity: there are no empty cells or zero values either biologically or by design for the probabilities of M_2 given M_1, A and C, of M_1 given A and C, of A given C
- no unmeasured and/or uncontrolled confounding of the exposure-outcome association, mediators-outcome association and exposure-mediators association
- cross-world independence assumption: it assumes no confounding of the effects of the mediators M_1 and M_2 on the outcome Y (i.e. no measured or unmeasured intermediate confounders) affected by the exposure A[21] (Figure 2,a). This is of particular interest because when the cross-world independence assumption does not hold, an option is to consider the intermediate confounder as an additional mediator and consider both mediators in the analysis (Figure 2,b), extending the cross-world independence to both mediators. The two mediators M_1, M_2 in Figure 2,b are sequential, i.e. M_1 causes M_2 and both M_1, M_2 may be directly affected by the exposure A.

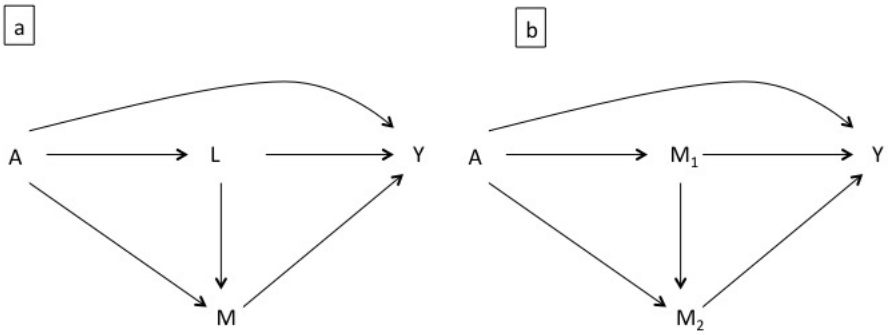

FIGURE 2 Panel a: DAG representing the simplified causal structure in presence of intermediate confounder. A: exposure, L: intermediate confounder, M: mediator, Y: outcome. Panel b: DAG representing the simplified causal structure where the intermediate confounder is modeled as additional mediator. M1: first mediator, M2: second mediator.

If the assumption of absence of confounding of the exposure-outcome association holds, the conditional total causal effect (4) can be estimated from the observed data being

$$g\{E[Y(a, M_1(a), M_2(a, M_1(a))|C = c)]\} - g\{E[Y(a^*, M_1(a^*), M_2(a^*, M_1(a^*))|C = c)]\} =$$
$$g\{E[Y|A = a, C = c]\} - g\{E[Y|A = a^*, C = c]\}$$

If, for example, $g\{E[Y|A = a, C = c]\} = \beta_0 + \beta_1 a + \beta_2 c$ and A is binary, the conditional total effect will be equal to $g^{-1}(\beta_1)$ and the marginal total effect can be estimated using methods like inverse-probability-weighting (IPW) or standardization[4,22]. For the estimation of the conditional and marginal direct and indirect effects, however, all additional assumptions reported above must hold and specific statistical approaches are needed, including the methods described in this paper.

2.3 | Effects decompositions

A two-way decomposition implies that, in presence of two sequential mediators, the total effect can be decomposed into the total indirect effect, i.e. the portion of the effect mediated through M_1 and M_2 jointly including the interaction between A and M_1 and M_2, and the pure direct effect, i.e. the portion of the effect not mediated from M_1 and M_2. However, as M_1 and M_2 are

sequential, one could first consider M_1 alone and estimate the portion of the effect mediated through M_1 and then consider M_1 and M_2 jointly and estimate the portion of the effect mediated through M_1 and M_2. This would still be a two-way decomposition: as M_1 and M_2 share a common pathway (i.e. the path going from A to M_1 and then to M_2 and Y jointly), the difference between the effects estimated by these two analyses may be different from the portion of the effect mediated through M_2 alone. Additionally to the assumptions mentioned above, it is necessary to assume the absence of unmeasured common causes U of the two mediators, because, if present, U would confound the $M_1 - Y$ association when considering M_1 alone, thus violating the assumption of absence of unmeasured confounding of the mediator-outcome association. Furthermore, for the cross-world independence assumption, those U should not be affected by the exposure irrespectively if they are measured or not. The same reasoning applies to the two-way decomposition of the total effect into the total direct and the pure indirect effects, namely when the effect of the interaction between the exposure and the mediators is assigned to the direct effect. However, it can be of interest to evaluate the additional contribution of M_2 beyond M_1 alone, and hence to decompose the indirect effect into the effect mediated through M_1 (i.e. the two pathways $A \rightarrow M_1 \rightarrow Y, A \rightarrow M_1 \rightarrow M_2 \rightarrow Y$) and the effect mediated through M_2 alone (i.e. the pathway $A \rightarrow M_2 \rightarrow Y$). As we will see in the next section, here too it is necessary to assume the absence of unmeasured confounders of the association between the sequential mediators and the absence of the confounders of this association if affected by the exposure.

2.4 | Selected methods for multiple mediation analysis

We will consider the following four methods: the inverse odds ratio weighting approach (IOR)[23,24], the inverse probability weighting approach (IPW)[13], the imputation approach[25] and the extended imputation approach[26]. The main characteristics of each approach are summarised in Table 1. Even if they differ for the estimation procedure, the IOR, IPW and the extended imputation approaches are weight-based approaches, and the IPW, the imputation and the extended imputation approaches rely on the imputation of counterfactuals.

In this section and next, when describing the implementation of the four methods, we consider that the exposure A, the mediators M_1 and M_2, and the outcome Y are all binary. However, these methods can be implemented in scenarios with different combinations of continuous, categorical, count and binary variables, as specified below.

The *inverse odds ratio weighting approach*[23,24] (IOR), estimates the conditional natural direct and indirect effects within the levels of the covariates C (expressions (4), (5), (6)). Specifically it estimates the counterfactual $g\{E[Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))|C]\}$ (i.e the scenario under which the population is exposed to $A = a$ but all the mediators take the natural value under the scenario $A = a^*$) by means of the following equality:

$$g\{E[Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))|C]\} = g[E(WY|A = a, C = c)] \tag{7}$$

where W is the inverse of the conditional odds ratio function relating M_1 and M_2 to A within the levels of C

$$W = \frac{P(M_1 = m_{01}, M_2 = m_{02}|A = a, C = c)P(M_1 = m_1, M_2 = m_2|A = a^*, C = c)}{P(M_1 = m_{01}, M_2 = m_{02}|A = a^*, C = c)P(M_1 = m_1, M_2 = m_2|A = a, C = c)} \tag{8}$$

$P(M_1 = m_1, M_2 = m_2|A; C)$ is the joint conditional probability of the two mediators conditional on the exposure and the covariates, and m_{01} and m_{02} are the reference values of the two mediators.

Because of the invariance property of the odds ratio, the weight W can be also expressed as follows:

$$W = \frac{P(A = a^*|M_1, M_2, C = c)P(A = a|M_1 = m_{01}, M_2 = m_{02}, C = c)}{P(A = a|M_1, M_2, C = c)P(A = a^*|M_1 = m_{01}, M_2 = m_{02}, C = c)} \tag{9}$$

where $P(A = a|M_1; M_2; C)$ is the conditional probability of the exposure given the two mediators and the covariates.

