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Mapping Causal Biology: Mendelian Randomization in the Era of Big Data

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ABSTRACT

Mendelian randomization (MR) is a method that utilizes genetic variants as instrumental variables to determine causal relationships between exposures and outcomes, thereby mitigating confounding bias and reverse causality inherent in observational studies. Rooted in Mendel's laws of inheritance, MR has undergone rapid development since Katan first introduced the concept in 1986, followed by the formalization of its methodology by George Davey Smith and Ebrahim in 2003. This review comprehensively summarizes the theoretical foundations, methodological innovations, and expanding applications of MR. This review discusses the three core assumptions underpinning MR methodologies—relevance, independence, and exclusion restriction—and examines advanced methodological extensions, two-sample MR, multivariable MR, bidirectional MR, nonlinear MR, *cis*-MR, mediation MR, multi-population MR, and cluster MR. It further provides an overview of key commonly used R packages, databases, and analytical workflows that facilitate the implementation of MR. Application domains spanning gene–environment interactions research, public health, complex diseases, drug target validation, and integration with other cutting-edge technologies are highlighted through representative case studies demonstrating their translational potential. Lastly, we critically assess methodological limitations, including weak instrument bias, horizontal pleiotropy, population stratification, data quality heterogeneity, lack of benchmark validation and comparison across MR methods, as well as selection and collider bias, while proposing future directions for improving robustness and expanding the applicability of MR through integration with multi-omics and cross-ancestry analyses. Overall, MR serves as a cornerstone in modern causal inference research, bridging genetics, epidemiology, and precision medicine to advance our understanding of disease etiology and therapeutic innovation.

1 | Introduction

Establishing causal relationships between exposures and complex diseases is a central challenge in biomedical and public health research. Traditional observational studies, although informative, are often plagued by confounding and reverse causation, which limits their causal interpretability. Although randomized controlled trials (RCTs) represent the gold standard for causal inference, their ethical, practical, and financial constraints preclude their application in many epidemiological contexts. Against this backdrop, Mendelian randomization (MR) has emerged as a powerful genetic epidemiological framework that uses germline genetic variants, typically single-nucleotide polymorphisms (SNPs), as instrumental variables (IVs) to estimate the causal effects of modifiable exposures on health outcomes. By exploiting the random allocation of alleles during meiosis, MR effectively mimics the randomization process of RCTs in natural populations, thereby providing a quasi-experimental design for causal inference.

Since the late 20th century, MR has undergone remarkable evolution. From Katan's seminal 1986 *Lancet* paper linking *APOE* genotypes with serum cholesterol and cancer risk to George Davey Smith and Ebrahim's 2003 systematic framework, MR has matured into a versatile methodological ecosystem. Advances in genome-wide association studies (GWAS) and computational resources have catalyzed the proliferation of MR approaches, including two-sample MR (TSMR), multivariable MR (MVMR), bidirectional MR (BiMR), and mediation MR (MMR), enabling the exploration of increasingly complex causal hypotheses. Moreover, the integration of MR with large-scale biobanks and multi-omics data has extended its reach beyond classical risk factor validation to encompass drug target discovery, systems biology, and population health research.

Nevertheless, the reliability of MR depends critically on the validity of its core assumptions—relevance, independence, and exclusion restriction—which are frequently challenged by weak instrument bias, horizontal pleiotropy, and population stratification. Addressing these challenges requires continuous methodological refinement, comprehensive sensitivity analyses, and triangulation with evidence from complementary study designs. This review aims to systematically elucidate the theoretical principles, core methodologies, and major application domains of MR, as well as its limitations and future prospects. By synthesizing advances in analytical frameworks and empirical research, we highlight how MR bridges the gap between genetics and causality, offering transformative insights into disease mechanisms, risk prediction, and precision therapeutics.

1.1 | Fundamental Principles of Mendelian Randomization

1.1.1 | Historical Development of Mendelian Randomization

MR [1] is an epidemiological method that utilizes genetic variants, typically SNPs, as IVs to infer causal effects between exposures and outcomes. As a recent advancement in genetic epidemiology, MR's theoretical foundation stems from Mendel's Second Law [2], the Principle of Independent Assortment. This method typically selects common genetic polymorphisms that are strongly associated with the exposure and have known functions as IVs [3]. Genetic variation is generally unaffected by confounding factors such as environmental influences or behavioral factors. This implies that association analyses based on genetic variation share characteristics similar to intention-to-treat analyses in RCTs.

The conceptual development of MR underwent a prolonged process from its inception to maturation. In the 1860s, Mendel discovered the Law of Segregation and Independent Assortment through pea experiments, revealing the random distribution of genetic variants and providing the core genetic basis for MR. In 1986, Katan [4] published a paper in *The Lancet* proposing the use of genotypes as IVs to infer causality between cholesterol and cancer, marking the first integration of Mendelian inheritance principles with causal inference. To investigate the cholesterol–cancer relationship, Katan suggested examining the association between cancer and the apolipoprotein E (*APOE*) gene, a gene known to influence serum cholesterol levels, to test causal links. The theoretical basis of this approach lies in the random assignment of alleles at conception [5]. This association is unaffected by confounding factors and avoids the issue of reverse causality. If a causal relationship between *APOE* and serum cholesterol is established, then the association between *APOE* and cancer would provide indirect evidence for a causal link between serum cholesterol and cancer. Although Katan did not use the term *Mendelian randomization*. This study is widely recognized as the origin of the MR concept.

In 2003, George Davey Smith and Ebrahim published the landmark review article [6] *"Mendelian randomization": can genetic epidemiology contribute to understanding environmental determinants of disease?* in the *International Journal of Epidemiology*. This paper systematically outlined the principles, assumptions, advantages, limitations, and application scenarios of MR, significantly promoting the method and marking the formal establishment of the MR methodology. In 2007, Vanessa Didelez and Nuala Sheehan [7] pioneered the integration of IV theory from econometrics with the MR framework, formally defining the IV within the context of genetic epidemiology. In 2013, Stephen Burgess et al. [8] proposed MR methods for utilizing summary-level data. Through simulation studies, they demonstrated that these methods yielded equivalent estimates to those obtained from individual-level data and analyzed the effects of gene-gene interactions, linkage disequilibrium (LD), and weak instruments on estimation accuracy. Their work established a practical analytical framework for performing causal inference using GWAS database summary statistics.

Early GWAS could only identify individual SNPs with large effect sizes, but such loci provided very limited explanatory power for the phenotypic variance. In 2010, Speliotes et al. [9] employed a large-scale meta-analysis system to construct a weighted genetic risk score (GRS) based on 32 BMI-associated SNPs, demonstrating that the cumulative effect of polygenic factors could explain 1.45% of BMI variation. Although the predictive accuracy remained low, this approach provided a template for subsequent studies to enhance the explanatory power of GRS by expanding the number of SNPs and sample sizes, thereby advancing the application of GRS in MR research.

Over the past decade, alongside the exponential growth of publicly available GWAS summary statistics, MR techniques have undergone significant diversification [10]. Methodologies such as TSMR, multivariate MR, non-linear MR (NLMR), and weighted median methods have continuously emerged. The software ecosystem has been continuously refined, with numerous researchers developing a wide array of user-friendly software tools, including R packages [11] (e.g., TwoSampleMR, MendelianRandomization, MR-PRESSO, and MendelR) and web-based platforms (Table 1). Numerous MR and genetic variant databases are now available for use in MR research (Supporting Information S1: Tables S1 and S2). These resources have greatly facilitated the application of MR methods for causal inference in complex traits, such as cardiovascular diseases (CVD), metabolic disorders, and psychiatric conditions.

1.1.2 | Fundamental Principles of Mendelian Randomization

1.1.2.1 | Three Core Assumptions of Mendelian Randomization. In MR studies, any IVs must satisfy three core assumptions [12] to be used for causal inference: the relevance, independence, and exclusion restriction (Figure 1). The relevance assumption requires that genetic variation must be robustly associated with exposure. This correlation strength is typically assessed using the *F*-statistic ($F = \beta^2/\mathrm{SE}^2$). The *F*-statistic serves as the core inferential measure for evaluating the issue of weak instruments. An *F*-value greater than 10 [13] is generally required to avoid estimation bias introduced by weak IVs effectively. Weak instruments ($F < 10$) produce different bias directions across study designs: In single-sample designs, they amplify the bias of observed associations, whereas in two-sample designs, they shift estimates toward zero effect. Among these, the genome-wide significance threshold ($p < 5 \times 10^{-8}$) and *F*-statistic (> 10) are not independent criteria; an indirect correlation exists between them. In common GWAS scenarios, SNPs achieving $p < 5 \times 10^{-8}$ typically exhibit *F*-statistics significantly greater than 10, rendering the simultaneous application of both criteria potentially redundant. However, divergence may occur in specific scenarios, such as differing genetic models [14], false positives from oversized samples [15], multiple testing corrections [14], rare variants [16], or ethnic heterogeneity. In such cases, the reliability of results requires a comprehensive evaluation using various statistical measures. Beyond the *F*-statistic, researchers should also consider the proportion of variance in exposure explained (R^2) by genetic variants. R^2 is primarily used to discuss statistical power and analyze the contribution of IVs; it is not sufficient to assess the presence of weak instruments on its own. For instance, the rs16969968-G allele in the *CHRNA3*/*CHRNA5* gene cluster is associated with increased daily smoking ($\beta \approx +1.0$ cigarettes/day), with this locus (or its linked region) accounting for approximately 0.5% of smoking variation [17]. This R^2 result can help illustrate the contribution of IVs to exposure, but the strength of the instrument requires a comprehensive assessment in conjunction with the *F*-statistic. Furthermore, selecting loci based on GWAS significance can inflate SNP-exposure association estimates [18]—a phenomenon termed the *winner's curse* [19]. This statistical significance filtering leads to overestimation of effect sizes, which in turn causes causal effect estimates to decay toward null values. Such overestimation may further compromise the reliability of the hypotheses of SNP-exposure associations. The independence assumption [20] is fundamental to distinguishing MR from traditional observational studies. This assumption posits that genetic variants follow Mendelian inheritance laws, undergo random segregation during meiosis, and are theoretically independent of potential confounding factors, such as environmental influences and social behaviors [21]. This makes genotypes analogous to random assignment in RCTs, thereby effectively mitigating confounding. However, in practice, this assumption may face challenges: When study populations include subgroups with different genetic backgrounds [22], population stratification may lead to allele frequency disparities between cases and controls, potentially generating spurious associations in disease research. Although methods such as principal component analysis (PCA) have been widely used to correct for major ancestral differences, recent studies indicate that sample structure [23] is a major source of confounding that links IVs to confounders, thereby violating the independence assumption. Residual confounding persists even within seemingly homogeneous populations due to subtle demographic structures or familial relationships [24]. Additionally, horizontal pleiotropy—where certain genetic variants may directly influence outcome variables through pathways unrelated to the target exposure—poses another issue. Furthermore, gene-environment (G × E) interactions can undermine the assumption of independence. For example, in regions with high PM 2.5 exposure, the effect of *APOE*ε4 on dementia risk is significantly amplified [25]. The exclusion restriction assumption requires that genetic variants influence outcomes solely through the target exposure, with no other direct or indirect pathways permitted. That is, the selection of genetic variants must exclude pleiotropy. The primary challenge to this assumption lies in genetic pleiotropy [26], which can influence multiple phenotypes. For instance, when studying the IL-6 signaling pathway and coronary heart disease risk [27], if the selected IL-6R gene variant simultaneously affects inflammation levels and regulates lipid metabolism, it violates the exclusion restriction assumption. This introduces parallel causal pathways independent of the target exposure, leading to severe bias in the final causal effect estimate. In recent studies, this hypothesis has been primarily evaluated through multiple sensitivity analyses to assess the extent to which the exclusion restriction assumption may be violated and its impact on results: MR-Egger regression [28] detects directional pleiotropy at the mean level by introducing an intercept term, whereas MR-PRESSO identifies and removes anomalous variants potentially exhibiting pleiotropy. Concurrently, researchers need to integrate sensitivity analyses with the GTEx database to confirm gene functional specificity. Beyond horizontal pleiotropy, vertical pleiotropy represents another critical form of genetic pleiotropy. It occurs when a genetic variant indirectly influences an outcome through an exposure variable, with the additional affected phenotype lying along the causal pathway between exposure and outcome. All genetic effects are ultimately transmitted to the outcome via the exposure. Consequently, vertical pleiotropy does not violate the exclusion restriction assumption nor introduce analytical bias. In a kidney disease study [29], polygenic scores for telomere shortening were associated with chronic kidney disease risk via vertical pleiotropy mechanisms, whereas

TABLE 1 | Summary of Mendelian randomization R packages.

