OXFORD

BIOLOGY Methods & Protocols

Biology Methods and Protocols, 2023, bpad024

https://doi.org/10.1093/biomethods/bpad024

Advance Access Publication Date: 17 October 2023

Review

Robustness of quantifying mediating effects of genetically regulated expression on complex traits with mediated expression score regression

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Abstract

Genetic association signals have been mostly found in noncoding regions through genome-wide association studies (GWAS), suggesting the roles of gene expression regulation in human diseases and traits. However, there has been limited success in colocalizing expression quantitative trait locus (eQTL) with disease-associated variants. Mediated expression score regression (MESC) is a recently proposed method to quantify the proportion of trait heritability mediated by genetically regulated gene expressions (GReX). Applications of MESC to GWAS results have yielded low estimation of mediated heritability for many traits. As MESC relies on stringent independence assumptions between *cis*-eQTL effects, gene effects, and nonmediated SNP effects, it may fail to characterize the true relationships between those effect sizes, which leads to biased results. Here, we consider the robustness of MESC to investigate whether the low fraction of mediated heritability inferred by MESC reflects biological reality for complex traits or is an underestimation caused by model misspecifications. Our results suggest that MESC may lead to biased estimates of mediated heritability with misspecification of gene annotations leading to underestimation, whereas misspecification of SNP annotations may lead to overestimation. Furthermore, errors in eQTL effect estimates may lead to underestimation of mediated heritability.

Keywords: eQTL; mediated heritability; genetically regulated expression; LDSC

Introduction

Recent years have seen many discoveries through genome-wide association studies (GWAS) [1], which aim to identify associations between genetic variants, particularly single-nucleotide polymorphisms (SNPs), and phenotypes. Despite these advances, a majority of trait-associated variants in GWAS lie in noncoding regions [2], making it challenging to interpret the roles of these variants and the underlying disease mechanisms. Because a GWAS variant in a noncoding region may affect disease via its regulation of gene expressions [3], integrating transcriptomics data may help us identify disease-associated genes and improve our understanding of disease mechanisms.

With the development of gene expression profiling technologies through microarrays and RNA-sequencing, expression quantitative trait locus (eQTL) studies have offered a valuable resource to link genetic variations with transcriptomes. For instance, the Genotype-Tissue Expression (GTEx) project [4, 5] has proved to be a valuable resource for interpreting GWAS results. Colocalization tests [6–8] have enabled scientists to identify genes with eQTLs colocalized with GWAS loci of many traits, suggesting the important roles of the genetic regulations of these genes in disease etiology. Transcriptome-wide association studies (TWAS) [9–11] "impute" the genetically regulated gene expression components (GReX) based on eQTL data and correlate the "imputed" GReX with phenotypes to gain insights into genetically regulated gene–trait associations.

Despite the great successes of using eQTL results to interpret GWAS signals, the relative contribution of genetically regulated expression to complex traits remains unclear and controversial. Although some studies have reported enrichment of disease heritability at eQTLs [12], others only found a small fraction of disease-associated loci colocalized to eQTLs [13, 14]. The lack of correspondence between GWAS and eQTL findings may be due to several factors. One possibility is that the eQTL effects are cell-type-specific [15] or context-specific [16], while most eQTL studies have been based on analyzing tissues, which only represent an average effect across cell types and contexts and may miss a large portion of eQTLs. Another explanation is the insufficient statistical power to detect disease-associated eQTLs due to the limited sample sizes of most eQTL studies [17]. However, it is not clear whether larger sample sizes can improve our understanding of the undiscovered regulatory associations [18]. Genetic regulation of complex traits can be driven by many processes beyond regulation of gene expression, such as the regulation of proteins, splicing, and DNA methylation, the effects of which can be studied through so-called xQTLs [19]. Therefore, it is important to quantify the extent to which GReX explain trait heritability to determine whether more and larger eQTL and eQTL-related

Received: May 10, 2023. **Revised:** October 8, 2023. **Accepted:** October 16, 2023. **Editorial decision:** October 9, 2023.

studies are worthwhile to understand the biological mechanisms underlying complex traits.

One method to quantify the trait heritability mediated by GReX is known as mediated expression score regression (MESC) [20], which performs multiple linear regression of GWAS summary statistics on linkage disequilibrium (LD) scores and expression scores (the weighted LD scores with respect to eQTL annotations) to estimate the nonmediated and mediated heritability. One advantage of MESC is that it can be applied when only GWAS and eQTL summary statistics are available. MESC has an extension that stratifies the regression across both gene and SNP categories, which offers two additional advantages: (1) it weakens the assumptions required for MESC, thereby improving the estimation accuracy, which will be discussed in detail in the "Results" section and (2) it enables enrichment analysis of certain gene or SNP sets to uncover biological mechanisms.

MESC has broad applications. It can quantify the proportion of the trait heritability mediated by GReX in preidentified gene categories, which identifies how much we can expect to learn about the biological mechanisms of complex traits through eQTL data. The application of MESC to different tissues in GTEx data and summary statistics of different traits found that, on average, only 11% of the trait heritability is mediated by *cis*-eQTLs [20]. Other studies also found a similarly low average fraction of mediated heritability [21–24]. MESC can also quantify the enrichment of mediated heritability in functional gene sets like enhancers, implying potential disease pathways [22, 24]. In addition to eQTL data, MESC has been applied to other QTL data, including splicing QTL (sQTL) [21, 23], RNA editing QTL (edQTL) [21], enhancer QTL (EeQTL) [22], and methylation QTL (mQTL) [24]. For example, a study found that a larger fraction of heritability of kidney diseases is mediated by methylation (30%–50%) than that by expression (10%–20%) [24], indicating that more information about complex traits may be gained with methylation data.

Despite its great potentials, MESC was developed under several stringent assumptions about the relationships between genetic variants, gene expression, and complex traits. In brief, it assumes that the eQTL effect sizes are uncorrelated with the nonmediated genetic effect sizes and the gene–trait effect sizes. It is of interest to investigate the impact of the violations of these assumptions on the accuracy of the mediated heritability estimation. In addition, it is also of great interest to understand how the prediction errors of the eQTL annotations, or equivalently, the prediction errors of the expression scores affect the estimated mediated heritability. Answering these two questions can help us better understand whether the low proportion of eQTL-mediated heritability for complex traits is due to their biological reality or due to model misspecifications in the applications of MESC. In this article, we will address these two questions through analytical and simulation studies and make recommendations for the applications and extensions of MESC.