Weighting each subject with the inverse odds ratio function (9) relating M_1 and M_2 to A within the levels of C makes A and $\{M_1, M_2\}$ independent. To obtain more stabilised weights, one can multiply each individual's exposure-mediator odds ratio by the predicted odds of the exposure when the mediators are fixed at their reference value obtaining an inverse odds weight instead of inverse odds ratio weight.

In practice (when considering A, M_1, M_2, Y binary), to estimate the conditional total effect (4) we model the mean observed outcome for each subject (Y) conditional on the observed exposure (A) and the covariates (C) using a generalized linear regression model. The conditional total effect is then equal to the exponentiated coefficient for the exposure A if the interactions

TABLE 1 Main characteristics of each of four approaches.

Decomposition of total effect	IORW*	IPW**	Imputation	Extended imputation
Two-way	√	√	√	√
Three-way				√
Type of estimated effects				
Marginal		√	√	√
Conditional	√		√	√
Models for				
Outcome	√	√	√	√
Mediators				√
Exposure	√	√		
Nested counterfactual			√	√
Exposure type				
Binary	√	√	√	√
Categorical	√	√	√	√
Count				
Continuous	√⁻	√⁻	√	√
Outcome type				
Binary	√	√	√	√
Categorical	√	√	√	√
Count	√	√	√	√
Continuous	√	√	√	√
Mediator type				
Binary	√	√	√	√
Categorical	√	√	√	√
Count	√	√	√	√
Continuous	√	√	√	√
Interactions				
Exposure-mediators		√	√	√
Exposure-covariates	√	√	√	√
Mediator-mediator	√	√	√	√
Mediators-covariates	√	√	√	√

*Inverse odds ratio weighting.

** Inverse probability weighting.

⁻The performance improves as the exposure is binary or categorical with few levels.

between A and C are not included in the model. To estimate the conditional pure direct effect (6) we model the mean observed outcome for each subject (Y) conditional on the observed exposure (A) and the covariates (C) using a weighted generalized linear regression model where weights W (9) are equal to 1 for unexposed subjects and equal to the inverse odds ratio predicted by the logistic regression model of A given M_1 and M_2 and C for the exposed subjects. The conditional pure direct effect is then equal to the inverse of the exponentiated coefficient for the exposure A if the interactions between A and C are not included in the model. Finally to estimate the conditional total indirect effect (5) we calculate the ratio between the estimated total effect and the estimated pure direct effect. Note that all the effects are estimated using the original data with no imputations (Table 2). Confidence intervals can be calculated by bootstrapping.

The main feature of this approach is that the mediators are not included in the regression model for the outcome, but they are only used to calculate the weights that, in their turn, are used to make the exposure and the mediators independent. Hence

TABLE 2 Inverse odds ratio weighting approach: example based on two subjects, one exposed (i=1) and the other one unexposed (i=2). A: exposure of the i-subiect, M_1: first mediator of the i-subiect, M_2: second mediator of the i-subiect, Y: outcome of the i-subiect.

i	A	$M_1(a)$	$M_2(a, M_1(a))$	$Y(a, M_1(a), M_2(a, M_1(a)))$
1	1	$M_1(1)$	$M_2(1, M_1(1))$	$Y(1, M_1(1), M_2(1, M_1(1)))$
2	0	$M_1(0)$	$M_2(0, M_1(0))$	$Y(0, M_1(0), M_2(0, M_1(0)))$

the implementation of this approach requires to specify a regression model for the exposure given the mediators and the covariates, and a weighted regression model for the outcome given the exposure and the covariates. The correct specification of these models is a requisite for the validity of the proposed method. It is the invariance property of the odds ratio that allows to perform a single regression model for the exposure conditional on the mediators and the covariates instead of modeling separately each mediator or the joint conditional probability of the multiple mediators. Interactions between the mediators can be included in the regression model for the exposure but exposure-mediator interactions do not need to be specified. It allows one to consider binary, categorical or continuous exposures (modeled via logistic regression, multinomial logistic regression and linear regression respectively), but being based on a weighting procedure its performance improves as the exposure is binary or categorical with few levels. Finally it can be used for any type of outcome through generalised linear models, including those with nonlinear link functions and quantile regression can be fitted.

The *inverse probability weighting approach*(IPW)[13] estimates the marginal pure direct and total indirect effects (expressions (1), (2) and (3)). Specifically it estimates the three counterfactuals, $g\{E[Y(a, M_1(a), M_2(a, M_1(a)))]\}$, $g\{E[Y(a^*, M_1(a^*), M_2(a^*, M_1(a^*)))]\}$ and $g\{E[Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))]\}$ by means of the following equalities:

$$g\Big\{E[Y(a, M_1(a), M_2(a, M_1(a)))]\Big\} = E\Big\{\frac{P(A=a)}{P(A=a|C)}g[E(Y|A=a, M_1, M_2, C)|A=a]\Big\} \tag{10}$$

$$g\Big\{E[Y(a^*, M_1(a^*), M_2(a^*, M_1(a^*)))]\Big\} = E\Big\{\frac{P(A=a^*)}{P(A=a^*|C)}g[E(Y|A=a^*, M_1, M_2, C)|A=a^*]\Big\} \tag{11}$$

$$g\Big\{E[Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))]\Big\} = E\Big\{\frac{P(A=a^*)}{P(A=a^*|C)}g[E(Y|A=a, M_1, M_2, C)|A=a^*]\Big\} \tag{12}$$

The two nested counterfactuals $g\{E[Y(a, M_1(a), M_2(a, M_1(a)))]\}$ and $g\{E[Y(a^*, M_1(a^*), M_2(a^*, M_1(a^*)))]\}$ can be estimated from the observed data. The third counterfactual $g\Big\{E[Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))]\Big\}$, which includes both two potential outcomes under $A = a$ and $A = a^*$ and cannot be obtained by the observed data, can still be estimated by standardising the mean outcome Y in each stratum defined by the mediators M_1 and M_2 and the confounders C among individuals exposed at the level $A = a$, to the mediator distribution of individuals exposed at the level $A = a^*$ and by weighting by the reciprocal of the conditional probability of the exposure A given the covariates C. This is an imputation procedure where the observed data are complemented with imputed data in which the same individual is evaluated at different exposure levels, a and a^*, but corresponding to the observed mediator levels and confounders. Applying inverse probability weighting entails calculating a weighted average of the imputed counterfactual outcomes to obtain marginal estimates of the effects.

In practice (when considering A, M_1, M_2, Y binary), we fit to the observed data i) an outcome model $g[E(Y|A = a, M_1 = m_1, M_2 = m_2, C = c)]$ conditional on the observed exposure A, the mediators M_1 and M_2, and covariates C using a generalized linear regression model and, ii) an exposure model $P(A|C = c)$ conditional on the observed covariates C using a logistic regression model to calculate the corresponding weights. We expand the observed data by repeating each observation in the original data set twice and we consider one additional variable A' which is equal to the observed exposure A for the first replication and equal to the opposite of the observed exposure for the second replication (Table 3). When A' is equal to the observed A, we estimate $g\{E[Y(1, M_1(1), M_2(1, M_1(1)))]\}$ and $g\{E[Y(0, M_1(0), M_2(0, M_1(0)))]\}$ from the outcome model by using the observed data. On the contrary, when A' is different from the observed A, we estimate $g\{E[Y(1, M_1(0), M_2(0, M_1(0)))]\}$ from the outcome model by using the individual's own values of mediators M_1 and M_2 and confounders C in the unexposed subjects (A=0), but using $A' = 1$, the opposite of the observed exposure A. Hence we calculate a weighted average of these predicted values for subjects with $A = 0$. Similarly we predict $g\{E[Y(0, M_1(1), M_2(1, M_1(1)))]\}$ from the outcome model by using the individual's own values of mediators M_1 and M_2 and confounders C in the exposed subjects (A=1), but using $A' = 0$,

the opposite of the observed exposure A, and we calculate a weighted average of these predicted values for subjects with $A = 1$.