R package	Description	Core features	Installation	Key dependencies
TwoSampleMR	The most popular two-sample MR package, supporting a full workflow from data retrieval to analysis	1. Access IEU OpenGWAS 2. Data harmonization & IV selection 3. Multiple MR methods (IVW, Egger, weighted median) 4. Sensitivity analysis & visualization	install.packages ("TwoSampleMR")	ieugwasr, dplyr, ggplot2
MendelianRandomization	Provides low-level control for MR methods, supports individual and summary data	1. Various MR methods (IVW, Egger, median) 2. IV strength assessment (F-statistic) 3. Flexible model specification	install.packages ("MendelianRandomization")	ggplot2, knitr
MR-PRESSO	Detects and corrects horizontal pleiotropy (PRESSO algorithm)	1. Global pleiotropy test 2. Outlier SNP detection 3. Corrected the MR analysis after outlier removal	install.packages ("MRPRESSO")	None
ieugwasr	The underlying data retrieval package for TwoSampleMR directly accesses the IEU OpenGWAS database	1. Query GWAS metadata 2. Extract IV and outcome data 3. LD clumping	install.packages ("ieugwasr")	httr, dplyr
phenoscanner	Phenotype scanning for IVs, checks SNP associations with other traits	1. Query SNP associations in GWAS Catalog 2. Identify pleiotropic pathways	install.packages ("phenoscanner") or devtools: install_github ("phenoscanner/phenoscanner")	httr, jsonlite
RadialMR	Radial regression-based MR with novel outlier detection perspective	1. Radial MR analysis 2. Outlier detection 3. Radial regression plots	install.packages ("RadialMR")	ggplot2, sandwich
mr.raps	Implements robust adjusted profile scoring (MR-RAPS), robust to weak instruments and pleiotropy	1. MR-RAPS estimation 2. Quantification of model uncertainty	install.packages ("mr.raps")	None
MRInstruments	Provides predefined IV sets for common exposures (e.g., lipids, BMI)	1. Precompiled IV datasets	devtools:install_github ("MRCIEU/MRInstruments")	TwoSampleMR

(Continues)

TABLE 1 | (Continued)

R package	Description	Core features	Installation	Key dependencies
cause	Bayesian approach to distinguish true causation from shared genetic architecture	1. CAUSE analysis 2. Confounding and causal effect estimation	devtools:install_github ("jean997/cause")	tidyverse, coda
MRlap	Specialized method for handling sample overlap issues	1. Corrects bias from sample overlap 2. Provides bias-adjusted MR estimates	devtools:install_github ("n-mounier/MRlap")	data.table, Rcpp

Note: (1) Installation priority: Prefer CRAN, use GitHub for the latest versions. (2) Recommended workflow: TwoSampleMR + MRPRESSO + phenoscanner. (3) All packages depend on base R; the table shows key functional dependencies.

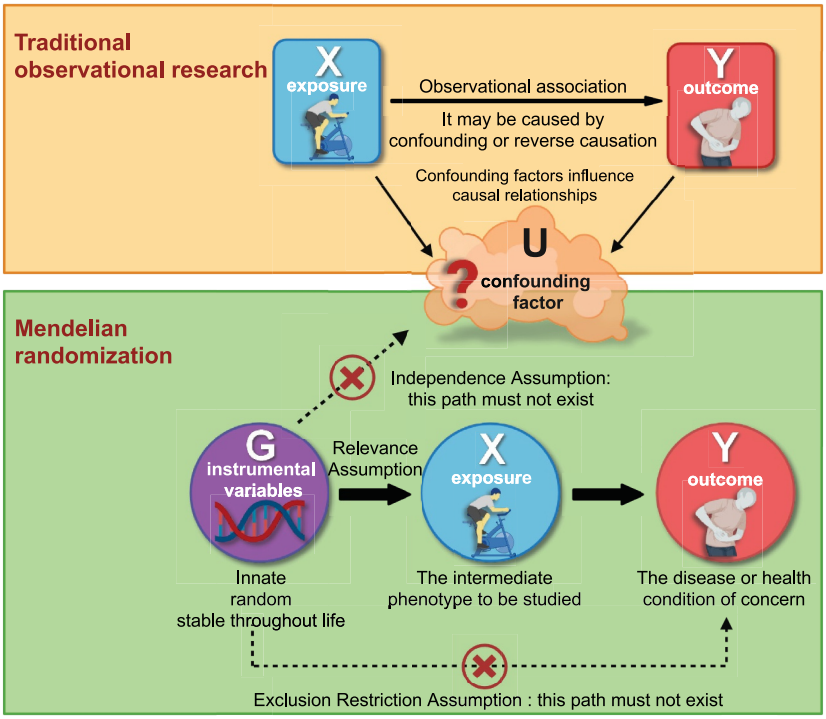

FIGURE 1 | Mendelian randomization causal diagram.

reverse MR analysis further validated the causal effect of declining renal function on telomere length. Vertical pleiotropy is generally recognized as a form of pleiotropy within MR that does not violate the exclusion restriction assumption. Its analysis requires integrating methods such as mediation models, co-localization, and pathway enrichment analysis [30] to elucidate complex biological mechanisms.

1.1.2.2 | *Genetic Variation as Instrumental Variables.* The primary advantage of genetic variants as an IV lies in their temporal precedence [31]. Traditional observational studies often cannot determine the temporal sequence between exposure and disease, making it impossible to distinguish the direction of causality [32]. However, genetic variation is innate and unaffected by environmental influences or disease states, whereas phenotypic traits or disease states manifest later in life. This biological characteristic effectively eliminates reverse causality arising from the "outcome-driven exposure" phenomenon commonly found in observational studies. Second, according to Mendel's Law of Segregation, parental alleles are randomly assorted at conception and largely independent of confounding factors, thereby mitigating common confounding biases in observational studies [33]. Third, the objectivity and stability of genotype data provide technical robustness for causal inference. Modern genotyping technologies (e.g., Illumina whole-genome chips) yield error rates as low as 0.001%–0.01%, whereas platforms such as Affymetrix typically maintain error rates below 0.1% [34]—far superior to the subjective reporting biases common in questionnaire surveys or environmental exposure assessments. Furthermore, except for rare somatic mutations, genotypes remain genetically stable throughout an individual's lifespan, unaffected by behavioral changes, memory biases, or fluctuations in time-dependent exposure [35]. Finally, biological mechanistic plausibility underpins this

application: Genetic variants identified through GWAS must directly alter phenotypes by regulating specific molecular pathways, with no pleiotropic effects or other confounding pathways involved. For example, Brian A. Ference et al. [36] constructed genetic scores based on variants in the *ACLY* and *HMGCR* genes, demonstrating that both regulate LDL-C levels through the cholesterol biosynthesis pathway (mevalonate pathway), thereby influencing cardiovascular disease risk. These characteristics collectively reinforce, collectively forming the theoretical foundation for genetic variants serving as MR IVs.

1.2 | Basic Methods and Primary Types of Mendelian Randomization

1.2.1 | Fundamental Steps in MR Analysis

The first step in MR analysis involves rigorously screening IVs to select genetic variants that are strongly correlated with the target exposure and satisfy three core assumptions. Researchers extract SNPs that reach genome-wide significance thresholds from the GWAS summary statistics of the exposure [37], and verify that they satisfy a strong correlation ($F > 10$). Clustering algorithms exclude LD clumping algorithms that exclude correlated genetic loci to ensure independence of the IVs; during data harmonization, the allele orientation of SNPs common to both exposure and outcome GWAS datasets is standardized. Sample overlap between exposure and outcome data sources is assessed, and confounding biases are corrected as much as possible. When estimating causal effects, IV effects on both exposure and outcome [38] are combined to calculate the causal impact of exposure on the outcome. The primary analysis employs inverse-variance weighting (IVW) to provide a point estimate, with robustness validated through sensitivity analyses including the weighted median method and MR-Egger regression [39]. Ultimately, three key modules [40]—multifactoriality testing, directionality verification, and leave-one-out analysis—systematically screen for potential violations of the IV assumptions, ensuring the reliability of causal inference.

1.2.2 | Basic Methods of Mendelian Randomization

As the MR research framework gains widespread adoption and deeper application, its methodology continues to evolve and be refined, giving rise to various advanced techniques tailored to distinct research needs and causal questions (Figures 2–3; Table 2). These advanced methodologies extend beyond the core assumptions of standard MR to address more complex scenarios encountered in real-world studies. Understanding the differences and connections among these approaches is crucial for correctly designing, implementing, and interpreting MR research.

1.2.2.1 | Two-Sample Mendelian Randomization. TSMR [56] is a methodological framework based on pooled data that utilizes two independent sets of GWAS summary statistics—one for the exposure and one for outcome—to assess the causal effect of exposure on outcome. The ideal design involves separately estimating the association between the IVs and the target exposure, and the association between the IVs and the outcome, in two independent samples. The causal effect of the exposure on the outcome is derived from the proportion of the IV effects across both sample groups [57]. TSMR requires that both samples originate from populations with consistent genetic backgrounds and that there is minimal participant to minimize bias [58].

TSMR's core strength lies in integrating large-scale public GWAS summary statistics [59] to substantially enhance the statistical power and reliability of causal inference. Simultaneously, because exposure and outcome measurements may originate from different cohorts, this high flexibility enables TSMR to perform collaborative analyses across studies without requiring all variables to be measured within a single cohort [60]. This significantly reduces research costs and is particularly well-suited for scenarios where it is challenging to measure both exposure and outcomes simultaneously within a single cohort.