Materials and methods

Overview of methods

We start with an overview of the MESC model. Suppose the sample size is N, the number of SNPs is M and the number of genes is G. The genetic regulation of the phenotype can be modeled as

$$\boldsymbol{y} = \boldsymbol{X}\boldsymbol{\gamma} + \boldsymbol{X}\boldsymbol{B}\boldsymbol{\alpha} + \boldsymbol{\epsilon}, \tag{1}$$

where $\boldsymbol{y} \in \mathbb{R}^N$ represents the standardized phenotype values, $\boldsymbol{X} \in \mathbb{R}^{N\times M}$ represents the standardized genotypes of the same individuals, $\boldsymbol{\gamma} \in \mathbb{R}^M$ is the nonmediated effect size vector of SNPs, $\boldsymbol{\alpha} \in \mathbb{R}^G$ is the effect size vector of genes, $\boldsymbol{B} \in \mathbb{R}^{M\times G}$ is the *cis*-eQTL effect size matrix and its (j,i)th element β_{ji} is the *cis*-eQTL effect size of SNP j on gene i, and $\boldsymbol{\epsilon} \in \mathbb{R}^N$ is noise. All effect sizes are treated as random effects with zero means. We further assume that $\boldsymbol{\alpha}, \boldsymbol{\gamma}, \boldsymbol{\epsilon}$ are mutually uncorrelated, and $\mathbb{E}(\beta_{ji}\beta_{ki}) = 0$ for $i = 1, \ldots, G$ and $j \neq k$, which means that $\mathbb{E}(\boldsymbol{B}\boldsymbol{B}^T)$ is a diagonal matrix.

Let $\boldsymbol{R} \in \mathbb{R}^{M\times M}$ represent the LD matrix and its (j,k)th element r_{jk} is the LD between SNP j and SNP k, i.e. $\boldsymbol{X} \sim \mathcal{N}(0, \boldsymbol{R})$. We can estimate the LD matrix from the study population as $\hat{\boldsymbol{R}} = \boldsymbol{X}^T\boldsymbol{X}/N$, where $\mathbb{E}\left[\hat{r}_{jk}^2\right] \approx r_{jk}^2 + 1/N + \mathcal{O}(1/N^2)$. (This approximation can be obtained via the Delta method [25].) Furthermore, we denote the GWAS χ^2 summary statistic of SNP k as $\chi_k^2 = N\hat{\omega}_k^2$, where $\hat{\omega}_k$ is the marginal least squares estimate of the total effect size of the SNP k. Given the LD estimates of the genotypes, $\hat{\boldsymbol{R}}$, and the *cis*-eQTL effect size matrix, $\boldsymbol{B}$, Equation (1) can be rewritten as

$$\mathbb{E}[\chi_k^2 \mid \boldsymbol{B}, \hat{\boldsymbol{R}}] = N\sum_{j=1}^{M}\mathrm{var}(\gamma_j)\hat{r}_{jk}^2 + N\sum_{i=1}^{G}\mathrm{var}(\alpha_i)\sum_{j=1}^{M}\beta_{ji}^2\hat{r}_{jk}^2 + 1. \tag{2}$$

Note that as each element of $\boldsymbol{B}$ is considered a random effect, a given $\boldsymbol{B}$ refers to given the variances of its elements. Under the assumption that the nonmediated effect sizes and the gene effect sizes are uncorrelated with the *cis*-eQTL effect sizes and LD, the mediated heritability is

$$h_{\mathrm{med}}^2 = \sum_{i=1}^{G}\sum_{j=1}^{M}\mathrm{var}(\alpha_i)\beta_{ji}^2,$$

and the nonmediated heritability is

$$h_{\mathrm{nmed}}^2 = \sum_{j=1}^{M}\mathrm{var}(\gamma_j).$$

Note that the term "mediated heritability" only refers to the trait heritability mediated by *cis*-eQTLs, as *trans*-eQTLs are not considered in MESC. Therefore, we may miss mediated heritability contributed by the *trans*-eQTLs.

This regression model (Equation 2) corresponds to a stratified MESC where each SNP or gene belongs to one and only one SNP or gene category. However, for real data, the number of predictors (the sum of the total number of SNPs and genes) could be much larger than the sample size, leading to a high-dimensional problem of estimating the regression coefficients (variances of the effect sizes) individually. For SNPs and genes, we have additional functional annotations to categorize them based on their specific functions. To address the high-dimensional problem, MESC assumes that the contributions to the variances of the effect sizes are the same for SNPs and genes in a certain annotated category and are also independent of the *cis*-eQTL effect sizes or LD. In the remaining part of this article, we will use the term "independence assumptions" to refer to these assumptions.

To be specific, suppose that we have C SNP categories and D gene categories, with $a_{j,c}$ denoting the annotation of SNP j to SNP category $\mathbb{C}\_c$ and $b_{i,d}$ denoting the annotation of gene i to gene category $\mathbb{D}\_d$. For binary annotations, $a_{j,c} = 1_{j\in\mathbb{C}\_c}$ and $b_{i,d} = 1_{i\in\mathbb{D}\_d}$. We need the following assumptions to estimate mediated heritability: (1) the per-SNP contribution to heritability with respect to one unit of the annotation is the same and denoted as τ_c; (2) the per-gene contribution with respect to

heritability to one unit of the annotation b_d is the same and denoted as π_d; and (3) the per-gene and per-SNP contributions are independent of each other. With these assumptions, the variances of the effect sizes are

$$\mathrm{var}(\gamma_j) = \sum_{c=1}^{C} a_{j,c}\tau_c,$$

$$\mathrm{var}(\alpha_i) = \sum_{d=1}^{D} b_{i,d}\pi_d.$$

Then, we have the following stratified MESC,

$$\mathbb{E}\left[\chi_k^2\right] = N\sum_{c=1}^{C}\tau_c l_{k,c} + N\sum_{d=1}^{D}\pi_d L_{k,d} + 1,$$

where

$$l_{k,c} = \sum_{j=1}^{M} a_{j,c} r_{j,k}^2$$

is the LD score of SNP k with respect to annotation a_c and

$$L_{k,d} = \sum_{i=1}^{G}\sum_{j=1}^{M} b_{i,d} r_{j,k}^2 \beta_{ji}^2$$

is the expression score of SNP k with respect to annotation b_d. The mediated heritability is

$$h_{\mathrm{med}}^2 = \sum_{d,i,j} b_{i,d}\pi_d \beta_{ji}^2,$$

and the nonmediated heritability is

$$h_{\mathrm{nmed}}^2 = \sum_{c,j} a_{j,c}\tau_c.$$

For unstratified MESC, there would be only one SNP and gene category containing all SNPs or genes, with stronger assumptions than the stratified version.