TABLE 3 Inverse probability weighting approach: example based on two subjects, one exposed (i=1) and the other unexposed (i=2). Bold quantities indicate the unobserved counterfactual values for each subject. A: exposure of the i-subiect, M_1: first mediator of the i-subiect, M_2: second mediator of the i-subiect, Y: outcome of the i-subiect.

i	A	A'	$M_1(a)$	$M_2(a, M_1(a))$	$Y(a', M_1(a, M_2(a, M_1(a))$
1	1	1	$M_1(1)$	$M_2(1, M_1(1))$	$Y(1, M_1(1), M_2(1, M_1(1)))$
1	1	**0**	$M_1(1)$	$M_2(1, M_1(1))$	$\boldsymbol{Y(0, M_1(1), M_2(1, M_1(1)))}$
2	0	0	$M_1(0)$	$M_2(0, M_1(0))$	$Y(0, M_1(0), M_2(0, M_1(0)))$
2	0	**1**	$M_1(0)$	$M_2(0, M_1(0))$	$\boldsymbol{Y(1, M_1(0), M_2(0, M_1(0)))}$

This approach does not require models for the mediators but only for the exposure conditional on confounders and for the outcome conditional on the exposure, the mediators, and the confounders. The correct specification of these models is a requisite for the validity of the proposed method. Exposure-mediator interactions and interactions between mediators can also be included. The use of weights makes the exposure and the covariates independent, deactivating pathways relating the exposure and the covariates. It allows one to consider binary, categorical or continuous exposures, but being based on a weighting procedure its performance improves as the exposure is binary or categorical with few levels. Finally it can be used for any type of outcome. Likewise to the IOR approach[23], one could first consider M_1, and then consider M_1 and M_2 jointly to evaluate the additional contribution of M_2 beyond M_1 alone.

The *imputation approach*[25] estimates both the marginal and conditional natural direct and indirect effects. We introduce it here focusing on the conditional effects. This approach is based on the so-called natural effects models, i.e. structural models for nested counterfactuals that directly parameterise the natural direct and indirect effects[27]. The natural effects models express the nested counterfactual $g\{E[Y(a'', M_1(a'), M_2(a', M_1(a')))|C = c]\}$ in terms of two newly defined '"exposure" variables A' and A'' (defined below) to compare as follows:

$$g\{E[Y(a'', M_1(a'), M_2(a', M_1(a')))|C = c]\} = \theta H(a'', a', c) \tag{13}$$

where $H(a'', a', c)$ is a vector depending on $A'' = a''$, $A' = a'$, $C = c$ and θ is a vector of regression parameters to estimate. For example, $\theta H(a'', a', c)$ could be $\theta_0+\theta_1 a''+\theta_2 a'+\theta_3 a'' a'+\theta_4 c$. A' and A'' are two variables with the same potential levels of A, and their inclusion in the regression model allows to encode two causal pathways: through neither mediator (i.e direct pathways $A \rightarrow Y$), or through at least one of the two mediators (i.e. indirect pathways $A \rightarrow M_1 \rightarrow Y$, $A \rightarrow M_1 \rightarrow M_2 \rightarrow Y$, $A \rightarrow M_2 \rightarrow Y$, for brevity: $A \rightarrow M_1 M_2 Y$). Suppose A is binary with two levels 0 and 1, A' and A'' are also binary and have two potential levels 0 and 1. If both A' and A'' are set to 1, the equation (13) is equal to $g\{E[Y(1, M_1(1), M_2(1, M_1(1)))|C = c]\} = \theta_0+\theta_1+\theta_2+\theta_3+\theta_4 c$, while, if both A' and A'' are set to 0, the equation (13) is equal to $g\{E[Y(0, M_1(0), M_2(0, M_1(0)))|C = c]\} = \theta_0 + \theta_4 c$. Hence the conditional total effect (4) is equal to:

$$g\{E[Y(1, M_1(1), M_2(1, M_1(1)))|C = c]\} - g\{E[Y(0, M_1(0), M_2(0, M_1(0)))|C = c]\} = \theta_1 + \theta_2 + \theta_3. \tag{14}$$

To decompose the total effect, it is necessary to consider scenarios in which A'' is set to a different value than A'. The conditional pure direct effect (6) is equal to:

$$g\{E[Y(1, M_1(0), M_2(0, M_1(0)))|C = c]\} - g\{E[Y(0, M_1(0), M_2(0, M_1(0)))|C = c]\} = \theta_1 \tag{15}$$

and the conditional total indirect effect (5) is equal to:

$$g\{E[Y(1, M_1(1), M_2(1, M_1(1)))|C = c]\} - g\{E[Y(1, M_1(0), M_2(0, M_1(0)))|C = c]\} = \theta_2 + \theta_3 \tag{16}$$

Similarly to IPW approach, the nested counterfactual $g\{E[Y(a'', M_1(a'), M_2(a', M_1(a')))|C = c]\}$ can be estimated from the observed data when a'' and a' equal the observed exposure A (a'' corresponds to a and a' to a^* in the IPW) . When a' is equal to the observed exposure A, while a'' differs from a', as in one of the terms in expressions (15) and (16), then

$g\{E[Y(a'', M_1(a'), M_2(a', M_1(a')))|C = c]\}$ can still be estimated according to the following equality:

$$g\{E[Y(a'', M_1(a'), M_2(a', M_1(a')))|C = c]\} = \sum_{m_1,m_2} g[E(Y|A = a'', M_1 = m_1, M_2 = m_2, C)]P(M_1 = m_1, M_2 = m_2|A = a', C) = E\Big\{ g[E(Y|A = a'', M_1, M_2, C)]|A = a', C \Big\} \quad (17)$$

It consists of standardising the mean outcome Y in each stratum defined by the mediators M_1, M_2 and the confounders C among individuals exposed at the level $A = a''$, to the mediator distribution of individuals exposed at the level $A = a'$.This gives arise to an imputation procedure where the observed data are complemented with imputed data in which the same individual is evaluated at different exposure levels, a' and a'', but corresponding to the observed mediator levels and confounders. The imputation approach differs from the IPW approach in the estimation of the effects: the former uses the natural effects model, while the latter calculates a weighted average of the imputed counterfactual outcomes. Note that the imputation approach could also be applied without resorting to natural effect models. However, this could make the analysis cumbersome as the direct/indirect effects would have to be estimated conditionally on specific combinations of confounder values (or specific reference values for a continuous exposure). Natural effect models enable to both summarise these conditional effects and to conduct specific hypothesis tests by also directly parameterizing the relation between the counterfactual outcomes and the confounders (and/or potentially continuous exposures).