Despite its numerous advantages, TSMR has several limitations. First, suppose two samples originate from different populations or exhibit differences in associations with the trait of interest. In that case, spurious associations may arise [61], which often need to be adjusted for using methods such as PCA. Second, sample overlap [62] is a common challenge in modern MR analysis: when GWAS summary statistics for exposures and outcomes originate from the same cohort or share samples, it undermines the assumption of "sample independence," leading to biased causal estimates. The direction of bias is complex, potentially skewing toward observational associations or toward zero bias. To address this issue, researchers should prioritize GWAS datasets with no or minimal overlap. If overlap cannot be avoided, researchers should model measurement error using bias correction methods such as MR-RAPS, MRlap [63], or GRAPPLE [64], and quantify the impact of overlap on results through stratified analysis and negative control analysis. Furthermore, the low statistical power and biased effects stemming from weak instrument issues are significant concerns. Gene pleiotropy, particularly horizontal pleiotropy, may violate the exclusion restriction assumption [65], thereby biasing estimates of the causal effect. Furthermore, hypothesis validation in TSMR is complex, typically requiring sensitivity analyses to indirectly assess the validity of these assumptions. In practical applications, sensitivity analysis constitutes a crucial component of TSMR research, enabling systematic evaluation of the robustness of IV assumptions. Researchers often employ multi-tiered strategies to detect and adjust for horizontal pleiotropy: MR-Egger regression detects directional pleiotropy, weighted median methods ensure more than 50% IV effectiveness, MR-PRESSO identifies and removes outlying variants likely influenced by pleiotropy, and instrument strength metrics combined with leave-one-out analysis assess the undue influence from individual SNPs. Concurrently, negative control studies [66] validate the IV assumptions. Future directions for TSMR include developing more robust statistical methodologies to address sample heterogeneity and pleiotropy, as well as advancing cross-ancestry analyses to ensure the generalizability of findings.

1.2.2.2 | Multivariable Mendelian Randomization. MVMR [67] extends standard MR by allowing for the estimation of the direct causal effects of multiple, potentially correlated exposures on an outcome within a single model. It employs a set of genetic variants as IVs for these exposures [68]. In practical applications, MVMR relies on the three core IV assumptions and further requires that the genetic instruments for each exposure are statistically independent of one another.

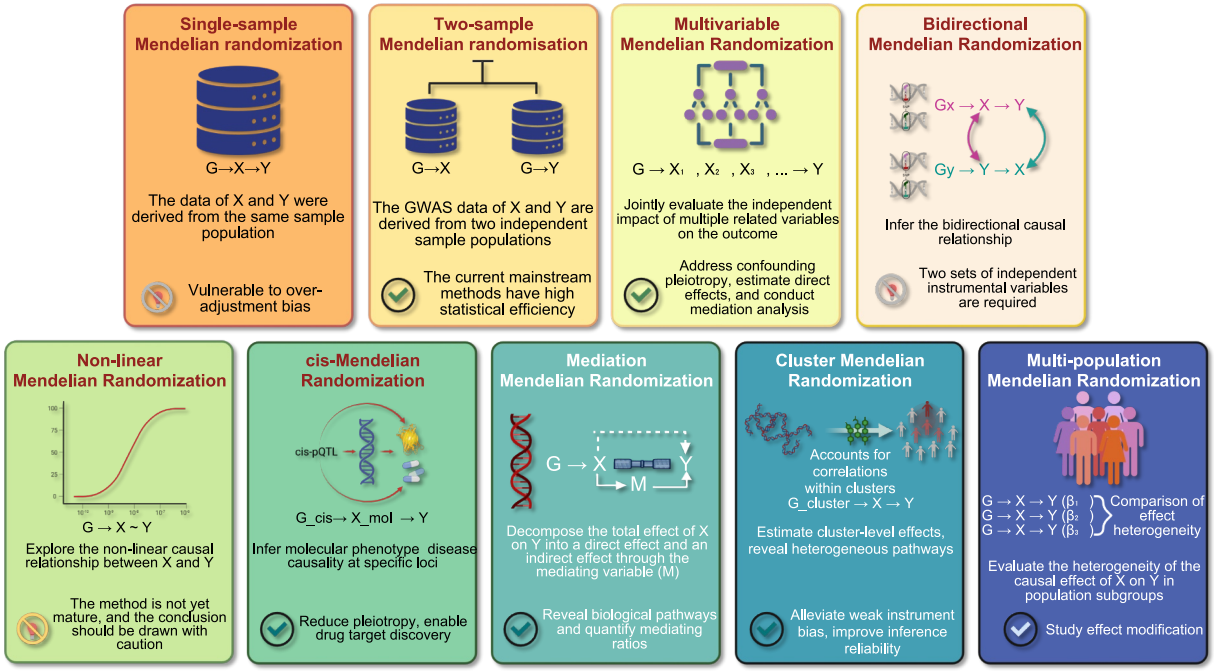

FIGURE 2 | Basic methods of Mendelian randomization.

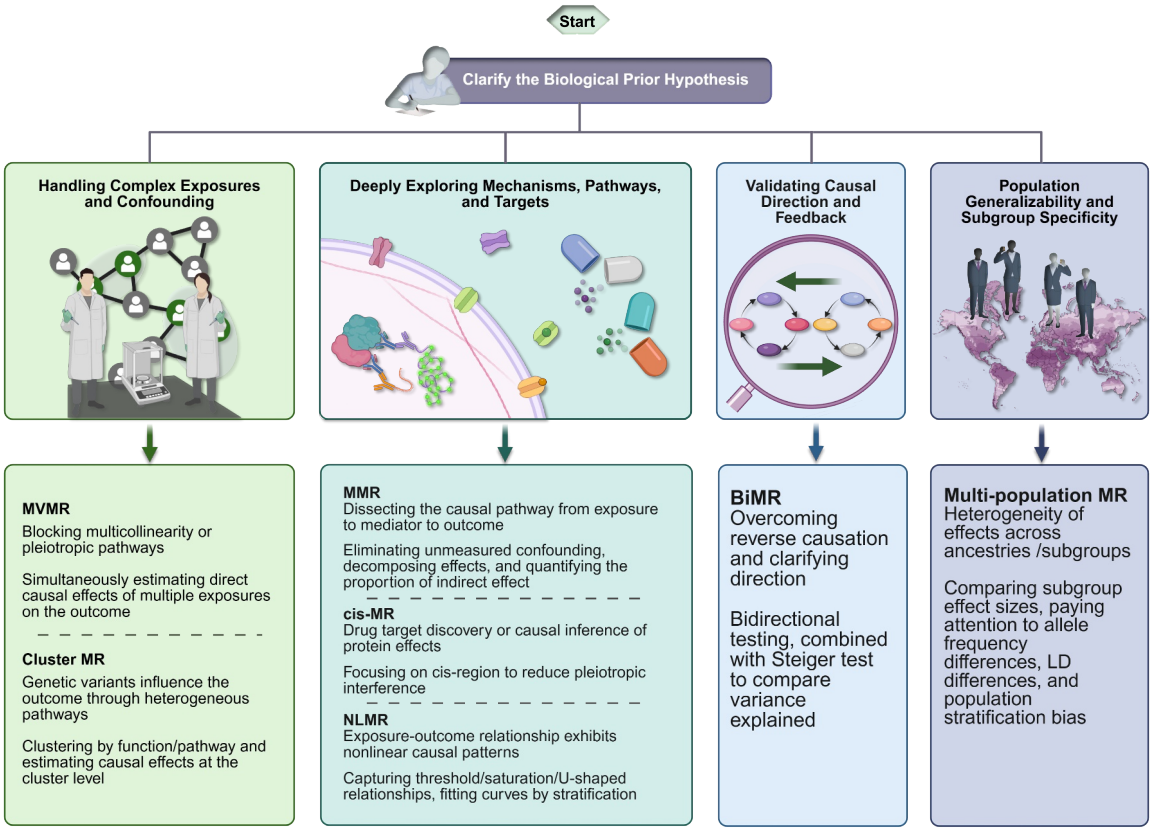

FIGURE 3 | Decision tree for advanced Mendelian randomization study designs.

Compared to traditional MR, MVMR offers significant advantages. MVMR resolves the multiple exposure problem [69], meaning it can simultaneously estimate the direct effect of each exposure when multiple exposure variables may causally influence the outcome, thereby reducing bias that arises when correlated exposures are omitted from the model [70]. Furthermore, MVMR incorporates correlated exposure variables into the model, which helps control for pleiotropic pathways where a genetic instrument might influence the outcome through an alternative exposure, thereby mitigating bias from

TABLE 2 | Summary of common Mendelian randomization methods.

Method name	Basic principle	Features	Limitations	Application fields
Two-sample Mendelian randomization	Uses genetic variants as IVs from two independent samples (exposure and outcome) to estimate the causal effect of exposure on outcome	1. Avoids sample overlap bias 2. High statistical efficiency 3. Suitable for GWAS summary data	1. Relies on three core IV assumptions 2. Potential bias if sample overlap exists	1. Epidemiology (cardiovascular [41], metabolic diseases [42]) 2. Drug target validation [43] 3. Behavioral factor research [44]
One-sample Mendelian randomization	Measures both exposure and outcome in the same cohort using genetic variants as IVs	1. Convenient data acquisition 2. Suitable for individual-level data	1. Susceptible to sample overlap and reverse causation 2. Lower statistical power	Small cohort studies for preliminary validation of specific exposure-outcome pairs [45]
Weighted median method	Ranks IV effect sizes by weight and takes the median estimate	1. Allows up to 50% invalid instruments 2. Robust to pleiotropy	1. Results may be biased if $>$ 50% invalid instruments 2. Requires a large sample size	Studies with partially invalid IVs [46] (e.g., complex pleiotropy scenarios)
MR-Egger regression	Introduces an intercept term to detect directional pleiotropy and estimates the causal effect through the regression slope	1. Lower requirement for IV validity 2. Can detect directional pleiotropy	1. Low statistical power 2. Causal estimates may be inaccurate if the intercept significant	Sensitivity analysis studies with potential pleiotropy [47] (e.g., socio-economic factors)
Multivariable Mendelian randomization	Simultaneously analyzes independent effects of multiple exposures while controlling for confounding exposures	Distinguishes independent causal effects of multiple exposures and avoids omitted variable bias	1. Requires exposure-specific IVs 2. Computationally complex	Complex exposure scenarios [48] (e.g., joint effects of BMI, blood glucose and blood pressure on cardiovascular disease)
MR-PRESSO	Detects and removes outlier IVs, corrects for pleiotropic bias	1. Sensitive to horizontal pleiotropy 2. Provides global and local outlier tests	1. Depends on the number of IVs 2. May over-exclude valid instruments	Studies with potential outlier IVs [49] (e.g., genetic variants associated with multiple phenotypes)
Lasso Mendelian randomization	Uses machine learning (Lasso regression) to select valid IVs	1. Automated IV selection 2. Suitable for high-dimensional genetic data	1. Requires large samples 2. High computational complexity	Studies with numerous candidate IVs where widespread pleiotropy or weak instruments may exist [50]
Non-linear Mendelian randomization	Estimates non-linear exposure-outcome relationships through segmented or locally weighted regression	Captures U-shaped, J-shaped and other non-linear effects, better reflects biological mechanisms	1. Needs numerous IVs 2. Strong model assumptions	Studies with complex dose-response relationships [51]
Longitudinal Mendelian randomization	Analyzes long-term causal effects of time-varying exposures on outcomes	1. Reveals time-dependent effects 2. Integrates life course epidemiology	Requires longitudinal genetic data IV stability over time difficult to ensure	Developmental origins hypothesis [52] (e.g., fetal nutrition and adult diseases) or aging research [53]
Reverse Mendelian randomization	Tests whether outcomes reversely affect exposures to rule out reverse causation	1. Validates causal direction 2. Complements forward MR findings	Results are unreliable if weak IV-exposure associations exist	Testing bidirectional causality [29] (e.g., telomere attrition and chronic kidney disease)

(Continues)

TABLE 2 | (Continued)

Method name	Basic principle	Features	Limitations	Application fields
Network Mendelian randomization	Integrates multi-omics data to construct causal networks	1. Reveals multi-level biological mechanisms 2. Systematic analysis	1. Data integration is challenging 2. Multiple testing issues are prominent	Complex disease mechanism studies [54] (e.g., protein–protein interaction network of multiple sclerosis)
Bayesian-based Mendelian randomization methods	Incorporates prior knowledge through Bayesian framework to optimize causal effect estimation	1. Flexibly handles uncertainty 2. Suitable for small samples or weak instruments	1. Subjective prior selection 2. Computationally intensive	Studies with well-defined prior information [55] (e.g., drug target validation)

Note: This table systematically summarizes core MR methods widely applied in causal inference research, with each entry detailing the method's basic principle, distinctive features, key limitations, and typical application scenarios. Representative references are provided for each method to enable readers to quickly locate and access relevant empirical studies, methodological validations, and practical examples for further reference.

certain forms of horizontal pleiotropy [71]. In scenarios with high exposure correlation or high dimensionality, MVMR combined with dimensionality reduction techniques can address multicollinearity issues and enhance estimation efficiency [72]. In mediation analysis [73], MVMR not only estimates the total effect of exposure on the outcome but also decomposes this effect into direct causal effects on the outcome and indirect effects acting through a specific mediator, thereby revealing causal pathways [74]. The extended multi-response MR (MR-MRM) [75] framework can simultaneously analyze multiple related outcomes, overcoming the limitation of traditional MR to a single outcome.