Datasets and simulation studies

In the "Results" section, we present some analytical results on the impact of the violation of independence assumptions and prediction errors in eQTL effect size estimation. To substantiate the analytical results, we conducted simulations to study potential biases of MESC.

We used imputed genotypes from the UK Biobank (UKBB v3) [26] restricted to HapMap 3 [27] SNPs on chromosome 1. We filtered out SNPs with missing call rates exceeding 5% or minor allele frequencies (MAFs) lower than 5%, resulting in $M = 84,202$ SNPs considered in simulations. To minimize population heterogeneity, we only used the UKBB European samples in our analysis.

We randomly sampled 1000 individuals as the reference samples to estimate the LD scores with annotations from the baselineLD model (v2.1) [28]. These functional annotations include coding, conserved, regulatory, and LD-related annotations. Like MESC method, we removed four categories related to QTL MaxCPP [29] as they were redundant to the *cis*-eQTL annotations for the expression scores. We used the LD Score Regression (LDSC) software [25] to calculate LD scores from summing LD with variants within a 1-cM window.

We randomly sampled another 1000 individuals and 1000 genes on chromosome 1 for the external expression panels. To generate the expression levels, we randomly designated five SNPs to be *cis*-eQTLs within 1 megabase (Mb) window around each gene, where one SNP had an effect size drawn from $\mathcal{N}(0,\ 0.8\ h_{\mathrm{cis,i}}^2)$ and the other four SNPs had effect sizes drawn from $\mathcal{N}(0,\ 0.05\ h_{\mathrm{cis,i}}^2)$. We then scaled these five random eQTL effect sizes to ensure that the sum of their squares was equal to the given value of $h_{\mathrm{cis,i}}^2$. The *cis*-heritability per gene had different structures in different simulation settings (see Table 1) with a mean of 10%. This *cis*-eQTL effect size setting followed the simulation setting of Yao *et al.* [20], which reflected the sparsity of *cis*-eQTLs after fine-mapping strategies, where top eQTLs have larger causal effects [4, 5]. The gene expression levels were then simulated using an additive model with normally distributed noise using the GCTA software [30]. The true expression scores were calculated using the LDSC software [25, 28, 31]; with annotations from true squared *cis*-eQTL effect sizes β_{ji}^2, while the estimated expression scores were estimated using the MESC software [20] with the individual-level genotype and expression level data from the expression panels.

We randomly sampled 10 000 individuals for GWAS analysis. The gene effect sizes and the nonmediated SNP effect sizes for all SNPs and genes were simulated with normal distributions. We then simulated the phenotypes using an additive model with normally distributed noise using the GCTA software [30]. We conducted association tests for each SNP to obtain GWAS summary statistics using PLINK (version 1.9-beta5.3) [32]. We then applied MESC on the external expression data and GWAS summary statistics to estimate the mediated heritabilities. The true total heritability, h_g^2, was fixed at 0.5 and the true mediated heritability, h_{med}^2, was varied in $\{0.1, 0.2, 0.3, 0.4\}$. We performed both unstratified and stratified MESC with both true and estimated expression scores using the MESC software [20]. All simulation settings were repeated 100 times.

We performed five sets of simulations, where the effect of gene i was drawn from $\mathcal{N}(0,\ \mathrm{var}(\alpha_i))$ and the nonmediated effect of SNP j was drawn from $\mathcal{N}(0,\ \mathrm{var}(\gamma_j))$. The variances of effect sizes were varied under different simulation settings (see Table 1).

Table 1. Summary table for the choices of the *cis*-heritabilities, $h_{\mathbf{cis,i}}^2$, the variances of the gene effect sizes, $\mathrm{var}(\boldsymbol{\alpha_i})$, and nonmediated SNP effect sizes, $\mathrm{var}(\boldsymbol{\gamma_j})$, in six simulation settings.

Setting	$h_{\mathbf{cis,i}}^2$	$\mathbf{var}(\boldsymbol{\alpha_i})$	$\mathbf{var}(\boldsymbol{\gamma_j})$
1	0.1	$\frac{h_{\mathrm{med}}^2}{G h_{\mathrm{cis,i}}^2}$	$\frac{h_{\mathrm{nmed}}^2}{M}$
2	$0.1G\frac{2^{i/200}}{\sum_k 2^{k/200}}$	$\frac{h_{\mathrm{med}}^2}{G h_{\mathrm{cis,i}}^2}$	$\frac{h_{\mathrm{nmed}}^2}{M}$
3	$\max\{0.7\mathrm{rexp}(7), 0.7\}$	$\frac{h_{\mathrm{med}}^2}{G h_{\mathrm{cis,i}}^2}$	$\frac{h_{\mathrm{nmed}}^2}{M}$
4	$0.1G\frac{2^{i/200}}{\sum_k 2^{k/200}}$	$\frac{h_{\mathrm{med}}^2 2^{-\frac{i}{200}}}{\sum_k h_{\mathrm{cis,k}}^2 2^{-\frac{k}{200}}}$	$\frac{h_{\mathrm{nmed}}^2}{M}$
5	$0.1G\frac{2^{i/200}}{\sum_k 2^{k/200}}$	$\frac{h_{\mathrm{med}}^2 2^{\left[\frac{5i-1}{G}\right]\frac{G}{50}\frac{i}{10}}}{\sum_k h_{\mathrm{cis,k}}^2 2^{\left[\frac{5k-1}{G}\right]\frac{G}{50}\frac{k}{10}}}$	$\frac{h_{\mathrm{nmed}}^2}{M}$
6	0.1	$\frac{h_{\mathrm{med}}^2}{G h_{\mathrm{cis,i}}^2}$	$\frac{h_{\mathrm{nmed}}^2}{2M} + \frac{h_{\mathrm{nmed}}^2 1_{j\in\mathrm{eQTL}}}{2\#\{\mathrm{eQTL}\}}$

Notations: The subscript i is the index for gene i and the subscript j is the index for SNP j. The sample size is N, the number of SNPs is M, and the number of genes is G. The mediated heritability is h_{med}^2 and the nonmediated heritability is h_{nmed}^2. The "rexp" represents a random sample from the exponential distribution, $\{t_1, t_2, \ldots, t_G\}$ is a random resample of $\{1, 2, \ldots, G\}$ without replacement, $[x]$ is a floor function, and $\#\{\mathrm{eQTL}\}$ represents the number of eQTLs.