In practice (when considering A, M_1, M_2, Y binary), we fit to the observed data an imputation model $g[E(Y|A = a, M_1 = m_1, M_2 = m_2, C = C)]$ to impute the outcome conditional on A, M_1, M_2 and C using a generalized linear regression model. The imputation model is used to complete an expansion of the data, in which (i) each observation in the original data set is repeated twice ii) two variables A' and A'' are added, and iii) A' is equal to the observed exposure A and A'' is equal to the observed exposure A for the first replication and equal to the opposite of the observed exposure for the second replication (Table 4). Only when A' and A'' are equal to the observed exposure A the counterfactual outcome $g\{E[Y(a'', M_1(a'), M_2(a', M_1(a')))|C = c]\}$ can be estimated from observed data, otherwise $g\{E[Y(a'', M_1(a'), M_2(a', M_1(a')))|C = c]\}$ can be imputed using the fitted values $\widehat{g}\{E[Y(a'', M_1(a'), M_2(a', M_1(a')))|C = c]\}$ obtained by the imputation model for the outcome with the exposure set to a'', the mediators M_1 and M_2 and the baseline covariates C set to their observed values. The imputed outcome is no longer binary, but is substituted by conditional mean imputations. Finally a natural effects model (13) has to be fitted to the imputed data and the conditional effects (14), (15), and (16) can be calculated.
The estimation of the marginal effects can be performed by weighting the marginal version of the natural effects model $g\{E[Y(a'', M_1(a'), M_2(a', M_1(a')))]\} = \theta H(a'', a')$ by the reciprocal of the conditional probability of the exposure A given the covariates C estimated using a logistic regression. Confidence intervals can be calculated by bootstrapping.

TABLE 4 Imputation approach: example based on two subjects, one exposed (i=1) and the other unexposed (i=2). Bold quantities indicate the unobserved counterfactual values for each subject. A: exposure of the i-subiect, M_1: first mediator of the i-subiect, M_2: second mediator of the i-subiect, Y: outcome of the i-subiect.

i	A	A'	A''	$M_1(a')$	$M_2(a', M_1(a'))$	$Y(a'', M_1(a', M_2(a', M_1(a'))))$
1	1	1	1	$M_1(1)$	$M_2(1, M_1(1))$	$Y(1, M_1(1), M_2(1, M_1(1)))$
1	1	1	**0**	$M_1(1)$	$M_2(1, M_1(1))$	$\boldsymbol{Y(0, M_1(1), M_2(1, M_1(1)))}$
2	0	0	0	$M_1(0)$	$M_2(0, M_1(0))$	$Y(0, M_1(0), M_2(0, M_1(0)))$
2	0	0	**1**	$M_1(0)$	$M_2(0, M_1(0))$	$\boldsymbol{Y(1, M_1(0), M_2(0, M_1(0)))}$

This approach does not require models for the mediators (averaging is performed over the empirical distribution of the joint mediators), while it requires a model for the outcome conditional on the exposure, the mediators, and the confounders (imputation model), and a model for the nested counterfactual (13) (natural effects model). The correct specification of these models is a requisite for the validity of the proposed method. Exposure-mediator interactions and interactions between the mediators can be included. The imputation approach allows binary, categorical or continuous exposures as well as binary, categorical, count

and continuous mediators and outcomes. Similarly to the other approaches, it is possible to first consider M_1 alone and then consider M_1 and M_2 jointly to evaluate the additional contribution of M_2 beyond M_1 alone.
The imputation approach has been implemented in the *medflex* package in R.[28].

The *extended imputation approach*[26] estimates both the marginal and conditional direct and indirect effects by further decomposing the indirect effect into the effect mediated through M_1 and the effect mediated through M_2 alone as follows:

$$\overbrace{g\{E[Y(a, M_1(a), M_2(a, M_1(a)))]\} - g\{E[Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))]\}}^{\text{Marginal indirect effect}} = \tag{18}$$

$$\overbrace{g\{E[Y(a, M_1(a), M_2(a, M_1(a)))]\} - g\{E[Y(a, M_1(a^*), M_2(a, M_1(a^*)))]\}}^{\text{Marginal indirect effect though } M_1} + \tag{19}$$

$$\overbrace{g\{E[Y(a, M_1(a^*), M_2(a, M_1(a^*)))]\} - g\{E[Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))]\}}^{\text{Marginal partial indirect effect through } M_2 \text{ alone}} \tag{20}$$

or

$$\overbrace{g\{E[Y(a, M_1(a), M_2(a, M_1(a)))|C]\} - g\{E[Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))|C]\}}^{\text{Conditional indirect effect}} = \tag{21}$$

$$\overbrace{g\{E[Y(a, M_1(a), M_2(a, M_1(a)))|C]\} - g\{E[Y(a, M_1(a^*), M_2(a, M_1(a^*)))|C]\}}^{\text{Conditional indirect effect though } M_1} + \tag{22}$$

$$\overbrace{g\{E[Y(a, M_1(a^*), M_2(a, M_1(a^*)))|C]\} - g\{E[Y(a, M_1(a^*), M_2(a^*, M_1(a^*)))|C]\}}^{\text{Conditional partial indirect effect through } M_2 \text{ alone}} \tag{23}$$

The indirect effect through M_1 captures all pathways along M_1 to Y further mediated or not mediated by M_2 ($A \rightarrow M_1 Y$). The partial indirect effect through M_2 captures all pathways along M_2 to Y not passing through M_1 ($A \rightarrow M_2 \rightarrow Y$). In order to estimate these effects, the two usual additional assumptions need to be satisfied, namely the absence of unmeasured confounding of the $M_1 - M_2$ association and the lack of confounders of this association in turn affected by the exposure.

Considering conditional effects, the nested counterfactual $g\{E[Y(a^{'''}, M_1(a^{'}), M_2(a^{''}, M_1(a^{'}))|C = c]\}$ is now defined in terms of three newly defined "exposure" variables $A^{'}$, $A^{''}$ and $A^{'''}$ (defined below) as follows:

$$g\{E[Y(a^{'''}, M_1(a^{'}, M_2(a^{''}, M_1(a^{'}))|C = c]\} = \theta H(a^{'''}, a^{'}, a^{''}, c) \tag{24}$$

where $H(a^{'''}, a^{'}, a^{''}, c)$ is a known vector depending on $a^{'''}, a^{'}, a^{''}, c$, and θ is a vector of unknown regression parameters. For example, $H(a^{'''}, a^{'}, a^{''}, c)$ could be $\theta_0 + \theta_1 a^{'''} + \theta_2 a^{'} + \theta_3 a^{''} + \theta_4 a^{'''} a^{'} + \theta_5 a^{'''} a^{''} + \theta_6 a^{'} a^{''} + \theta_7 a^{'''} a^{'} a^{''} + \theta_8 c$. $A^{'}$, $A^{''}$ and $A^{'''}$ are three variables with the same potential levels of A (if A is binary with two levels 0 and 1, then $A^{'}$, $A^{''}$ and $A^{'''}$ have also two hypothetical levels 0 and 1), and their inclusion in the regression model allows to encode the three causal pathways of interest, through neither of the mediators (i.e. the direct pathway $A \rightarrow Y$), through M_1 or M_1 and then M_2 (i.e. the indirect pathway through M_1: $A \rightarrow M_1 \rightarrow Y$, $A \rightarrow M_1 \rightarrow M_2 \rightarrow Y$) or through M_2 alone (i.e. the partial indirect pathway through M_2: $A \rightarrow M_2 \rightarrow Y$).