Despite the powerful capabilities of MVMR, researchers must remain mindful of the potential for weak instrument bias when employing this method [76]. Although MVMR can adjust for measured horizontal pleiotropy via included exposures, this approach may still yield biased causal estimates when the IV affects the outcome through unmodeled confounding exposures. In practice, especially when analyzing multiple exposures, MVMR requires a sufficient number of strong genetic instruments relative to the number of exposures. As the number of included exposure variables increases, the model may experience multicollinearity [77], which reduces the precision of the effect estimates. When exposure or mediator variables are dichotomous, a two-stage regression approach is required; however, estimation efficiency and consistency may be compromised.

1.2.2.3 | *Bidirectional Mendelian Randomization.* BiMR [78] is a causal inference approach consisting of two independent TSMR analyses, which enables the simultaneous assessment of potential bidirectional causal effects between two phenotypic traits. Its core principle [79] involves alternately treating each trait as the exposure and the other as the outcome, using independent genetic instruments to separately estimate the potential bidirectional causal relationship between the exposure and outcome. This approach avoids the biases arising from unclear causal direction or reverse causality inherent in simple correlation analyses.

BiMR employs a bidirectional causality testing framework to address the reverse causality issue in traditional observational studies, clearly defining causal direction. Quantifying the relative strength of causal effects in both directions reveals dynamic feedback mechanisms between variables. BiMR employs independent sets of genetic instruments ($G\_X \cap G\_Y = \varnothing$) [80] to analyze bidirectional effects. It combines Steiger's [81] directionality test to compare the variance explained by genetic instruments for each variable. When confronting complex scenarios involving bidirectional causality, BiMR serves as a pre-validation tool. Through its bidirectional causality testing framework, it first clarifies the primary causal direction between variables, thereby eliminating reverse causality confounding for subsequent mediation analyses [82].

In practice, BiMR still faces multiple challenges. The method requires the independent selection of valid genetic instruments, which requires the independent screening of genetic variants that satisfy the three core IV assumptions for each analysis direction. Regarding statistical power, BiMR requires large sample sizes [83], particularly when addressing causal relationships with small effect sizes, where power is often inadequate. When analyzing multiple exposure-outcome pairs, multiple comparison issues increase the risk of false positives, necessitating rigorous *p*-value correction. Sample overlap across data sources may bias estimates toward observational associations, and population stratification confounding is particularly prevalent in cross-ancestry analyses. Although the Steiger directionality test can be used to infer the likely direction of causality, its statistical power significantly declines when bidirectional effect sizes are similar or when a common genetic basis exists [80], making it difficult to distinguish true bidirectional causation accurately. These limitations fundamentally stem from the tension between the biological complexity of genetic instruments and the simplifying assumptions required by statistical models. Addressing this requires methodological innovations—such as longitudinal MR frameworks—and more rigorous sensitivity analyses, coupled with interpretation grounded in biological plausibility.

1.2.2.4 | *Non-linear Mendelian Randomization.* NLMR [84] is designed to investigate potential nonlinear causal relationships between exposure and outcomes. Whereas traditional MR typically assumes linear associations, NLMR employs more flexible modeling approaches [85] to capture complex nonlinear effects, providing a more nuanced perspective for causal inference. The fundamental steps of NLMR include [86]: Using

genetic instruments to predict exposure levels, stratifying the population based on predicted exposure levels, independently estimating the local average causal effect within each stratum, and finally combining the stratum-specific estimates to characterize the overall nonlinear dose–response relationship.

Compared to traditional MR, NLMR theoretically offers significant advantages. NLMR can capture nonlinear causal relationships between exposure and outcome [87], including threshold, saturation, U-shaped, and J-shaped dose-response curves. Methodologically, in contrast to residual-based methods and other approaches reliant on linear modeling assumptions, NLMR's fractional polynomial or spline-based methods [88] provide greater flexibility for modeling nonlinear associations even when genotype-exposure associations are nonlinear, thereby demonstrating greater robustness. However, NLMR faces fundamental methodological limitations and empirical shortcomings that undermine the reliability of its inferences. The core issue lies in the introduction of uncontrollable biases when attempting to capture complex curvilinear relationships [89]. Regarding the stratification step, the stratification based on genetic predictors may induce "collider bias" if there is heterogeneity in the genetic effects or if the genetic instruments are associated with unmeasured confounders within exposure strata. Simultaneously, the effect size of genetic instruments on exposure is not constant but varies with exposure levels. This "non-constant genetic instrument effect" is amplified within the stratified framework, leading to biased estimates of local causal effects. These limitations have been demonstrated in simulation studies and practical applications. An NLMR study [90] on vitamin D and mortality reported a counterintuitive U-shaped association, namely, extremely high vitamin D levels were associated with an increased mortality risk. This finding prompted the publishing journal to issue an editorial expression of concern. This demonstrates that current mainstream stratified NLMR methods face considerable challenges in meeting their underlying assumptions, and are thus prone to generating misleading results. Until fundamental methodological issues are resolved and effective validation tools are developed, relying on existing NLMR methods to infer nonlinear causal patterns between exposure and outcomes carries significant risks. Caution is warranted regarding their conclusions.

Future development in the NLMR method should prioritize methodological innovation, including the development of more robust estimators, the construction of novel stratification strategies that do not rely on the rank-preserving assumption, and the direct estimation of nonlinear causal curves based on semiparametric models. It should integrate G × E interaction detection techniques to identify and exclude SNPs with heterogeneous effects before stratification. A standardized simulation validation process should be established, where methods are tested against simulated datasets with known nonlinear causal structures in NLMR analysis.

1.2.2.5 | *Cis*-Mendelian Randomization (*cis*-MR). *Cis*-MR is a method that utilizes *cis*-single nucleotide polymorphisms (*cis*-SNPs) near a specific genomic locus as IVs to investigate causal relationships between phenotypic traits. Unlike traditional genome-wide MR, *cis*-MR focuses on a single genomic region and acts solely by regulating target gene expression, thereby reducing interference from pleiotropy [18]. This unique characteristic confers distinct advantages for drug target discovery [91].

At the application level, *cis*-MR is commonly used to assess the impact of protein expression on disease risk, aiding in understanding the molecular pathways through which drug interventions may alter disease risk. This holds significant importance for target mechanism research and preclinical drug development [92]. For example, in drug target analysis for coronary artery disease (CAD), *cis*-protein quantitative trait loci (*cis*-pQTLs)-based MR studies have identified potential drug targets such as PCSK9, COLEC11, and FGFR1 [91]. Concurrently, *cis*-MR can also be employed to more precisely localize potential causal molecules and infer the causal effects of proteins on disease [93].

Methodologically, genetic variation within cis regions exhibits high LD. When combined with specialized *cis*-MR methods for LD modeling and correction, *cis*-MR still provides relatively robust causal inferences when handling correlated IVs. By leveraging the highly structured nature of genetic correlations within single gene regions, *cis*-MR can reduce the dimensionality of genetic variation to some extent [94]. This enhancement improves the accuracy of causal effect testing and mitigates potential instability arising from weak IV effects on exposure. *Cis*-MR is also frequently combined with colocalization analyses to verify whether the same genetic variation simultaneously influences molecular traits and phenotypes, thereby strengthening the credibility of causal interpretations [91].

Addressing pleiotropy and LD in *cis*-SNPs remains a major challenge for *cis*-MR [95]. Several specialized statistical methodologies tailored for *cis*-MR have been developed in recent years: MR-link-2 [96] corrects for bias by modeling LD structures using external reference panels; cisMRcML [91] employs constrained maximum likelihood estimation, offering greater robustness against weak instrumental variables and correlated pleiotropy; PCGMM [97] employs factor analysis and Bayesian variable selection for efficient handling of multiple IVs; and cisMRBEE [98] specifically corrects biases caused by measurement errors, among others. Collectively, these methods form a modern *cis*-MR methodological framework for addressing complex genetic architectures in cis regions. However, limited *cis*-SNP availability may result in insufficient IV strength, requiring larger sample sizes to enhance detection power [99]. Additionally, certain genes may undergo concurrent cis and trans regulation, necessitating careful differentiation of effects from distinct regulatory sources during analysis and interpretation [100].

By focusing on individual gene regions, *cis*-MR provides a powerful and precise framework for causal analysis from genetic variation to molecular phenotypes and ultimately to disease risk. With the maturation of specialized statistical methodologies and the expansion of large-scale multi-omics databases, *cis*-MR is poised to play an increasingly critical role in systematically decoding the biological mechanisms of diseases and accelerating therapeutic target evaluation.

1.2.2.6 | *Mediation Mendelian Randomization.* MMR [101] is an analytical framework that integrates causal effect decomposition with IV methods, designed to dissect causal pathways through which exposure influences outcomes via mediating variables. Traditional causal mediation analysis typically estimates three parameters [102]: the total effect, representing the exposure variable's influence on the outcome variable through all potential pathways; the direct effect, denoting the residual impact of the exposure variable on the outcome through pathways other than the specified mediator; and the natural indirect effect, reflecting the pathway through which the exposure variable influences the outcome via the mediator. When the total effect, direct effect, and indirect effect share the same direction, the "mediation proportion" can be calculated [103].

The core advantages of MMR lie in its ability to address key limitations inherent in traditional methods [104]. Leveraging the random assignment characteristic of IVs, MR eliminates unmeasured confounding bias between exposure and mediators [105], and outcomes-an inherent limitation unresolvable by traditional regression methods. Furthermore, MR estimates are generally robust to non-differential measurement errors in exposure or mediators [81], whereas such errors in conventional mediation analysis lead to biased estimates of indirect effects. By integrating MR's causal inference capabilities with the mediation analysis framework, MMR provides evidence on whether a mediator explains part of the exposure-outcome relationship, and quantifies the magnitude of indirect effects [106].

In practical applications, the validity of MMR's IVs relies heavily on the strength of the IVs, and it remains susceptible to weak instrument bias; horizontal pleiotropy may also be amplified during the two-step estimation process [65]. In the MVMR scenario [107], sufficiently strong IVs are required to satisfy the association requirements for both exposure and mediators simultaneously. Each additional mediator variable may weaken the conditional *F*-statistic, necessitating larger sample sizes to ensure statistical power. Methodologically, most MMR methods assume linear relationships between exposure and mediator, mediator and outcome, and exposure and outcome. When encountering nonlinear or time-varying effects [86], estimation accuracy declines. Additionally, binary exposure or mediator variables may also violate model assumptions in MMR. Because mediation effects are typically components of total effects [108], they exhibit smaller effect sizes. Detecting small mediation effects requires extremely large sample sizes, and issues with weak instruments further reduce power. When the total effect is close to null, the estimate of the proportion mediated can become unstable, leading to false positive inferences.