In the first setting, all the independence assumptions were satisfied, where we expect that even unstratified MESC can obtain unbiased estimates of h^2_{med}. In the second setting, the *cis*-eQTL effect sizes were negatively correlated with the gene effect sizes, but after partitioning genes with similar *cis*-eQTL heritabilities into the same gene categories, the gene effect sizes were similar in the same category. This setting was used in Yao *et al.* [20] to illustrate that the stratified MESC can retain its unbiasedness even when the independence assumptions are violated. However, this setting did not violate the independence assumptions after stratifying the genes (see more details in the Results section). In the third setting, the *cis*-eQTL effect sizes were still negatively correlated with the gene effect sizes, but the *cis*-eQTL heritabilities were drawn from a different distribution to introduce randomness. This violated the independence assumptions, and we expect that even stratified MESC may lead to biased estimates of h^2_{med}. In the fourth setting, the *cis*-eQTL heritabilities were the same as the second setting while the variances of the gene effect sizes were randomly chosen, where the *cis*-eQTL heritabilities were uncorrelated with the gene effect sizes (cor = −0.049). In the fifth setting, the *cis*-eQTL effect sizes were negatively correlated with the gene effect sizes within each gene category, and the default stratification recommendation in MESC failed to group genes with similar gene effect sizes into the same categories, corresponding to a strong violation of the independence assumptions.

We used a negative correlation between *cis*-eQTL effect sizes and gene effect sizes in these settings, considering that evolutionarily constrained genes tend to have fewer eQTLs [5]. The violations observed in the third and fourth settings might represent more general scenarios where the independence assumptions fail. On the other hand, the correlation relationship in the fifth setting was a numerical example and might not be typical of real-world situations. However, it may serve the purpose of evaluating the performance of MESC under extremely challenging conditions.

In the sixth setting, the *cis*-eQTL effect sizes were positively correlated with the nonmediated SNP effect sizes and the correlation structure could not be characterized by the SNP category structure in the baselineLD model. We adopted a positive correlation between *cis*-eQTL effect sizes and nonmediated effect sizes, as genetically regulatory variants have been found to be highly implicated in biological activities of both transcriptome and proteome [33]. Across all the simulation settings, we compared the performance of MESC with true and predicted expression scores.

Results

Violation of the independence assumptions leads to biased estimates of mediated heritability

MESC requires strict independence assumptions for its accuracy. The violation of these assumptions may cause biased estimation. We present an overview of conditions where MESC is no longer consistent.

Since MESC is an extension of LDSC that models mediation by GReX, we first consider LDSC, which also requires a similarly strong assumption that all the SNP effect sizes have the same variances. However, it has been shown that LDSC leads to consistent estimators under model misspecification [34]. To be more specific, suppose the true contribution to the total heritability of SNP j is τ_j, which may be different across SNPs, we have

$$\mathbb{E}[\hat{h}^2] = M\mathbb{E}[\hat{\tau}] = \sum_j \tau_j = h^2.$$

This result can be extended to a binary annotation a_c in stratified S-LDSC. Suppose that the true contribution to heritability with one unit of the annotation a_c of SNP j is $\tau_{j,c}$, we have

$$\mathbb{E}[\hat{h}^2_c] = \sum_j a_{j,c}\mathbb{E}[\hat{\tau}_c] = |\mathbb{C}_c|\mathbb{E}[\hat{\tau}_c] = \sum_{j\in\mathbb{C}_c}\tau_{j,c} = h^2_c.$$

Essentially, if for all SNP categories, the equality

$$\sum_j a_{j,c}\frac{\sum_{k\in\mathbb{C}_c}\tau_{k,c}}{|\mathbb{C}_c|} = \sum_j a_{j,c}\tau_{j,c} \quad (3)$$

holds, S-LDSC will lead to consistent estimates even when SNPs in the same SNP category have different degrees of contributions to heritability. Equation (3) is a weakened assumption for S-LDSC compared with the independence assumption. Both independence assumptions (where $\tau_{j,c}$ are the same) and binary annotations (where $a_{j,c} = 1$ represents SNP j belongs to category $\mathbb{C}_c$) are special cases satisfying Equation (3).

However, if we have continuous annotations in S-LDSC, Equation (3) may not hold for all categories and the partitioned heritability may have biased estimates. Unfortunately, mediated heritability is a special case of heritability with continuous annotations, as

$$\hat{h}^2_{med,d} = \sum_{i=1}^{G} b_{i,d}\left(\sum_{j=1}^{M}\beta^2_{ji}\right)\hat{\pi}_d = \sum_{j=1}^{M}\left(\sum_{i=1}^{G} b_{i,d}\beta^2_{ji}\right)\hat{\pi}_d.$$

Even with binary annotations of genes, $\sum_{i=1}^{G} b_{i,d}\beta^2_{ji}$ are still continuous annotations of SNP j, which can potentially lead to a biased estimate of mediated heritability.

Prediction error in *cis*-eQTL effect sizes leads to underestimation of h^2_{med}

The derivation of the MESC model is conditioned on a given set of *cis*-eQTL effect sizes. However, the true *cis*-eQTL effect sizes are unknown for real data. MESC uses LASSO [35] to infer the causal *cis*-eQTL effect sizes and adjusts their scales by the restricted maximum likelihood (REML) [36] estimate of the *cis*-heritability for genes. Although the estimated *cis* heritability and the expression scores were reported to be accurate [20], the errors in prediction may affect the mediated heritability estimation. This corresponds to the problem of high-dimensional linear mixed model with measurement errors in the covariates having random effects. To the best of our knowledge, there has not been any study on this problem. However, we can qualitatively analyze the effects from the prediction error.