Suppose A is binary, the conditional total effect is equal to:

$$g\{E[Y(1, M_1(1), M_2(1, M_1(1)))|C = c]\} - g\{E[Y(0, M_1(0), M_2(0, M_1(0)))|C = c]\} = \theta_1 + \theta_2 + \theta_3 + \theta_4 + \theta_5 + \theta_6 + \theta_7 \tag{25}$$

the conditional pure direct effect is equal to:

$$g\{E[Y(1, M_1(0), M_2(0, M_1(0)))|C = c]\} - g\{E[Y(0, M_1(0), M_2(0, M_1(0)))|C = c]\} = \theta_1 \tag{26}$$

the conditional total indirect effect through the mediators jointly is equal to:

$$g\{E[Y(1, M_1(1), M_2(1, M_1(1)))|C = c]\} - g\{E[Y(1, M_1(0), M_2(0, M_1(0)))|C = c]\} = \theta_2 + \theta_3 + \theta_4 + \theta_5 + \theta_6 + \theta_7 \tag{27}$$

the conditional total indirect effect through M_1 is equal to:

$$g\{E[Y(1, M_1(1), M_2(1, M_1(1)))|C = c]\} - g\{E[Y(1, M_1(0), M_2(1, M_1(0)))|C = c]\} = \theta_2 + \theta_4 + \theta_6 + \theta_7 \quad (28)$$

and the partial total indirect effect through M_2 is equal to:

$$g\{E[Y(1, M_1(1), M_2(1, M_1(1)))|C = c]\} - g\{E[Y(1, M_1(1), M_2(0, M_1(1)))|C = c]\} = \theta_3 + \theta_5 \quad (29)$$

The nested counterfactual $g\{E[Y(a''', M_1(a'), M_2(a'', M_1(a')))|C = c]\}$ can be estimated from the observed data when a''', a'' and a' equal the observed exposure A. When a''', a'' and a' differ one from others, the nested counterfactual can be estimated according to the following equality:

$$g\{E[Y(a''', M_1(a', M_2(a'', M_1(a')))|C = c]\} = \\ = \sum_{m_1, m_2} g[E(Y|A = a''', M_1 = m_1, M_2 = m_2, C)]P(M_1 = m_1|A = a', C)P(M_2 = m_2|A = a'', M_1 = m_1, C) \quad (30)$$

which is equal to

$$\sum_{m_1, m_2} g[E(Y|A = a''', M_1 = m_1, M_2 = m_2, C)]\frac{P(M_1 = m_1|A = a', C)}{P(M_1 = m_1|A = a'', C)}P(M_1 = m_1, M_2 = m_2|A = a'', C) = \\ = E\left\{\frac{P(M_1|A = a', C)}{P(M_1|A = a'', C)}g[E(Y|A = a''', M_1, M_2, C)]|A = a'', C\right\} \quad (31)$$

or

$$\sum_{m_1, m_2} g[E(Y|A = a''', M_1 = m_1, M_2 = m_2, C)]\frac{P(M_2 = m_2|M_1 = m_1, A = a'', C)}{P(M_2 = m_2|M_1 = m_1, A = a', C)}P(M_1 = m_1, M_2 = m_2|A = a', C) = \\ = E\left\{\frac{P(M_2|M_1, A = a'', C)}{P(M_2|M_1, A = a', C)}g[E(Y|A = a''', M_1, M_2, C)]|A = a', C\right\} \quad (32)$$

In practice, we fit to the observed data a model for the probability of either M_1 conditional on A and C or M_2 conditional on M_1, A and C (according to the researchers' preference, see the next paragraph), and we fit to the observed data an imputation model for the outcome $g[E(Y|A = a, M_1 = m_1, M_2 = m_2, C = C)]$ conditional on A, M_1, M_2 and C using a generalized linear regression model. The imputation model is used to complete the expansion of the data, in which each observation in the original data set is repeated four times and three variables A''', A'' and A' are added to the original exposure variable A. If we are interested in estimating the expression (31), A' is equal to the observed exposure level for the first two replications and equal to the opposite of the observed exposure for the third and fourth replications, A'' is equal to the observed exposure level for all four replications, and A''' is equal to the observed exposure level for the first and third replications and equal to the opposite of the observed exposure for the second and fourth replication (Table 5). Only when A''', A'' and A' are equal to the observed exposure A the counterfactual outcome $g\{E[Y(a''', M_1(a'), M_2(a'', M_1(a')))|C = c]\}$ can be estimated from the observed data, otherwise $g\{E[Y(a''', M_1(a'), M_2(a'', M_1(a')))|C = c]\}$ can be imputed using the fitted values $\hat{g}\{E[Y(a''', M_1(a'), M_2(a'', M_1(a')))|C = c]\}$ obtained on the extended dataset by the imputation model for the outcome with the exposure set to a''', the mediators M_1 and M_2 and the baseline covariates C set to their observed values. Similarly the weights $\frac{P(M_1|A=a', C)}{P(M_1|A=a'', C)}$ can be computed on the extended dataset by the model for the probability of M_1 with the exposure set to a' and the baseline covariates C set to their observed values in the numerator and the exposure and the baseline covariates C set to their observed values at the denominator. If we are interested in estimating the expression (32), A' is equal to the observed exposure level for all four replications, A'' is equal to the observed exposure level for the first two replications and the counterfactual exposure $1 - A$ for the third and fourth replications, and A''' is equal to the value of the observed exposure A for the first and third replications and of the counterfactual exposure $1 - A$ for the second and fourth replication (Table 6). Now the counterfactual outcome $g\{E[Y(a''', M_1(a'), M_2(a'', M_1(a')))|C = c]\}$ can be imputed using fitted values $\hat{g}\{E[Y(a''', M_1(a'), M_2(a'', M_1(a')))|C = c]\}$ obtained on the extended dataset by the imputation model for the outcome with the exposure set to a''', the mediators M_1 and M_2 and the baseline covariates C set to their observed values. The weights $\frac{P(M_2|M_1, A=a'', C)}{P(M_2|M_1, A=a', C)}$ can be computed on the extended dataset by the model for the probability of M_2 with the exposure set to a'', the mediator M_1 and the baseline covariates C set to their observed values in the numerator and the exposure, the mediator M_1 and the baseline covariates C set to their observed values at the denominator. Finally the natural effects model (24) can be fitted by regressing the imputed outcomes on a', a'' and a''' and the covariates C and weighting by weights described above.

The estimation of the marginal effects can be performed by weighting the marginal version of the natural effects model $g\{E[Y(a''', M_1(a'), M_2(a'', M_1(a')))]\} = \theta H(a''', a', a'')$ by the reciprocal of the conditional probability of the exposure A given the covariates C estimated using a logistic regression. Confidence intervals can be calculated by bootstrapping.