1.2.2.7 | *Multi-Population Mendelian Randomization (Multi-Population MR).* Multi-population MR [109] aims to utilize IVs to explore whether the causal effect of the same exposure on the same outcome exhibits heterogeneity across different ancestral backgrounds or population subgroups. It encompasses either cross-ancestry MR or subgroup analyses stratified by characteristics such as sex or age within the same ancestral group; the sources of bias and methodological approaches for addressing them are not entirely identical. Beyond relying on the three core assumptions, multi-population MR additionally incorporates subgroup stratification and cross-group effect comparisons: The overall population is divided into mutually exclusive subgroups based on characteristics. Standard MR analysis is conducted independently within each subgroup to estimate the causal effect between exposure and outcome. When these estimates are compared, statistically significant differences indicate that the causal effect is modified by subgroup characteristics. In cross-ancestry scenarios, the more common objective is to assess effect consistency and transferability while addressing biases arising from allele frequency differences, LD structures, and population stratification. Multi-population MR requires that genetic instruments exhibit consistent correlation across subgroups and approximately meet independence and exclusion restriction assumptions within each subgroup.

Why do we need multi-population MR? Its core utility lies in its capacity to detect heterogeneity in causal effects. Simply put, traditional MR estimates the average causal effect across the entire population, yet the impact of many exposures on disease may vary significantly across different population subgroups. Multi-population MR quantifies effect modification by comparing causal effects across subgroups under specific conditions [110], thereby meeting the demands of precision medicine. In practical applications, it can help identify biological heterogeneity, for example, differences in a biological pathway by sex or age [111]. Grouping subjects based on environmental exposure levels enables an indirect investigation of environmental exposure modification effects and facilitates the formulation of G $\times$ E interaction hypotheses. Multi-population MR enables more refined causal inference by transcending the limitations of a single average effect [109], thereby enhancing the robustness of research findings. In recent years, multi-population MR methodology has transcended the traditional subgroup comparison framework, evolving into a series of targeted techniques: Integrating multi-ancestry GWAS summary statistics or constructing pan-ancestry IVs to balance instrument validity and population applicability; leveraging transfer learning-based MVMR to utilize shared information across ancestries, enhancing effect estimation precision for small-sample ancestries [112]; and correcting sample mismatch bias through quantifying ancestry-specific differential contributions, thereby reducing residual population stratification bias in results [113].

Multi-population MR faces challenges due to contradictions between methodological assumptions and practical applications. When an IV exhibits subgroup heterogeneity in its direct effect on outcomes [114], observed causal effect differences may be entirely driven by this subgroup-specific pleiotropy rather than genuine effect modification. This assumption cannot be directly validated and relies on biological plausibility or indirect inference via sensitivity analyses. Furthermore, the strength of the IV-exposure association may be weakened in certain subgroups, or the independence assumption may be violated due to factors such as population stratification, family structure, and selection bias, further undermining the credibility of the results. Subgroup stratification reduces the sample size per group, leading to weak instruments, imprecise effect estimates, and diminished power of heterogeneity tests. Multiple comparison corrections further amplify the risk of false negatives. Subjective subgroup allocation may introduce bias, whereas the discretization of continuous variables causes information loss. For

cross-ancestry MR studies, differences in allele frequencies and LD structures may alter instrument validity. Ancestry mismatch and subtle population structures may also introduce confounding through residual population stratification, thereby distorting effect estimates [115]. In practice, population stratification can be corrected via PCA, LD pruning strategies can optimize the independence of cross-ancestry IVs, and overlap-robust methods such as GRAPPLE [116] can be combined to mitigate the combined effects of sample overlap and ancestry confounding. Collectively, these findings caution us: Multi-population MR discoveries must undergo rigorous validation through large independent cohorts, be constrained by prior biological hypotheses, and undergo multidimensional sensitivity analyses to avoid misinterpreting methodological artifacts as scientific discoveries.

Future multi-population MR studies should prioritize addressing the following issues: establishing standards for verifying the consistency of genetic effects across populations to address estimation bias caused by genetic heterogeneity; developing an evidence-based framework that integrates molecular, cellular, animal, and quasi-experimental studies to systematically validate the reliability of MR hypotheses; developing temporal MR methods to parse dynamic causal pathways and exploring algorithms for identifying effect modifiers; strengthening multidisciplinary collaboration to translate MR evidence into the basis for drug target discovery and RCT design; and prioritizing the resolution of practical bottlenecks such as survival bias in late-onset diseases and modeling the interpretability of high-dimensional exposures.

1.2.2.8 | *Cluster Mendelian Randomization (Cluster MR).* Cluster MR [117] is an innovative method developed from traditional MR, and its core concept leverages the clustering characteristics of genetic variants to enhance the accuracy and robustness of causal inference. By grouping genetic variants with similar biological functions, molecular pathways, or chromosomal locations, this approach estimates causal effects at the cluster level, thereby overcoming the limitation of traditional methods that treat each variant independently.

Since the introduction of MR-Clust [117] in 2020, cluster MR methodologies have continued to evolve. MR-Clust addresses estimation uncertainty and false positive findings by incorporating zero clusters and junk clusters; MR-AHC [118] significantly improves computational efficiency; MR-PATH introduces the concept of mechanistic heterogeneity; and PCMR [119] focuses on resolving multi-effect issues at the correlation level. These advancements collectively drive continuous progress in analytical capabilities within this research field.

Cluster MR offers distinct advantages over traditional MR methods. In terms of statistical power, it significantly enhances the precision and robustness of causal effect estimates by integrating multiple relevant genetic variant sources [120], effectively mitigating estimation biases caused by weak instrumental variables. The core methodological breakthrough of cluster MR lies in resolving effect heterogeneity. Whereas traditional methods assume homogeneous causal effects across all genetic variants, the clustering approach identifies subgroups of variants with distinct causal effects. This reveals multiple biological pathways through which exposure influences outcomes, greatly enhancing the biological interpretability of results. Compared to other causal inference methods, cluster MR excels at handling multivariate causality and horizontal pleiotropy [119]. It enhances the reliability of causal inference by identifying clusters with similar pleiotropic patterns.

Despite the significant advantages of cluster MR, its application still faces multi-level constraints and challenges. Methodologically, clustering strategies for genetic variants lack unified standards [120], and existing approaches often rely on model assumptions that may compromise result robustness [121]. Implementation demands exceptionally high data quality and a large sample size, whereas algorithms face computational complexity with large-scale genomic data. The intricate result structure further complicates interpretation. Additionally, clustering outcomes frequently exhibit population specificity, limiting their generalizability across cohorts. This approach primarily reveals the long-term cumulative effects of genetic variation, which may differ from the outcomes of short-term clinical interventions, necessitating careful consideration during clinical translation. Translating complex clustering results into clear biological mechanisms or clinically actionable strategies is particularly challenging [122] and requires researchers to possess advanced multidimensional interpretive skills. These limitations suggest that when applying cluster MR, its underlying assumptions should be rigorously evaluated, and findings should be interpreted judiciously.

1.3 | Application Areas of Mendelian Randomization

In recent years, against the backdrop of the exponential growth of large-scale GWAS summary statistics and the rapid advancement of MR methodologies, MR applications have expanded beyond their initial role in validating causal relationships for classical risk factors. They now extend into biomedicine, public health, and even the social sciences [123], demonstrating unique value in elucidating disease etiology, identifying actionable targets, verifying drug mechanisms of action, and guiding public health policies.

1.3.1 | *Gene-Environment Interaction*

G × E [124] refers to the phenomenon in which an individual's genetic factors and environmental factors interact to influence phenotypic traits such as disease risk, behavioral characteristics, and physiological indices. Practically speaking, genetic effects may vary across different environments, while the impact of the environment on individuals may also differ due to genetic variations [125]. G × E research is crucial for comprehensively understanding the origins of complex human phenotypes [126]. It sheds light on the heterogeneity of genetic effects, explaining why identical environmental conditions yield divergent phenotypic outcomes for individuals with varying genotypes. Simultaneously, it lays the foundational framework for precision medicine by identifying high-risk populations and developing personalized intervention strategies. G × E research also drives innovation in statistical methodologies [127, 128], enabling the robust detection of G × E interactions within large-scale genetic datasets.

Traditional G × E studies directly test interaction terms in the regression model $Y = G + E + G \times E + \varepsilon$ to identify interactions. However, due to the inherent multicollinearity between the genotype G and the interaction term G × E, the standard error of the interaction effect estimate increases, thereby reducing the statistical power of the test [129]. To circumvent this issue, researchers have introduced the MR framework into G × E studies, developing multiple methodologies. Among these, the $T_{\text{MR-GxE}}$ test [130] leverages summary-level GWAS statistics (i.e., marginal effect estimates) to enhance genome-wide screening efficiency, whereas in-depth exploration of causal heterogeneity increasingly relies on methods such as stratified MR, MVMR, and structural equation modeling. These methods assess how the causal effects of an exposure are modified by environmental or genetic backgrounds, utilizing individual-level data. Collectively, these methods form the methodological framework for MR-based G × E research; they provide powerful tools for comprehensively revealing complex G × E interactions, from large-scale screening to refined mechanism validation.

Theoretical advantages require practical validation. A study on physical activity and glaucoma [131] within the UK Biobank (UKB) serves as a prime example. The research first employed traditional regression models to examine associations between physical activity and glaucoma-related traits such as intraocular pressure and retinal thickness. It then constructed a polygenic risk score to assess gene-physical activity interactions. Subsequently, the researchers employed the TSMR method, utilizing large-scale GWAS summary statistics to construct genetic IVs for inferring causal relationships between physical activity and glaucoma-related traits. Although the study did not identify significant G × E interactions or MR-supported causal effects, it comprehensively demonstrated how to integrate polygenic risk scores with the MR framework for a systematic exploration of G × E—progressing from observation to causation and from screening to validation.

1.3.2 | Epidemiology and Public Health

MR has become a pivotal method in establishing causal relationships within epidemiology and public health [132]. The core challenge [127] in this field lies in establishing reliable causality based on observational associations, thereby providing evidence for the development of effective prevention strategies. Traditional observational studies are susceptible to confounding factors and reverse causality. Although RCTs [133] represent the gold standard, they are often constrained by ethical, cost, and feasibility limitations. MR methods utilize genetic variants as IVs, simulating the randomization process inherent in RCTs [134]. This provides a powerful methodological tool for effectively controlling confounding bias and reverse causality, thereby enhancing the credibility of causal inferences between exposure and outcomes.