We denote the true *cis*-eQTL effect size matrix as $\boldsymbol{B}$ and the estimated one as $\hat{\boldsymbol{B}}$. Let $\boldsymbol{C} = \mathbb{E}\left(\hat{\boldsymbol{B}}^T\hat{\boldsymbol{B}}\right)^+\mathbb{E}\left(\hat{\boldsymbol{B}}^T\boldsymbol{B}\right)$ and $\boldsymbol{D} = \boldsymbol{B} - \hat{\boldsymbol{B}}\boldsymbol{C}$, where $\mathbb{E}\left(\hat{\boldsymbol{B}}^T\hat{\boldsymbol{B}}\right)^+$ represents the Moore–Penrose inverse of $\mathbb{E}\left(\hat{\boldsymbol{B}}^T\hat{\boldsymbol{B}}\right)$. Then

$$\begin{aligned}\mathbb{E}(\boldsymbol{D}^T\hat{\boldsymbol{B}}\boldsymbol{C}) &= \mathbb{E}\left(\boldsymbol{B}^T\hat{\boldsymbol{B}}\boldsymbol{C} - \boldsymbol{C}^T\hat{\boldsymbol{B}}^T\hat{\boldsymbol{B}}\boldsymbol{C}\right)\\ &= \mathbb{E}(\boldsymbol{B}^T\hat{\boldsymbol{B}})\mathbb{E}\left(\hat{\boldsymbol{B}}^T\hat{\boldsymbol{B}}\right)^+\mathbb{E}\left(\hat{\boldsymbol{B}}^T\boldsymbol{B}\right)\\ &\quad - \mathbb{E}(\boldsymbol{B}^T\hat{\boldsymbol{B}})\mathbb{E}\left(\hat{\boldsymbol{B}}^T\hat{\boldsymbol{B}}\right)^+\mathbb{E}\left(\hat{\boldsymbol{B}}^T\hat{\boldsymbol{B}}\right)\mathbb{E}\left(\hat{\boldsymbol{B}}^T\hat{\boldsymbol{B}}\right)^+\mathbb{E}\left(\hat{\boldsymbol{B}}^T\boldsymbol{B}\right)\\ &= \mathbb{E}(\boldsymbol{B}^T\hat{\boldsymbol{B}})\mathbb{E}\left(\hat{\boldsymbol{B}}^T\hat{\boldsymbol{B}}\right)^+\mathbb{E}\left(\hat{\boldsymbol{B}}^T\boldsymbol{B}\right) - \mathbb{E}(\boldsymbol{B}^T\hat{\boldsymbol{B}})\mathbb{E}\left(\hat{\boldsymbol{B}}^T\hat{\boldsymbol{B}}\right)^+\mathbb{E}\left(\hat{\boldsymbol{B}}^T\boldsymbol{B}\right) = 0_{G\times G}.\end{aligned}$$

Next, we denote $\boldsymbol{\gamma}' = \boldsymbol{\gamma} + \boldsymbol{D}\boldsymbol{\alpha}$ and $\boldsymbol{\alpha}' = \boldsymbol{C}\boldsymbol{\alpha}$, where $\boldsymbol{\gamma}'$, $\hat{\boldsymbol{B}}\boldsymbol{\alpha}'$, and $\boldsymbol{\epsilon}$ are also mutually uncorrelated. Notably, $\boldsymbol{\gamma}'$and $\hat{\boldsymbol{B}}\boldsymbol{\alpha}'$ are uncorrelated since

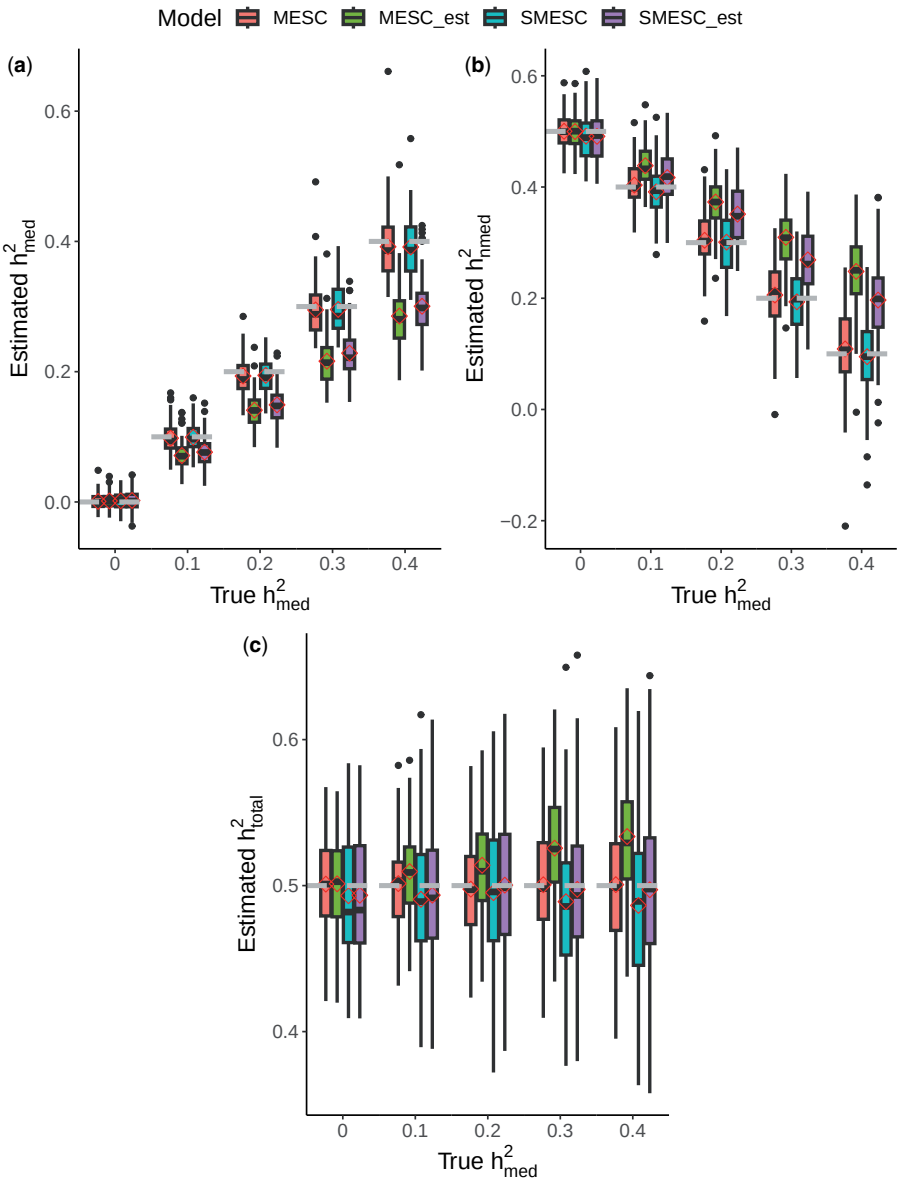

Figure 1. Comparisons of the estimation of (a) mediated heritability, (b) nonmediated heritability, and (c) total heritability between unstratified and stratified MESC with true and estimated expression scores under the simulation setting 1. The total heritability was fixed at 0.5, and the x-axis represents different simulation settings where the mediated heritability was varied from 0 to 0.4. The label "S" means stratified and the label "est" represents models using estimated expression scores. The dashed gray lines represent the true values and the red rhombuses represent the means of the estimates.