TABLE 5 Extended imputation approach (expression (31)): example based on two subjects, one exposed (1) and the other unexposed (2). Bold quantities indicate the unobserved counterfactual values for each subject. A: exposure of the i-subiect, M_1: first mediator of the i-subiect, M_2: second mediator of the i-subiect, Y: outcome of the i-subiect.

i	A	A'	A''	A'''	$M_1(a')$	$M_2(a'', M_1(a'))$	$Y(a''', M_1(a', M_2(a'', M_1(a'))))$
1	1	1	1	1	$M_1(1)$	$M_2(1, M_1(1))$	$Y(1, M_1(1), M_2(1, M_1(1)))$
1	1	1	1	**0**	$M_1(1)$	$M_2(1, M_1(1))$	$\boldsymbol{Y(0, M_1(1), M_2(1, M_1(1)))}$
1	1	**0**	1	1	$\boldsymbol{M_1(0)}$	$\boldsymbol{M_2(1, M_1(0))}$	$\boldsymbol{Y(1, M_1(0), M_2(1, M_1(0)))}$
1	1	**0**	1	**0**	$\boldsymbol{M_1(0)}$	$\boldsymbol{M_2(1, M_1(0))}$	$\boldsymbol{Y(0, M_1(0), M_2(1, M_1(0)))}$
2	0	0	0	0	$M_1(0)$	$M_2(0, M_1(0))$	$Y(0, M_1(0), M_2(0, M_1(0)))$
2	0	0	0	**1**	$M_1(0)$	$M_2(0, M_1(0))$	$\boldsymbol{Y(1, M_1(0), M_2(0, M_1(0)))}$
2	0	**1**	0	0	$\boldsymbol{M_1(1)}$	$\boldsymbol{M_2(0, M_1(1))}$	$\boldsymbol{Y(0, M_1(1), M_2(0, M_1(1)))}$
2	0	**1**	0	**1**	$\boldsymbol{M_1(1)}$	$\boldsymbol{M_2(0, M_1(1))}$	$\boldsymbol{Y(1, M_1(1), M_2(0, M_1(1)))}$

TABLE 6 Extended imputation approach (expression (32)): example based on two subjects, one exposed (1) and the other unexposed (2). Bold quantities indicate the unobserved counterfactual values for each subject. A: exposure of the i-subiect, M_1: first mediator of the i-subiect, M_2: second mediator of the i-subiect, Y: outcome of the i-subiect.

i	A	A'	A''	A'''	$M_1(a')$	$M_2(a'', M_1(a'))$	$Y(a''', M_1(a', M_2(a'', M_1(a'))))$
1	1	1	1	1	$M_1(1)$	$M_2(1, M_1(1))$	$Y(1, M_1(1), M_2(1, M_1(1)))$
1	1	1	1	**0**	$M_1(1)$	$M_2(1, M_1(1))$	$\boldsymbol{Y(0, M_1(1), M_2(1, M_1(1))}$
1	1	1	**0**	1	$M_1(1)$	$\boldsymbol{M_2(0, M_1(1))}$	$\boldsymbol{Y(1, M_1(1), M_2(0, M_1(1))}$
1	1	1	**0**	**0**	$M_1(1)$	$\boldsymbol{M_2(0, M_1(1))}$	$\boldsymbol{Y(0, M_1(1), M_2(0, M_1(1))}$
2	0	0	0	0	$M_1(0)$	$M_2(0, M_1(0))$	$Y(0, M_1(0), M_2(0, M_1(0))$
2	0	0	0	**1**	$M_1(0)$	$M_2(0, M_1(0))$	$\boldsymbol{Y_2(1, M_1(0), M_2(0, M_1(0))}$
2	0	0	**1**	0	$M_1(0)$	$\boldsymbol{M_2(1, M_1(0))}$	$\boldsymbol{Y(0, M_1(0), M_2(1, M_1(0))}$
2	0	0	**1**	**1**	$M_1(0)$	$\boldsymbol{M_2(1, M_1(0))}$	$\boldsymbol{Y(1, M_1(0), M_2(1, M_1(0))}$

Contrarily to other approaches described above, this approach allows to estimate the mediating contribution of the second mediator alone. However this estimate requires to model the distribution of one of the two mediators. According to the confidence on the model's correct specification, one can choose as where to model the distribution of the first or the second mediator. This approach requires also models for the outcome conditional on the exposure, the mediators, and the confounders (imputation model), and models for the nested counterfactuals (24) (natural effects model). The correct specification of these models is a requisite for the validity of the proposed method. Exposure-mediator interactions and interactions between mediators can also be included. It is possible to consider binary, categorical or continuous exposures as well as binary, categorical, count and continuous mediators and outcomes.

3 | RESULTS

3.1 | Participants

To illustrate the approaches described in this article we used data from 4797 infants of the Ninfea cohort[18]. Ninfea is a web-based birth cohort with the aim of investigating the effects of early-life exposures on the health of newborns, children, adolescents, and adults. Cohort members are children of mothers recruited between 2005 and 2016 in Italy who completed a first online questionnaire at any time during their pregnancy and are invited to complete six follow-up questionnaires when their child turn 6 months, 18 months, 4, 7, 10 and 13 years of age. The study was approved by the Ethical Committee of the University Hospital Città della Salute e della Scienza di Torino (project n. 45). Informed consent was obtained from all subjects and/or their parents. All methods were performed in accordance with the relevant guidelines and regulations.

3.2 | Exposure, mediators, confounders, and outcome

Our aim was to investigate the mediating role of adverse reproductive outcomes and infant respiratory infections underlying the effect of maternal mental health during pregnancy on infant wheezing between 6 and 18 months.
In particular, we consider a binary exposure A indicating whether or not the woman had depression or anxiety in pregnancy; a binary mediator M_1 that indicates the occurrence of at least one between low birth weight, preterm birth, or delivery with cesarean section (hereafter collectively referred as to "adverse reproductive outcomes"); a binary mediator M_2 for the occurrence of lower respiratory infections in the first 6 months of infant life, as reported at the 6-month follow-up questionnaire; and an outcome Y for the occurrence of wheezing between 6 and 18 months of infant life, as reported at the 18-month follow-up questionnaire. Maternal age, education, residence, and pre-pregnancy body mass index, parity and child's sex are considered as baseline confounders C. The underlying hypothesized causal structure is represented in Figure 3, in which M_1 and M_2 are assumed to be sequential. Although the example is necessarily simplified, we assume that the selected set of confounders is sufficient to satisfy the assumptions defined above. The variables involved in the analysis are described in the Supplementary Material.

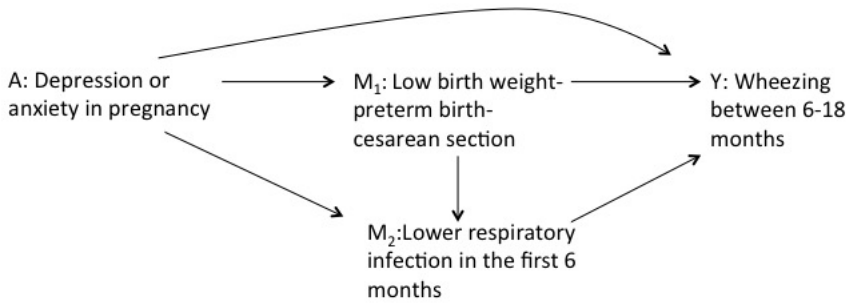

FIGURE 3 DAG representing the hypothesized causal structure of the case study. For the sake of simplicity the confounders C are not shown.