The aforementioned methodological advantages were demonstrated in a recent large-scale study by Ran et al. [135], which integrated observational research with MR analysis. This study aimed to elucidate the biological mechanisms by which air pollution ($PM_{2.5}$, PM_{10}, NO_2, NOx) leads to metabolic dysfunction-associated steatohepatitis (MASLD), employing a multi-methodological approach to establish a comprehensive evidence chain. Leveraging data from over 240,000 participants in the UKB, researchers first employed elastic net regression to construct "metabolic signatures" specific to each air pollutant using 251 plasma metabolites. These signatures essentially represent weighted combinations of multiple lipids, amino acids, and lipoproteins. Traditional Cox proportional hazards model observational analyses revealed that elevated air pollution exposure and its associated metabolic signatures were both significantly associated with an increased risk of MASLD. To overcome the limitations of residual confounding and reverse causality in observational analyses, researchers further performed TSMR analysis. They utilized genetic variants significantly associated with these "air pollution-related metabolic features" as IVs to investigate their causal effects on MASLD risk. MR analysis results confirmed that metabolic features related to $PM_{2.5}$, NO_2, and NO_x are indeed causal risk factors for MASLD. This not only provides genetic-level causal evidence for the hypothesis that "air pollution causes liver disease by disrupting specific metabolic pathways", but more critically, mediation analyses further demonstrated that these metabolic signatures exert a significant mediating effect in the process by which air pollution elevates MASLD risk, mediating approximately 0.5%–1.1% of the total risk effect. The innovation of this study lies in expanding the scope of MR analysis beyond traditional single exposure factors [136] (such as BMI and blood lipids) to comprehensive "metabolic signatures" derived from multi-omics technologies. This significantly enhances the ability of MR to decipher complex disease mechanisms, aligning with the principles of systems biology. Notably, this study stands as an exemplary case of deep integration with the UKB. Key steps—including observational analysis, metabolic signature construction, and IV identification via GWAS—all benefited from the statistical power and rich multi-omics data layers provided by this database's vast sample size. Furthermore, as one of the core challenges in public health, infectious diseases have long relied on traditional observational epidemiological studies to assess their transmission dynamics, host susceptibility, and intervention effectiveness. The development of MR offers a unique perspective for deciphering causality in infectious diseases [137] and optimizing prevention and control strategies, achieving key advances in recent years in areas such as pathogen susceptibility mechanisms, vaccine efficacy validation, and drug target screening. Taking a recent MR study by Wang et al. [138] as an example, researchers systematically investigated causal associations between gut microbiota and nine infectious diseases. Using gut microbiota GWAS summary statistics from the MiBioGen Consortium and infectious disease GWAS summary statistics from UKB, they conducted a TSMR analysis. Results revealed that the abundance of specific microbial taxa, such as Coriobacteria, is positively associated with an increased risk of lower respiratory tract infections, whereas the abundance of the Lentisphaeria phylum reduced the risk of sepsis. This study provides genetic-level causal evidence for the role of the gut-organ axis in the pathogenesis of infectious diseases and demonstrates the practical value of MR in identifying microbe-mediated infection mechanisms and discovering potential intervention targets. This highlights a significant trend in modern public health research [139]: leveraging large-scale biobanks to integrate epidemiological design, multi-omics technologies, and MR, thereby providing robust scientific evidence for developing precise public health intervention strategies.

1.3.3 | *Research on Complex Diseases*

Complex diseases [140], also known as multifactorial diseases, refer to a category of disorders caused by multiple genetic variants, environmental factors, and their intricate interactions. Complex diseases such as CVD [141], cancer [142], diabetes [143], and mental disorders [144] are the leading causes of disability and death worldwide [145], accounting for the vast majority of the global disease burden. With accelerating population aging, the incidence of age-related complex diseases such as Alzheimer's disease [146] and Parkinson's disease [147] is rising sharply. Research aimed at preventing complex diseases and advancing precision medicine is, therefore, of critical importance. As a methodological innovation in genetic epidemiology, MR is widely applied in etiological studies of various complex diseases, including mental disorders, cancer, and CVD. It provides causal evidence chains that help us understand disease mechanisms, formulate prevention strategies, and develop novel therapeutic approaches.

Recently, Bowden et al. [148] published a large-scale study investigating the genetic susceptibility to cervical cancer and its precursor lesions, as well as the underlying etiological mechanisms. Utilizing GWAS summary statistics from the UKB and the Finnish Biobank (FinnGen), the study analyzed over 9.6 million genetic variants. The study identified six independent genetic loci associated with cervical cancer and cervical intraepithelial neoplasia grade 3 (CIN3), including variants in the *PAX8*, *CLPTM1L*, and *HLA* regions. Through TSMR analysis, the study further validated the causal relationship between smoking, number of sexual partners, and cervical cancer risk, while also identifying a protective association of older age at first birth. Researchers integrated MR with multiple bioinformatics methodologies to comprehensively analyze the functional significance and potential mechanisms of genetic variations. By combining data from the PanCanQTL and GTEx databases, they discovered that genetic variations at the *PAX8* locus were significantly associated with gene expression levels in cervical cancer tissues, suggesting that *PAX8* may influence cervical cancer development by regulating apoptosis and proliferation pathways. Functional annotation of non-coding region genetic variants was performed using databases such as HaploReg and ENCODE, revealing that relevant variants may regulate gene expression by affecting histone marks, transcription factor binding sites, and chromatin accessibility. Using conditional analysis methods, the study evaluated complex genetic signals in the HLA region, elucidating the contributions of multiple independent variants to disease risk and revealing the key roles of genes such as *HLA-DQA1* and *HLA-B* in adaptive immune responses. In the FinnGen dataset, the study independently validated the genetic signals for *CLPTM1L*, *PAX8*, and *HLA-DQA1*, further enhancing the reliability and broad applicability of the findings. This integrated analytical framework not only offers novel insights for etiological research and precision prevention of cervical cancer but also provides robust support for the advancement of precision medicine.

1.3.4 | *Drug Target Validation and Interdisciplinary Collaboration*

Drug development is a capital-intensive, time-consuming, and high-risk process [149]. On average, it takes 10–15 years for a new drug to progress from the laboratory to the market, with total investment costs probably exceeding $2 billion [150]. The clinical development phase [151] (Phase I–III trials) represents the most time-consuming and costly segment, accounting for the majority of total expenses. Moreover, over 90% of drug candidates entering clinical trials ultimately fail to gain approval [152]. This high failure rate stems primarily from biological complexity, species differences, and the limitations of traditional preclinical models in predicting human responses. These factors collectively form the well-known “Eroom's Law” [153]: whereas R&D investment in the pharmaceutical industry continues to grow, output efficiency steadily declines. To address these challenges, the pharmaceutical sector is actively exploring new approaches such as artificial intelligence (AI), adaptive platform trials, and MR to enhance target validation accuracy, reduce failure risks, and control R&D costs.

Against this backdrop, MR has demonstrated tremendous potential for drug target validation and therapeutic development. A recent study by Mazidi et al. [154] serves as a prime example of MR's successful application in drug target validation. This study combined cohort data from the China Kadoorie Biobank (CKB) and UKB, integrating proteomics and genomics data. Through observational analysis and MR analysis, it validated multiple potential drug targets associated with ischemic heart disease (IHD). The strength of MR in this research lies in its synergy with large-scale cohort studies and multi-omics data. Cohort studies provide rich contextual information on disease-exposure associations through long-term follow-up and systematic data collection. MR integrates genetic and molecular data from these cohorts, transforming observational associations into causal inferences. By incorporating multi-omics data such as proteomics and transcriptomics, MR analysis enables deeper insights into the biological mechanisms of targets, providing critical support for precision medicine. Mazidi et al. employed *cis*-pQTLs as IVs and identified 13 protein targets causally associated with IHD through TSMR analysis. These included novel targets such as FURIN and known targets such as MMP3. By integrating tissue-specific expression analysis and gene knockout models, they further elucidated the potential mechanisms of these targets in cardiovascular disease. Through multi-level data integration, the researchers not only demonstrated the causal relationship between these targets and IHD but also provided crucial theoretical support for their application in drug development. This innovative approach offers a viable strategy for pharmaceutical R&D, highlighting the potential of MR to reduce late-stage clinical attrition and inform decision-making [155].

This study also demonstrates that MR serves not only as a method for causal inference but also as a research platform that fosters interdisciplinary collaboration. It provides a standardized analytical framework for integrating data, technologies, and perspectives from diverse fields, including the following: Epidemiology [77] contributes large-scale prospective cohort designs, phenotypic data, and follow-up resources; proteomics [123] enables extensive quantitative analysis of plasma proteins through high-throughput technologies, establishing a molecular phenotypic foundation; genetics [91] identifies *cis*-pQTLs and performs MR analyses to provide genetic causal evidence for protein-disease associations; bioinformatics [156] further

integrates multi-source biological databases to perform functional annotation, pathway enrichment, and expression pattern analysis of candidate targets, thereby elucidating their biological mechanisms; and finally, clinical medicine and pharmaceutical R&D experts conduct translational assessments of these findings to clarify their development potential and clinical application directions.

As demonstrated, MR extends beyond the role of traditional statistical tools in this context, thereby establishing a structured interdisciplinary framework that integrates an evidentiary chain from population phenomena, molecular phenotypes, genetic tools, and mechanism analysis to clinical translation. This multidisciplinary collaborative approach centered on MR significantly enhances the efficiency and reliability of target validation, providing a systematic and robust solution for reducing drug development failure risks.

1.3.5 | *Integration With Other Cutting-Edge Technologies*

With the rapid advancement of frontier technologies, including multi-omics, AI, and spatial biology, MR continues to integrate deeply with these methodologies, expanding the dimensions and precision of causal inference while unveiling significant potential.

In multi-omics integration, MR combines with transcriptomic, proteomic, and metabolomic data to form a multi-level causal inference framework. For example, by integrating pQTLs with disease GWAS summary statistics, researchers can validate the causal roles of specific proteins in diseases such as glioblastoma [157]. Such studies rely on tissue-specific expression quantitative trait loci (eQTLs/pQTLs) and employ colocalization analysis to ensure shared causal variants among genetic signals, molecular phenotypes, and diseases [158]. However, multi-omics exposures are often highly correlated, and the performance of traditional multivariate MR is compromised by multicollinearity [159]. Furthermore, complex causal structures—such as bidirectional causality, mediating pathways, and temporal dynamics—are difficult to capture [160]. Moreover, most studies lack orthogonal validation through molecular, cellular, or animal experiments, resulting in insufficient reproducibility and biological interpretability of causal inferences. Future efforts should prioritize methodological innovation, developing MR frameworks capable of simultaneously handling nonlinear effects, time-varying exposures, and multi-level mediation pathways [160], while promoting the integration of multi-omics data with experimental validation and combining MR findings with cutting-edge technologies such as single-cell multi-omics and organoid models; efforts should be made to enhance reproducibility and transparency by promoting standardized analytical platforms [161] and strengthening cross-cohort validation [162]; efforts should focus on drug target validation and early biomarker warning [163]; and efforts should strengthen interdisciplinary collaboration across genetics, bioinformatics, clinical medicine, and AI to assess the biological plausibility of causal hypotheses and expand clinical translation applications.

The introduction of AI and machine learning (ML) methods has further enhanced MR's efficacy in integrating high-dimensional data and optimizing IVs. In liver cancer risk prediction studies [164], LASSO regression and Cox models were employed to screen key genes from multi-omics data, with causal contributions subsequently validated via MR; ML methods such as ColocBoost and mvSuSiE efficiently handle complex genetic architectures. However, these approaches demand large sample sizes and high data quality, carrying risks of overfitting and interpretability limitations, necessitating validation with independent samples and biological plausibility assessments.

The emergence of spatial transcriptomics and single-cell technologies enables MR to perform causal inference at cellular and tissue spatial resolutions. In a glioma study, Liao et al. [165] integrated single-cell RNA sequencing with spatial transcriptomics data to identify the *CHST11* gene's specific expression pattern within the tumor microenvironment. They validated its causal relationship with immunotherapy response via MR, offering new insights into understanding disease spatial heterogeneity and cell type-specific mechanisms.