$$\mathbb{E}\Big((\boldsymbol{\gamma}+\boldsymbol{D\alpha})^T\hat{\boldsymbol{B}}(\boldsymbol{C\alpha})\Big) = \mathbb{E}\big(\boldsymbol{\gamma}^T\hat{\boldsymbol{B}}\boldsymbol{C\alpha}+\boldsymbol{\alpha}^T\boldsymbol{D}^T\hat{\boldsymbol{B}}\boldsymbol{C\alpha}\big) = 0_{G\times M}.$$

Thus, we can express the additive model of the phenotype based on $\boldsymbol{B}$ or $\hat{\boldsymbol{B}}$ as

$$\begin{aligned}\boldsymbol{y} &= \boldsymbol{X\gamma}+\boldsymbol{XB\alpha}+\boldsymbol{\epsilon}\\ &= \boldsymbol{X\gamma}+\boldsymbol{X}\big(\hat{\boldsymbol{B}}\boldsymbol{C}+\boldsymbol{D}\big)\boldsymbol{\alpha}+\boldsymbol{\epsilon}\\ &= \boldsymbol{X}(\boldsymbol{\gamma}+\boldsymbol{D\alpha})+\boldsymbol{X\hat{B}}(\boldsymbol{C\alpha})+\boldsymbol{\epsilon}\\ &= \boldsymbol{X\gamma}'+\boldsymbol{X\hat{B}\alpha}'+\boldsymbol{\epsilon}.\end{aligned}$$

Here, $\boldsymbol{\gamma}'$, $\boldsymbol{\alpha}'$, and $\boldsymbol{\epsilon}'$ can be regarded as the effect sizes given $\hat{\boldsymbol{B}}$ rather than $\boldsymbol{B}$. The total heritability of the phenotype, $\boldsymbol{y}$, is the ground truth and thus will not change due to the prediction error, which means

$$\mathrm{var}(\boldsymbol{X\gamma}+\boldsymbol{XB\alpha}) = \mathrm{var}(\boldsymbol{X\gamma}'+\boldsymbol{X\hat{B}\alpha}') = h^2,$$

i.e. the prediction error will only change the proportions of the mediated and nonmediated heritability among the total heritability. Therefore, the nonmediated heritability with the predicted expression scores will be overestimated:

$$\begin{aligned}\mathbb{E}\Big[\hat{h}^2_{\mathrm{nmed,est}}\Big] &= \mathrm{var}(\boldsymbol{X\gamma}')\\ &= \mathrm{var}(\boldsymbol{X\gamma}+\boldsymbol{XD\alpha})\\ &= \mathrm{var}(\boldsymbol{X\gamma})+\mathrm{var}(\boldsymbol{XD\alpha})\\ &\geq \mathrm{var}(\boldsymbol{X\gamma}) = h^2_{\mathrm{nmed,true}}\end{aligned} \tag{4}$$

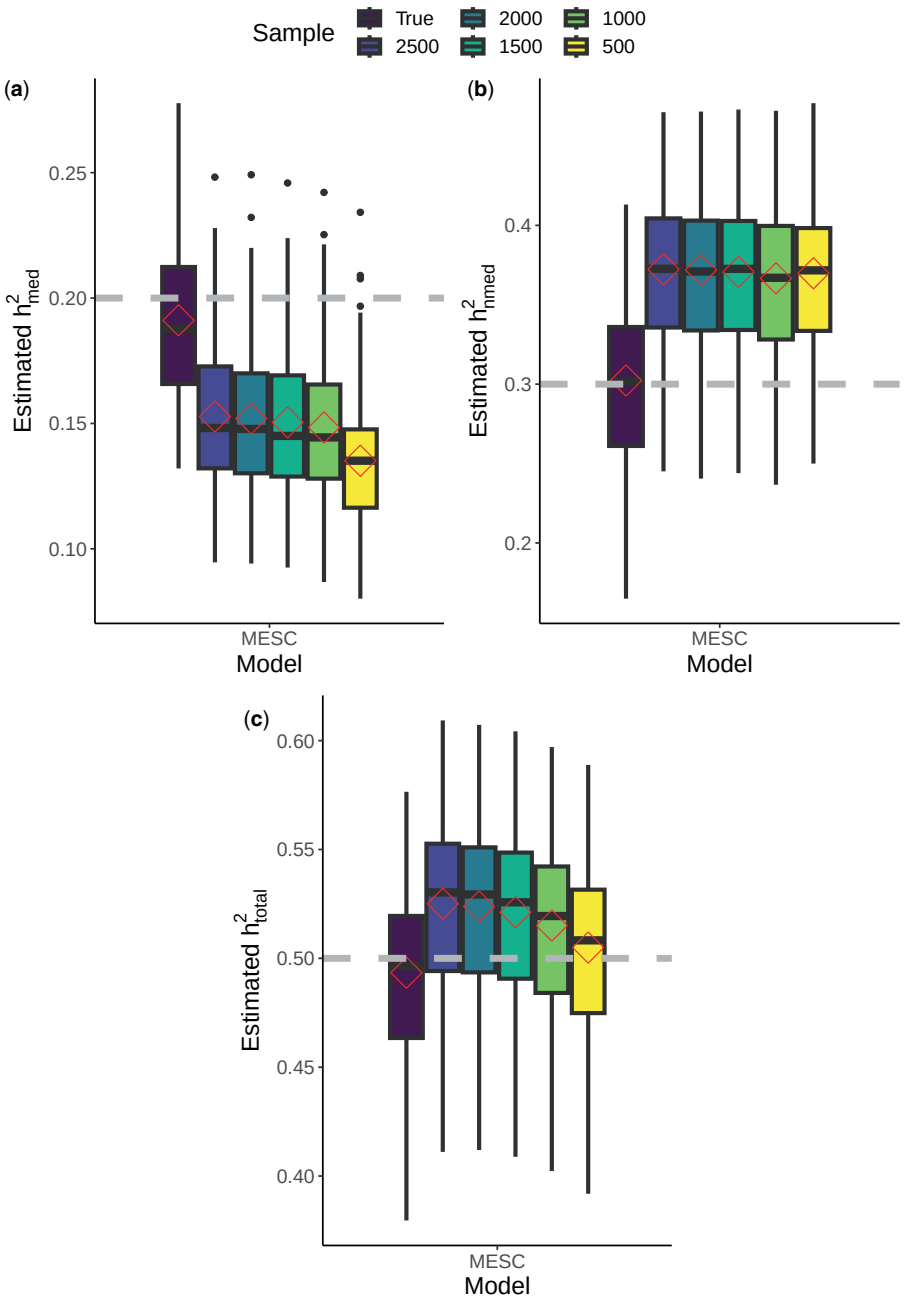

Figure 2. Comparisons of the estimation of (a) mediated heritability, (b) nonmediated heritability, and (c) total heritability between unstratified MESC with true and estimated expression scores with varied eQTL sample size under the simulation setting 1. The total heritability was fixed at 0.5 and the mediated heritability was fixed at 0.2. The numbers represent models using the estimated expression scores with corresponding eQTL sample sizes and "True" represents using the true expression scores. The dashed gray lines represent the true values and the red rhombuses represent the means of the estimates.