3.3 | Marginal and conditional effects

Let a and a^* correspond to the levels of the variable depression or anxiety in pregnancy (presence *vs* absence); $M_1(a^*)$ to the level of the adverse reproductive outcomes (presence *vs* absence) that would have been observed had the mother not suffered from depression or anxiety in pregnancy (if A were set to a^*); $M_2(a^*, M_1(a^*))$ to the level of the occurrence of lower respiratory infections (presence *vs* absence) that would have been observed if the mother had not suffered from depression or anxiety in pregnancy (if A were set to a^*) and the adverse reproductive outcomes were set to the level that would have been observed if the mother had not suffered from depression or anxiety in pregnancy (A were set to a^*). $Y(a, M_1(a^*), M_2(a^*, M_1(a^*))$ corresponds

to the level of the occurrence of wheezing between 6 and 18 months of infant life (presence *vs* absence) that would have been observed if i)the mother had suffered from depression or anxiety in pregnancy (A were set to a); ii)the adverse reproductive outcomes were set to the level that would have been observed if the mother had not suffered from depression or anxiety in pregnancy (A were set to a^*); and iii)the occurrence of lower respiratory infections were set to the level that would have been observed if the mother had not suffered from depression or anxiety in pregnancy (A were set to a^*) and the adverse reproductive outcomes were set to the level that would have been observed if the mother had not suffered from depression or anxiety in pregnancy (A were set to a^*). Finally $Y(a, M_1(a), M_2(a^*, M_1(a))$ corresponds to the level of the occurrence of wheezing between 6 and 18 months of infant life that would have been observed if i)the mother had suffered from depression or anxiety in pregnancy (A were set to a); ii)the adverse reproductive outcomes were set to the level that would have been observed if the mother had suffered from depression or anxiety in pregnancy; and iii)the occurrence of lower respiratory infections were set to the level that would have been observed if the mother had not suffered from depression or anxiety in pregnancy but the adverse reproductive outcomes were set to the level that would have been observed if the mother had suffered from depression or anxiety in pregnancy.

The marginal total effect expresses how much the occurrence of wheezing between 6 and 18 months of infant life would differ when comparing two hypothetical scenarios in which all women suffered from depression or anxiety in pregnancy versus all women did not suffer from depression or anxiety in pregnancy. The marginal pure direct effect expresses how much the occurrence of wheezing between 6 and 18 months of infant life would differ comparing two hypothetical scenarios in which all women suffered from depression or anxiety in pregnancy versus all women did not suffer from depression or anxiety in pregnancy but both adverse reproductive outcomes and occurrence of lower respiratory infections were kept at the level they would have naturally taken in absence of depression or anxiety in pregnancy. The marginal total indirect effect expresses how much the occurrence of wheezing between 6 and 18 months of infant life would differ if women suffered from depression or anxiety in pregnancy but both adverse reproductive outcomes and occurrence of lower respiratory infections were shifted from the level they would have taken if women did not suffer from depression or anxiety in pregnancy to the level they would have taken if women suffered from depression or anxiety in pregnancy. Alternatively we can define the effects conditional on maternal age, education, residence and body mass index at the beginning of pregnancy, parity and child's sex.

3.4 | Estimated effects

Out of 4797 mother-child pairs, 7% of mothers had depression or anxiety during pregnancy. The prevalence of adverse reproductive outcomes, as defined above, was 31% and the prevalence of lower respiratory infections in the first 6 months of infant life was 11%. The prevalence of wheezing between 6 and 18 months of infant life was 17%. Some 26% of the infants born to mothers affected by depression or anxiety during pregnancy had wheezing between 6 and 18 months of life *vs* 16% of those born to mothers without depression or anxiety during pregnancy. We used a Poisson regression to model risk ratio and prevalence ratio. We found a 37% increased prevalence of adverse reproductive outcomes in women with depression or anxiety in pregnancy compared to those without these conditions (PR: prevalence ratio, PR adjusted for C: 1.37, CI: confidence interval, 95% CI: 1.20;1.55), a 29% increased risk of lower respiratory infection in the first 6 months (RR: risk ratio, RR adjusted for C: 1.29, 95% CI: 0.95;1.76) and a 64% increased prevalence of wheezing (PR adjusted for C: 1.64, 95% CI: 1.35: 1.99). Adverse reproductive outcomes were associated with a 19% increased risk of lower respiratory infections in the first 6 months (RR adjusted for A and C: RR=1.19, 95% CI: 0.98; 1.43) and a 23% increased prevalence of infant wheezing (PR adjusted for A and C: PR=1.23, 95% CI: 1.06;1.40). Finally lower respiratory infections in the first 6 months double the prevalence of infant wheezing between 6 and 18 months of life (PR adjusted for A, M_1 and C: PR=2.03, 95% CI: 1.75;2.35).

A summary of the fitted regression models to implement each of the four approaches to sequential mediation analysis is reported in the Supplementary Material.
Results of the sequential analyses performed using the inverse odds ratio weighting, the inverse probability weighting and the imputation approaches are reported in Table 7, while results obtained using the extended imputation approach are reported in Table 8.

The *inverse odds ratio weighting approach* suggests that being born to a mother with depression or anxiety in pregnancy compared to a mother not suffering from these disorders increases the prevalence of infant wheezing (PR=1.64, 95% CI:

TABLE 7 Estimates of total, direct and indirect effects of maternal depression or anxiety in pregnancy on the risk of infant wheezing between 6 and 18 months of age from inverse odds ratio weighting, inverse probability weighting and imputation approach. M_1: adverse reproductive outcomes. M_2: infant lower respiratory infections.

	Through M_1		Through M_1 and M_2	
	PR	**95% CI***	**PR**	**95% CI**
Conditional effect		**IOR* approach**		
Pure direct effect	1.59	1.27-1.94	1.57	1.25-1.92
Total indirect effect	1.03	0.94-1.12	1.05	0.95-1.15
Total effect	1.64	1.33-2.00	1.64	1.33-1.97
Marginal effect		**IPW** approach**		
Pure direct effect	1.60	1.30-1.94	1.57	1.27-1.87
Total indirect effect	1.02	0.99-1.04	1.04	0.99-1.09
Total effect	1.63	1.33-1.98	1.63	1.31-1.95
Conditional effect		**Imputation approach**		
Pure direct effect	1.60	1.31-1.94	1.57	1.26-1.90
Total indirect effect	1.02	1.01-1.05	1.05	1.01-1.09
Total effect	1.64	1.33-1.99	1.64	1.33-1.99
Marginal effect		**Imputation approach**		
Pure direct effect	1.60	1.30-1.91	1.57	1.24-1.88
Total indirect effect	1.02	1.00-1.04	1.04	0.99-1.09
Total effect	1.63	1.33-1.95	1.62	1.29-1.95

PR: prevalence ratio; CI: confidence interval calculated by bootstrap.

*Inverse odds ratio weighting.

** Inverse probability weighting.

TABLE 8 Estimates of conditional total, direct and indirect effects by extended imputation approach. M_1: adverse reproductive outcomes. M_2: infant lower respiratory infections.

	Extended imputation approach	
Conditional effect	**PR**	**95% CI**
Pure direct effect	1.57	1.28-1.86
Total indirect effect through M_1 and M_2 jointly	1.05	1.00-1.09
Total indirect effect through M_1	1.00	0.99-1.00
Partial total indirect effect through M_2	1.05	1.00-1.09
Total effect	1.64	1.34-1.96

PR: prevalence ratio; CI: confidence interval calculated by bootstrap.

1.33-2.00). Being born to a mother with depression or anxiety in pregnancy compared to a mother not suffering from these conditions, while setting presence of adverse reproductive outcomes as naturally observed in the absence of maternal depression or anxiety in pregnancy, increases the prevalence of infant wheezing (pure direct effect when only M_1 is considered: PR=1.59, 95% CI: 1.27-1.94). Comparing levels of adverse reproductive outcomes that would have been observed in presence of maternal depression or anxiety in pregnancy to levels that would been observed in absence of maternal depression or anxiety in pregnancy, while setting maternal depression or anxiety in pregnancy as present, increases only minimally the prevalence of infant wheezing (total indirect effect when only M_1 is considered PR=1.03, 95% CI: 0.94, 1.12). Similarly being born to a mother with depression or anxiety in pregnancy compared to a mother not suffering from these disorders, while setting the presence of adverse reproductive outcomes and lower respiratory infections as naturally observed in absence of maternal depression or anxiety in pregnancy, increases the prevalence of infant wheezing (pure direct effect when M_1 and M_2 are considered jointly: PR=1.57, 95% CI: 1.25-1.92). Comparing levels of adverse reproductive outcomes and lower respiratory infections that would

have been observed in presence of maternal depression or anxiety in pregnancy to levels that would been observed in absence of maternal depression or anxiety in pregnancy, while setting the maternal depression or anxiety in pregnancy as present, increases only minimally the prevalence of infant wheezing (total indirect effect when M_1 and M_2 are considered jointly: PR=1.05, 95% CI: 0.95, 1.15).