Additionally, MR is frequently combined with other causal inference methods such as colocalization analysis [166], mediation models [167], and phenotype-associated scanning [121] methods to form more rigorous inference frameworks. The use of these complementary approaches aids in distinguishing direct from indirect effects and identifying confounding pathways, but also imposes higher demands on the strength and specificity of the IVs. Given the core strengths and challenges of MR, future efforts should focus on developing more robust cross-omics MR analysis frameworks, promoting algorithm transparency and the standardization of workflows, while strengthening validation across diverse populations and dynamic biological processes. This will enhance its scientific value and clinical translation potential.

1.4 | Challenges and Limitations of Mendelian Randomization

MR serves as a core tool bridging genetics and epidemiology, yet its robustness is constrained by multifaceted challenges. This chapter systematically addresses key limitations, including IV validity, genetic complexity, data quality issues, statistical constraints, selection and collider bias, as well as benchmarking needs. The core challenges and corresponding strategies are concisely summarized in Figure 4.

1.4.1 | *Validity of Instrumental Variables*

The validity of IVs in MR primarily rests on three core assumptions [168]: the assumptions of relevance, independence, and exclusion restriction. Taking studies on alcohol consumption's impact on CVD as an example, Roerecke [169] explicitly states in his paper that the MR approach “depends on several assumptions that are not easily met in a complex relationship.” This case reveals that genetic variants affecting alcohol metabolism (e.g., *ALDH2* and *ADH1B*) may be correlated with other CVD risk factors such as socioeconomic status and lifestyle, thereby violating the independence assumption and introducing potential confounding. Furthermore, these alcohol metabolism genes may directly influence CVD pathophysiological processes through pleiotropic effects—specifically, acetaldehyde metabolism may directly affect blood pressure, lipid levels, or

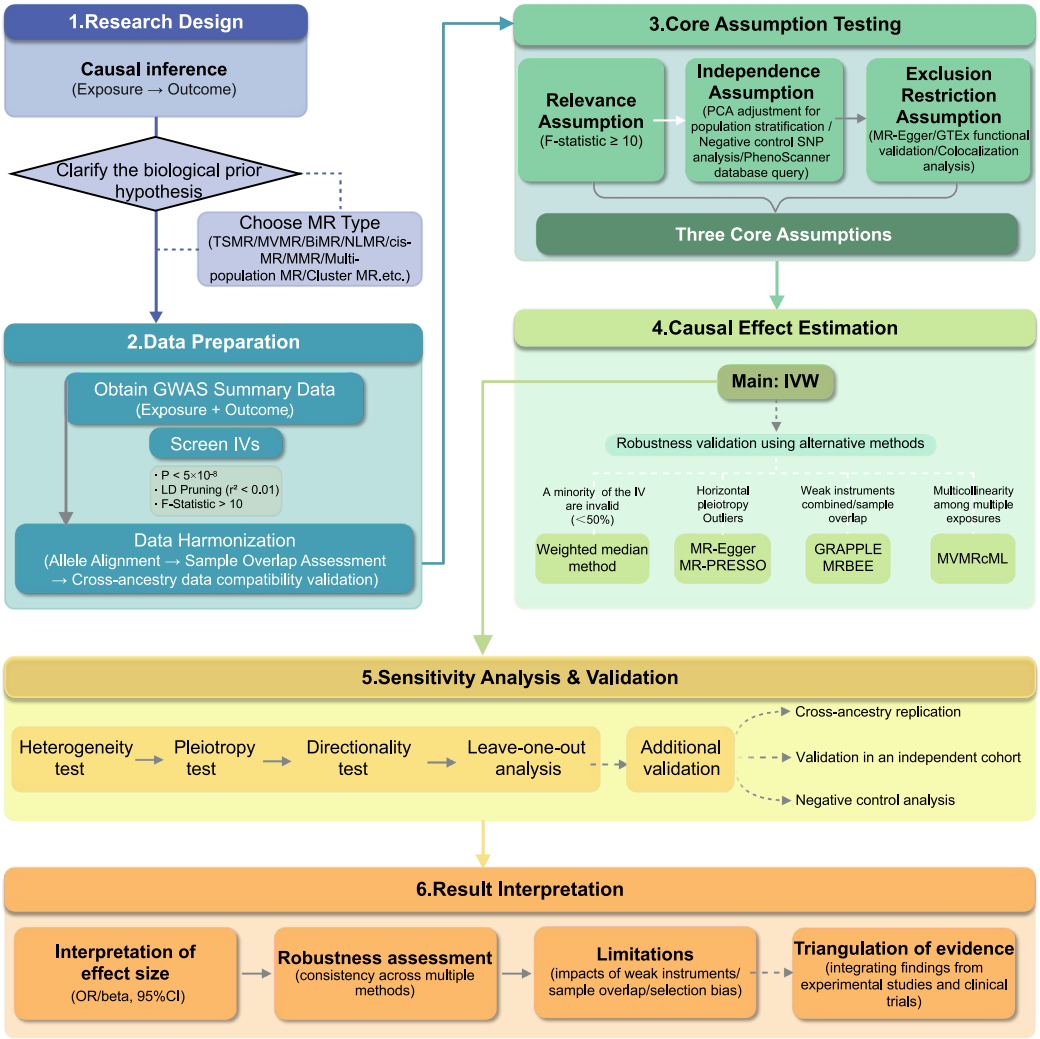

FIGURE 4 | Mendelian randomization "issue-diagnosis-mitigation" flowchart.

inflammation. This creates an alternative pathway through which the IV influences the outcome, directly violating the exclusion restriction assumption and leading to biased causal effect estimates. It is precisely due to the significant controversy surrounding these fundamental assumptions [170] that different MR studies addressing the same scientific question yield inconsistent and even contradictory results. This indicates that even when using genes as IVs, their application to complex behavioral exposures may still be confounded by residual confounding and multivariate confounding, thereby undermining the reliability of causal inference. To address these challenges, a multi-tiered solution has emerged in recent years: to ensure the relevance of IVs and tackle issues of weak instrumental variables and multicollinearity, it is necessary to compute *F*-statistics [171] and select robust genetic IVs from large-scale GWAS summary statistics. A series of mutually validating sensitivity analyses [172] is employed, including MR-Egger regression, weighted median methods, MVMR, and MR-PRESSO. Some methods specifically address heritable confounding at the model specification level; for example, CAUSE distinguishes between horizontal polygenic effects and genetic sharing, whereas LCV can be used for causal inference when potential heritable confounding is present, and LHC-MR explicitly models unmeasured heritable confounding factors. Likelihood-based or estimating equation methods, such as GRAPPLE [64], correct for weak instrumental variable effects by modeling measurement errors; MVMRcML [173] is suitable for multivariate scenarios; and MRBEE [116] can simultaneously address weak instruments and sample overlap. Comprehensive diagnostics are performed using Cochran's Q test [174] for genetic heterogeneity, the MR-Egger intercept test for directional horizontal pleiotropy, and leave-one-out analyses with funnel plots. Causal inferences drawn from MR analyses are considered the most robust when multiple methods yield consistent results and sensitivity analyses reveal no substantial violations of the core assumptions.

1.4.2 | *Complexity of Genetic Structure*

Traditional MR models often treat genetic instruments as a single homogeneous entity and assume that all IVs uniformly influence exposure. However, this assumption is frequently violated for complex polygenic exposures, which are influenced by factors such as pleiotropy, G × E interactions, and population stratification. Parra-Soto et al. [175] found that although height shows an observational association with multiple cancer types,

insulin-like growth factor-1 (IGF-1) levels do not modify this association. This finding directly highlights a challenge in MR studies: IVs may influence outcomes through multiple heterogeneous biological pathways, and MR estimates may capture only a confounded "average causal effect." Furthermore, because height is highly polygenic, the explanatory power for phenotypic variation of individual genetic variants is limited. This can lead to unstable or biased MR estimates due to weak instrument bias. Second, the study found that the strength of genetic associations varied across subgroups, such as smokers versus non-smokers, indicating the presence of G × E interactions. Because MR analysis cannot capture these environmental modification effects, it limits the guiding value of its findings for public health intervention policies. To address these challenges, beyond resolving issues of weak instrument bias and pleiotropy, MVMR incorporates multiple related exposures simultaneously into the model at the methodological level to isolate confounding pathways and estimate the direct causal effect of the target exposure [71]. At the study design level, conducting validation analyses across ancestral groups or different environmental subgroups is crucial for assessing G × E interactions. Concurrently, researchers must carefully interpret heterogeneous results in light of biological knowledge to avoid simplistic extrapolation of MR findings to short-term intervention effects. In summary, overcoming challenges posed by genetic complexity has no single shortcut. Instead, it demands a systematic evaluation that integrates data resources, analytical methodologies, and iterative frameworks to minimize bias risks and yield more accurate and reliable causal inferences.

1.4.3 | Data Quality and Availability

The open sharing of GWAS summary statistics has fueled the rapid expansion of MR studies. However, the inconsistent quality and inherent limitations of these datasets [176] pose potential risks to MR analyses. Insufficient statistical power, inconsistent variable definitions and measurement errors, population representativeness bias, and sample overlap are the most prominent issues. Statistical power is the foundation of MR analysis and remains a widespread challenge. Many MR methods require extremely large sample sizes [177], and sufficient data are key to addressing this issue. In their study exploring the causal relationship between depression and CAD, Lu et al. [178] utilized exposure GWAS summary statistics from over 800,000 individuals and outcome data from more than 180,000 individuals. This statistical power was sufficient to detect a small effect with an odds ratio (OR) of 1.10, successfully revealing that genetically predicted depression significantly increased the risk of CAD and myocardial infarction (MI). However, data availability depends not only on sample size but also critically on the quality. Despite the substantial scale of the data, the authors highlighted several data-related challenges in their discussion. The core IV for depression in this study integrated GWAS summary statistics from UKB, 23andMe, and the Psychiatric Genomics Consortium (PGC). These sources do not uniformly define or measure depression, and such discrepancies may introduce bias in the exposure represented by the IV, affecting the interpretation of results. The data primarily originate from populations of European ancestry, necessitating further validation of the study's conclusions in other ethnic groups. Additionally, the researchers noted sample overlap between GWAS studies of depression and certain secondary outcomes, which could constitute a potential source of confounding. These issues necessitate rigorous data quality control and comprehensive sensitivity analyses. Researchers must clearly specify the population to which findings apply, avoid overgeneralizing discoveries from a single cohort, and replicate results across diverse populations. Where feasible, sensitivity analyses should utilize completely independent sample sets. If sample overlap cannot be avoided, specialized statistical methodologies (e.g., MR-RAPS, a cross-sample correction technique) should be employed for quantitative adjustment. These challenges are particularly pronounced in non-European populations; however, biobanks containing comprehensive phenotypic and genomic data have also been established in Asian populations, providing crucial support for addressing the issue of insufficient population representativeness.