The third equality of Equation (4) holds because γ is uncorrelated with $\boldsymbol{D\alpha}$. The final inequality of Equation (4) is only strict in some special cases, for example, the gene–trait–effect sizes are zero (i.e. $\text{var}(\boldsymbol{\alpha}) = 0$) or the correlation between the true and predicted *cis*-eQTL effect sizes is one (i.e. $\boldsymbol{D} = 0_{M\times G}$).

Thus, if S-LDSC gives an unbiased estimator for the total heritability under certain conditions, i.e.

$$\mathbb{E}\left[\hat{h}^2_{\text{nmed,est}}\right] + \mathbb{E}\left[\hat{h}^2_{\text{med,est}}\right] = h^2 = h^2_{\text{nmed,true}} + h^2_{\text{med,true}},$$

we can infer $\mathbb{E}\left[\hat{h}^2_{\text{med,est}}\right] \leq h^2_{\text{med,true}}$. That means the imperfect prediction will result in probable underestimation of the mediated heritability, even when the independence assumptions are satisfied. However, if S-LDSC overestimates the total heritability, we may not be able to know whether the mediated heritability is overestimated or underestimated.

Simulation results of evaluating MESC with estimated and true expression scores

We first evaluated the performance of MESC with estimated and true expression scores in the first simulation setting, where all the independence assumptions were satisfied (Fig. 1). We chose to present the comparison under this setting to avoid the potential confounding effects from the violation of the assumptions.

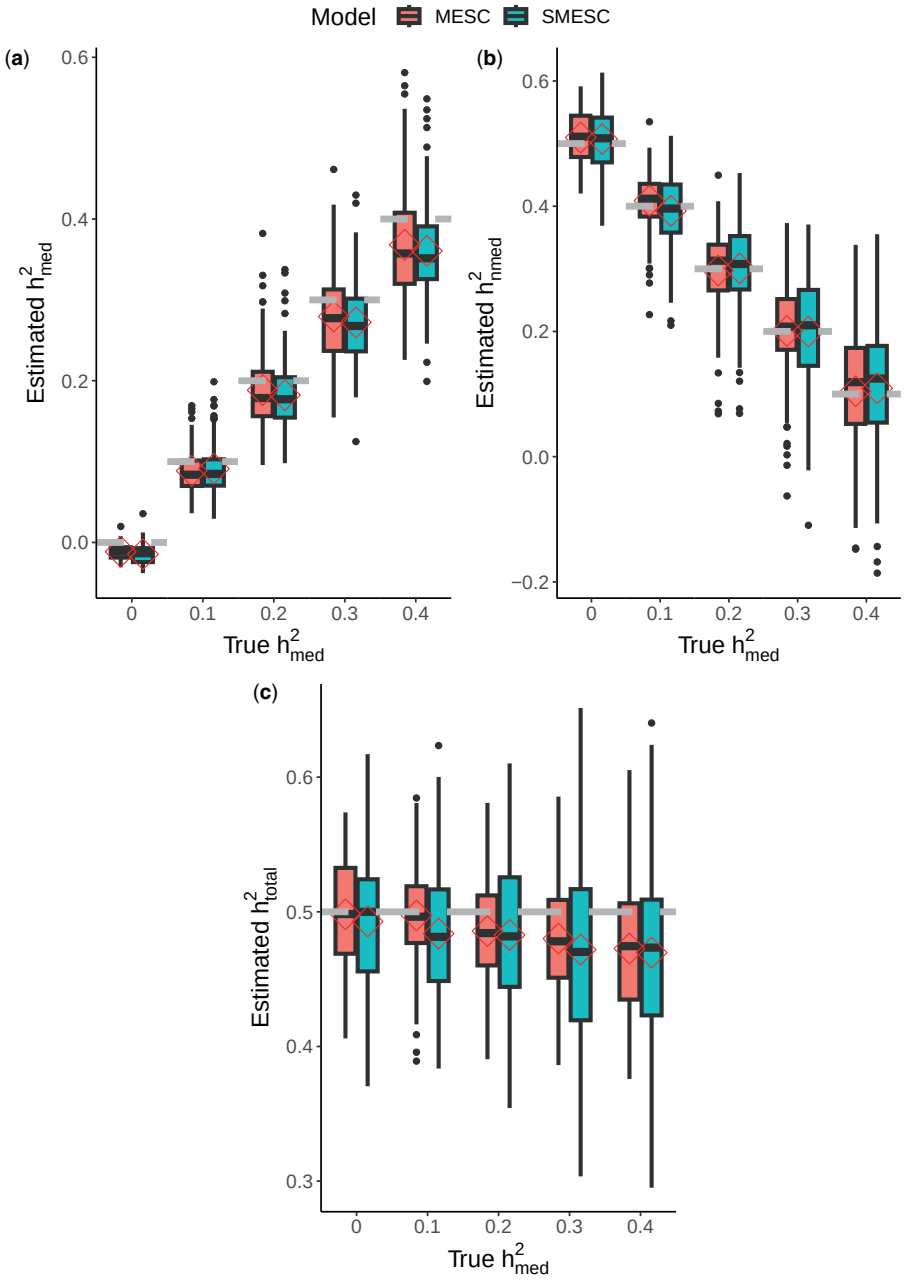

Figure 3. Comparisons of the estimation of (a) mediated heritability, (b) nonmediated heritability, and (c) total heritability between unstratified and stratified MESC with true expression scores under the simulation setting 2. The total heritability was fixed at 0.5, and the x-axis represents different simulation settings where the mediated heritability was varied from 0 to 0.4. The label "S" means stratified. The dashed gray lines represent the true values and the red rhombuses represent the means of the estimates.