In summary, the direct effect of maternal depression or anxiety in pregnancy is equal to a 59% (95% CI: 27%-94%) increased prevalence of infant wheezing and the mediated effect through adverse reproductive outcomes is equal to a 3% (95% CI: -6%-12%) increased prevalence of infant wheezing. When including infant lower respiratory infections in the mediation pathway, the direct effect decreases slightly to 57% (95% CI: 25%-92%) and consequently the indirect effect increases slightly to 5% (95% CI: -5%,15%). Hence although adverse reproductive outcomes and infant lower respiratory infections are both risk factors for infant wheezing and are affected by maternal depression or anxiety in pregnancy, they explain only minimally the observed increased risk of infant wheezing associated with maternal depression or anxiety in pregnancy[29,30,31]. This exposure may act on infant wheezing through other mechanisms/pathways that are not considered in our case-study analysis.

The corresponding estimates of the natural direct and indirect effects obtained using the *weighting approach* and the *imputation approach* are very similar to those described above, although the inverse odds ratio weighting approach has slightly larger confidence intervals for the direct and indirect effects. The *extended imputation approach* suggests further that the small joint indirect effect through adverse reproductive outcomes and lower respiratory infections (PR=1.05, 95% CI:1.00-1.09) is due entirely to the contribution of infant lower respiratory infections (PR=1.05, 95% CI: 1.00,1.09), independently from the increased prevalence of adverse reproductive outcomes.

Conditional and marginal effects are expected not to differ because interactions between the exposure and the baseline covariates and between covariates are not included in the regression models. In our case-study we considered the interaction between the two mediators, while we assumed absence of the interaction between the exposure and the baseline covariates and the three-way interaction between the exposure and the mediators. However, all methods can further consider these interactions with the exclusion of the inverse odds ratio weighting approach that cannot specify the three-way interaction. When we included the latter in the analysis, similar results of lack of indirect effects were obtained.

Note that the estimated effects can be considered as causal only if the assumptions specified in the Background and Notation section hold, and in particular if there is no residual confounding of all considered associations $(A-Y, A-M_1, A-M_2, M_1-Y, M_2-Y, M_1-M_2)$.

4 | DISCUSSION

In this paper we have reviewed four different estimation approaches recently developed to answer research questions involving sequential mediation analysis. We also provided the codes to run the analyses applied to a specific case-study of interest for birth cohort research. The interest in using these methods can be twofolds: on the one hand they allow the study of multiple mechanisms underlying the association between an exposure and an outcome, on the other they provide a possible solution for the problem of intermediate confounding by considering the intermediate confounder as a sequential mediator in the analysis. However, the correct estimation of natural direct and indirect effects relies on several assumptions (on the top of the issue of intermediate confounder): the absence of unmeasured confounders of the exposure-outcome, exposure-mediators, mediators-outcome associations in all four approaches, the absence of unmeasured confounders of the association between the sequential mediators and the absence of the confounders of this association if affected by the exposure in the extended imputation approach, the correct specification of the models for i)the outcome in all four approaches, ii)the exposure in the inverse odds ratio and inverse probability weighting approaches, iii)at least one mediator in the extended imputation approach, and iv)the nested counterfactual in the imputation and the extended imputation approaches. The choice of the method may depend on the nature of the variables involved in the analysis: for example, the inverse odds ratio and the inverse probability weighting could be preferred when the mediators are more difficult to model than exposure (e.g. continuous mediators and binary exposure), while the imputation approaches may be the first option when it is more difficult to specify the model for the exposure than for mediators (e.g continuous exposure and binary mediators). It is also important to consider what is the effect of main interest: the inverse odds ratio approach estimates the conditional direct and indirect effects, the inverse probability weighting estimates the marginal direct and indirect effects, while the imputation and the extended imputation approaches can estimate both conditional and marginal direct and indirect effects. Finally, the extended imputation approach is the only method that allows the

decomposition of the total indirect effect into the effect mediated through the first mediator and the effect mediated through the second mediator alone. Despite these differences, the four estimation methods led to similar conclusions in our case-study. This is reassuring for what regards the underlying assumptions used in mediation analysis, including for example the assumption on the correct specification of the model.

In this article we focused on the application of the methods to the context with two sequential mediators. In presence of multiple mediators, one could for simplicity consider a group of mediators as a joint mediator as we did for adverse reproductive outcomes. Alternatively the above illustrated approaches can be extended to settings with more than two mediators with caution in underlying identification and estimation assumptions and modelling. Steen at al (2017)[26] showed how to fit the extended imputation approach to these contexts. Some other methods provide a finer decomposition of the total effect than the methods addressed in this paper, yet stronger assumptions may be required[2,16].

For the sake of completeness, it is worth mentioning here an approach that was not applied in this tutorial. Vansteenlandt and Daniel (2017)[32] revisited and refined the interventional direct and indirect effects[33] in presence of multiple mediators, and showed how the total effect can be decomposed into these effects. Briefly, the interventional effects differ from the natural effects because, instead of setting the mediator to the counterfactual level it would have naturally taken under different scenarios of the exposure, it sets the mediator for each subject to a random draw from the counterfactual distribution of mediator given the covariates under different scenarios of the exposure. Loh et al (2020)[34] generalized the interventional effects framework for multiple mediators to high-dimensional mediators.

In the sequential mediation analysis there are still unsolved methodological issues, which, although of interest, go beyond the scope of this work, for example the degree of bias in the estimates when the underlying assumptions of each approach are violated or when the variables involved in the mediation pathways are poorly measured.

5 | CONCLUSIONS

As the need to use sequential mediation analysis is becoming increasingly common in epidemiology and the proposed methods are not easy to implement, the aim of this work is to help applied epidemiologists to run valid sequential mediation analysis whenever required by their research hypothesis. It provides a detailed overview and step-by-step implementation with the statistical software R of four weight-based and/or imputation-based methods to analyse multiple sequential mediators in a causal inference framework using a case-study of interest for birth cohort research.

List of abbreviations

IOR: inverse odds ratio weighting approach
IPW: inverse probability weighting approach
PR: prevalence ratio
CI: confidence interval

Declarations

Ethics approval and consent to participate

The study was approved by the Ethical Committee of the University Hospital Città della Salute e della Scienza di Torino (project n. 45).

Informed consent was obtained from all subjects and/or their parents.

All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication

Consent for publication was obtained from all subjects and/or their parents.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

The code implemented in R is available in the Appendix material.

Competing interests
The authors declare that there is no conflict of interest.

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Authors' contribution
DZ had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
DZ, LR, MP, GS, FF, BH conceptualized the study. DZ, LR, MP analyzed and interpreted the data. DZ implemented the statistical methods. DZ, LR drafted the first version of the manuscript. All authors read and approved the final manuscript.
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