BioBank Japan (BBJ) is a core biobank representing the genetic background of East Asian populations. It primarily [179] includes native East Asian residents in Japan and provides aggregated GWAS statistics, genetic variant information, and clinical diagnostic records to support cross-ethnic genetic analysis and the construction of IVs [180]. In the field of MR research, BBJ is widely used across various disease domains; for example, Liu et al. [180] successfully validated pathways linking gut microbiota metabolites to corresponding metabolic phenotypes. BBJ also supports the development of cross-ethnic MR methods [181] and the optimization of polygenic risk scores [182], providing essential tools for genetic analysis in non-European populations. The CKB covers Han Chinese adults across 10 geographic regions in China, accurately reflecting the demographic characteristics of the Chinese population. The data types are extremely diverse: Baseline data include questionnaires, physical measurements, and biological samples; regarding genetic data [183], over 100,000 participants have completed custom chip genotyping; follow-up data [184] enable long-term tracking of health outcomes through the national health insurance system and mortality and morbidity registries; and the study has expanded to include multi-omics information such as plasma metabolomic, metagenomic, and proteomic profiles [185]. Based on these data, the CKB produced a series of findings within the MR framework: Clarke et al. [186] elucidated the causal effects of lipoprotein (a) levels on MI and atherosclerotic stroke, with effects comparable to those observed in European populations; and O'Loughlin et al. [187] found that genetically predicted BMI was positively correlated with subjective health satisfaction, but significant differences existed between East Asians and Europeans. As a vital resource for East Asian populations, the CKB frequently collaborates with BBJ and is widely utilized to validate the cross-ethnic generalizability of European findings, optimize MR methods for non-European populations, and support genetic research, drug target development, and precision public health decision-making.

1.4.4 | Limitations of Statistical Methodologies

As previously discussed, a series of statistical methodologies have been developed and applied to address challenges such as IV validity, complex genetic architectures, and data limitations. However, each method has its limitations, with the validity of its

findings predicated upon specific, idealized assumptions. MR-Egger regression [188] detects directional horizontal pleiotropy by incorporating an intercept term. However, it is more sensitive to weak instruments and typically exhibits relatively low statistical power, which can lead to false negative results. Its estimates are also susceptible to outliers. The weighted median method [189] requires that at least 50% of the IVs are valid, an assumption that is untestable in practice. Moreover, this method cannot fully correct biases introduced by invalid IVs. MR-PRESSO [190] can identify and exclude outliers related to horizontal pleiotropy, but its test power depends on the magnitude of differences between outlier effects and other IV effects. Furthermore, the exclusion process may introduce model selection bias. More importantly, these methods implicitly assume that the causal effect of exposure on the outcome is homogeneous across all individuals [191]. In reality, however, causal effects are likely heterogeneous due to factors such as age, sex, environment, or genetic background. Standard MR methods cannot capture this heterogeneity, potentially obscuring important subgroup-specific causal effects of exposure. Particularly in dynamic causal analysis, because MR results reflect the effects of changes in genetically determined "exposure-specific susceptibility" rather than direct causality at a specific point in time, estimates may vary significantly across different time points [192]; at the same time, polygenic effects and confounding are further exacerbated in dynamic settings, with associated polygenic effects, population stratification, and heritable confounders more likely to lead to false positives or attenuated effects [170]; moreover, traditional IVs struggle to capture the temporal dynamics of exposure, and the trade-off between statistical power and model complexity is particularly pronounced in high-dimensional dynamic analyses [193]. Furthermore, the distinction between vertical and horizontal pleiotropy in MR analysis relies on biological evidence, and the absence of unified statistical diagnostic criteria may lead to misclassification of certain pleiotropic effects. Functional genomic data should be integrated to assist in validation. Given these methodological limitations, current practice often employs a "multiple methods triangulation" strategy to enhance the robustness of conclusions. Significant discrepancies between different methods can reveal potential pleiotropy or other biases, prompting further investigation. Targeted strategies have also emerged to address these dynamic challenges; for example, the FLOW-MR [160] framework uses spiking–smoothing priors to mitigate the weak instrumental variable problem and estimate time-series-path-specific effects, whereas MR-DEG [194] integrates differentially expressed genes to identify time-dependent effects. Meanwhile, the rapid development of methods such as MVMR, stratified MR, and MR-G × E interaction analysis is progressively enabling more accurate and robust causal inference.

1.4.5 | *Selection and Collider Bias*

Large biological sample repositories are widely used in GWAS and MR studies. However, an individual's selective participation, sustained engagement, or attrition often correlates with health status, socioeconomic factors, and other variables. This can introduce selection bias [195, 196], leading to biased causal effect estimates and increased Type I error rates. It also creates spurious associations between IVs and confounding factors, undermining the validity of IVs. This is particularly pronounced in studies of behavioral traits, where notable participation biases in databases such as the UKB significantly distort genetic associations and MR outcomes related to education, smoking, and other factors [197]. Single-sample MR (SSMR) analyses are particularly vulnerable to selection bias [198] due to the lack of independence between estimated gene-exposure and gene-outcome associations. Collider bias [199] arises when conditioning on a common effect of two variables induces spurious statistical associations between them, thereby distorting causal estimates through non-causal pathways. At the diagnostic level, the presence and magnitude of these biases can be jointly assessed through the following methods: First, causal graph visualization employs directed acyclic graphs to clearly depict causal relationships among variables, enabling precise identification of collider variables and potential non-causal pathways; second, statistical testing methodologies, such as MR-Egger regression, Cochran's Q test, and the I^2 statistic, verify the validity of the IV assumptions and indirectly indicate potential bias signals; and third, sensitivity analyses, including negative control analysis, effect size simulation, and validation in sample subsets, assess the robustness of results against bias assumptions. In practical applications, hierarchical study designs [200] can reduce selection bias: Constructing a "residual collider" layer and estimating causal effects stratified by residual quantiles avoids bias caused by direct stratification [199]. Alternatively, the MR-horse method [201] within a Bayesian framework enables valid causal inference under generalized settings where IV assumptions are violated. Dual-ranking MR (DRMR) and residual-stratified MR can also identify nonlinear causal relationships, mitigating bias risks from inappropriate conditioning to some extent [200]. Leveraging the multi-phenotype data advantage of large biological cohorts, combined with life-course MR analysis of age-dependent effects [202], can further reduce selection bias interference at the data dimension.

1.4.6 | *Benchmark Validation and Comparison of MR Methods*

This review covers multiple core challenges and approaches in MR, yet has not systematically explored comparative evaluations of mainstream MR methods under conditions approximating real-world scenarios. Core MR challenges often coexist in analyses, making large-scale benchmark validation studies crucial for systematically assessing and enhancing method reliability. Such studies can quantitatively evaluate the comprehensive performance of different methods by comparing simulated and real-world data [115]. An MR study [192] on time-varying exposure factors validated the validity of MR estimation results under the "exposure-latent liability" framework through a combination of simulation experiments and empirical analysis, providing a representative reference for method interpretation and validation in specific scenarios. In recent years, benchmark validation of MR methods has yielded a series of outcomes. Hu et al.'s study [115] stands as a representative work in recent MR method benchmarking. By integrating large-scale simulated data with real GWAS meta-analysis summary statistics, it comprehensively evaluated the performance of 16 mainstream MR methods. Through multi-gradient IV threshold screening, it delineated the strengths, limitations, and applicability boundaries of different methods across complex scenarios and four core dimensions: type I error

control, accuracy of causal effect estimation, replicability, and statistical power. Cui et al. [203] combined transcriptome-level MR with single-cell analysis, integrating dynamic eQTL data, SMR, and colocalization analysis to investigate causal associations between gene expression during $CD4^+$ T cell activation and colorectal cancer. They successfully identified 28 potential immunotherapy targets, providing a practical case study for applying and validating MR methods in complex real-world scenarios involving cell specificity and dynamic exposure. As GWAS datasets expand, MR applications have extended to multi-trait causal network analysis [204]. However, large-scale data may amplify errors from weak instrumental variable bias or genetic correlations [205, 206]. Benchmark validation guides parameter optimization for new methods in large samples [207]. Large-scale benchmark studies are crucial for ensuring the reliability of MR methods and advancing their translation from theory to clinical practice, particularly requiring attention to hypothesis relaxation methodologies under complex genetic structures [23, 115, 208] and cross-study consistency validation [209].

1.5 | Conclusion and Outlook

This review systematically outlines the core principles, methodological advancements, and applications of MR methods in the biomedical field. Since Katan's pioneering proposal in 1986 to use genetic variants for causal inference, MR has evolved considerably, spurred by both the formalization of its framework by George Davey Smith and Ebrahim in 2003 and the subsequent expansion of GWAS summary statistics resources. MR technology has evolved from single-instrument MR analysis into a diversified methodological system encompassing SSMR, TSMR, MVMR, BiMR, NLMR, MMR, and multi-population MR. These methodological advancements have significantly expanded MR's capacity to address complex scientific questions, thereby linking genetics, epidemiology, and clinical medicine. In practice [121, 192, 210], MR is now applied across numerous frontiers, including G × E interaction research, the etiology of complex diseases, drug target validation, and precision public health. By integrating multi-omics data with large-scale biological databases, MR research has uncovered causal chains linking genetic variation to molecular phenotypes and clinical outcomes, providing genetic evidence to understand disease mechanisms and develop novel therapeutic interventions. It is worth noting that although the large-scale data era presents growth opportunities for MR, it also poses challenges stemming from methodological misuse [211]. With the public availability of GWAS summary statistics, MR analyses based on pooled two-sample GWAS summary statistics have become highly accessible. Simplified methodologies [121], disregard for assumptions [192], and data misuse have led to a proliferation of low-quality, rigor-deficient studies, threatening the overall credibility of MR [210]. Researchers should adopt rigorous study designs. Enhancing methodological training, implementing multidimensional validation, and embracing open science practices can improve the reliability of MR causal inferences. Concurrently, journal editors and reviewers must develop the ability to identify low-quality submissions. They should focus on the validity of research questions, the rationale for IV selection, robustness testing of results, appropriate interpretation, and dialogue with existing literature, exercising caution with every manuscript.

The validity and reliability of MR methodologies are highly dependent on the IVs satisfying three core assumptions [212]: relevance, independence, and exclusion restriction. In practice, causal inference can be biased by issues such as weak instruments [213], horizontal pleiotropy, population stratification [214], and data quality limitations. To address these challenges, researchers have developed multi-level sensitivity analysis methodologies—including MR-Egger regression, weighted median method, and MR-PRESSO—to assess and correct potential biases. Additionally, techniques such as cross-ancestry validation and negative control analysis [215] enhance the robustness of findings.

Future research still faces several key challenges [216]: Developing more robust statistical methodologies to identify and mitigate pleiotropy, particularly in cross-population settings; expanding genetic data resources for understudied non-European populations through multi-ancestry MR analyses to improve the generalizability of findings; and promoting the deep integration of MR with multi-omics and time-series data to develop next-generation analytical methodologies capable of resolving dynamic causal relationships. Despite these challenges, MR holds considerable promise for drug target discovery, precision prevention, and elucidating disease etiology [59, 95]. With the accumulation of proteomic, metabolomic, and single-cell sequencing data, MR is advancing in deciphering causal temporal sequences within biological pathways and cell-type-specific effects. A recent study on primary sclerosing cholangitis [158] successfully identified seven core causal genes, including *MMEL1* and *FUT2*, by integrating genomic, transcriptomic, gut microbiome, and metabolomic data. Employing transcriptome-wide association study (TWAS), colocalization, and multidimensional MR analysis, the research systematically revealed key pathways through which gut microbiota mediate disease development via the gut–liver axis. This marks the gradual emergence of an MR framework integrating multi-omics and spatiotemporal dynamic data, facilitating the discovery and validation of precision medicine intervention targets.

With the continuous expansion of GWAS sample sizes, deep integration of multi-omics data, and ongoing innovation in statistical methodologies, MR will play a pivotal role in deciphering the causal architecture of complex traits, identifying actionable targets, and advancing precision medicine. As an indirect inference method based on genetic proxies, MR conclusions must be corroborated by experimental studies, clinical trials, and other epidemiological evidence to collectively construct a causal evidence framework [217]. Only through rigorous methodological application and deepening multidisciplinary collaboration can MR truly realize its value in translational medicine and public health decision-making.

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Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

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