We found that only when there were no mediating effects of GReX, MESC with both estimated and true expression scores could unbiasedly estimate $h^2_{med} = 0$. Otherwise, MESC with estimated expression scores underestimated h^2_{med}, while MESC with true expression scores is unbiased as expected. Correspondingly, MESC with estimated expression scores overestimated h^2_{nmed} when there was mediating effects of GReX. As for the total heritability, interestingly MESC with estimated expression scores also overestimated h^2 when there was mediating effects of GReX. This result showed that the overestimation of h^2_{nmed} outweighed the underestimation of h^2_{med}. There was no systematic difference between the results of using unstratified or stratified MESC, except for a slight inflation of the standard deviations of the estimators across simulations due to the inflation of the number of predictors in the regression. We also compared the performance of MESC with estimated and true expression scores in other simulation settings, and found that the estimated expression scores all led to underestimation of h^2_{med} and overestimation of h^2_{nmed} and h^2 in MESC compared to these with true expression scores, although the estimators of MESC with true expression scores might not be on target in these settings due to the violations of the independence assumptions (see Supplementary Figs S1–S4).

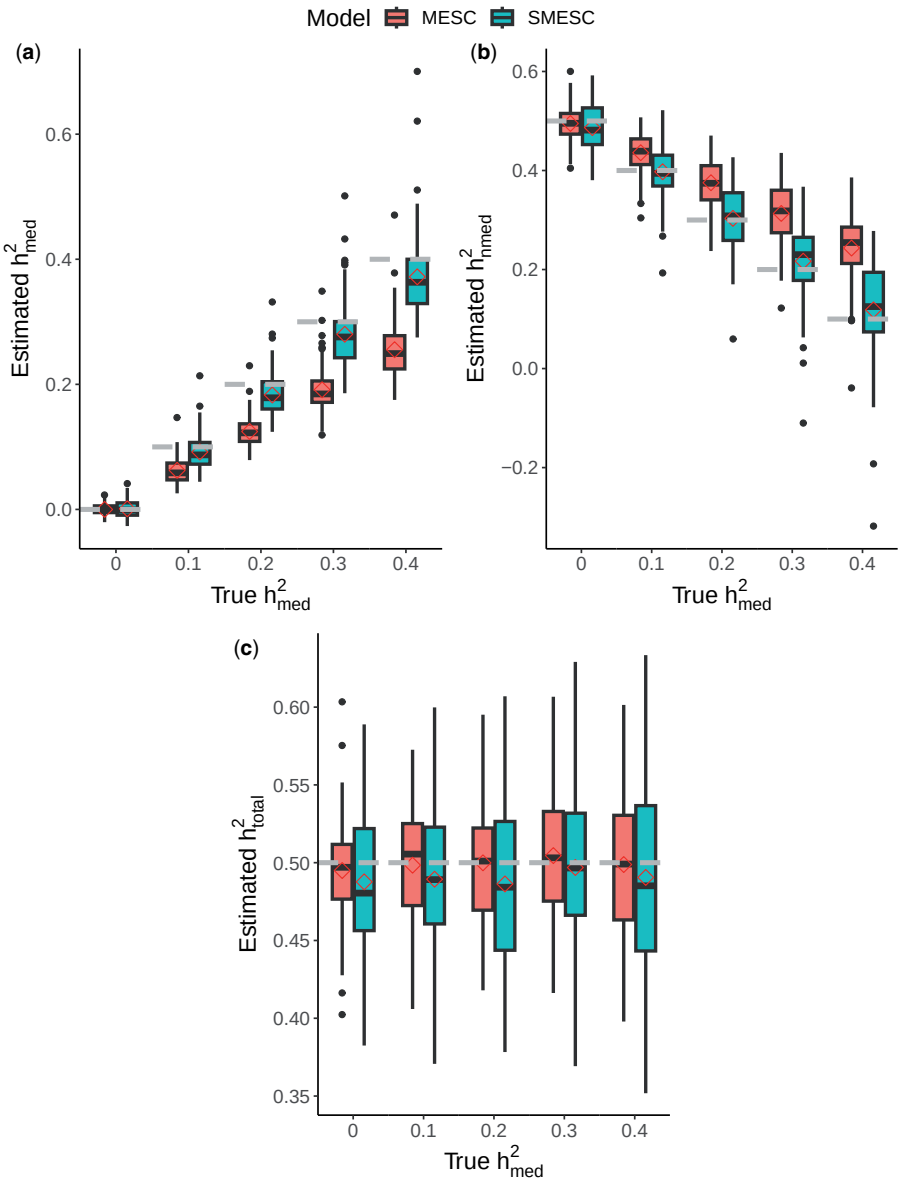

Figure 4. Comparisons of the estimation of (a) mediated heritability, (b) nonmediated heritability, and (c) total heritability between unstratified and stratified MESC with true expression scores under the simulation setting 3. The total heritability was fixed at 0.5, and the x-axis represents different simulation settings where the mediated heritability was varied from 0 to 0.4. The label "S" means stratified. The dashed gray lines represent the true values and the red rhombuses represent the means of the estimates.

Yao *et al.* [20] demonstrated that increasing the eQTL sample size could lead to improved expression score estimation. Therefore, we investigated how the eQTL sample size could affect the biases in MESC heritability estimation. We conducted a simulation study under setting 1, where we fixed $h^2_{med} = 0.2$ while varying the eQTL sample size. As shown in Fig. 2, as the eQTL sample size increased, the biases of the mediated heritability decreased. However, even with a relatively large sample size of 2500, there was still a noticeable bias in the heritability estimation. Interestingly, the biases of the nonmediated heritability did not exhibit significant changes as the eQTL sample size varied. This observation can be attributed to the fact that, even with varying sample sizes, the noise terms in gene expressions remained similar in the simulation setting, so the prediction errors as represented by the matrix $\boldsymbol{D}$ were similar for different sample sizes. As a result, the bias term of the nonmediated heritability, var($\boldsymbol{XD\alpha}$), in Equation (4) remained similar.

These simulation results are consistent with our analytical results that MESC leads to biased estimate of mediated heritability when we use the expression scores predicted from an external eQTL data set, even when all the modeling assumptions are satisfied. The magnitude of this impact is difficult to assess for real data as we do not know the true *cis*-eQTL effect sizes. Although MESC has compared several expression score prediction models through simulation studies to figure out the empirically best prediction model to mitigate the accuracy loss, we should still be cautious about the impact of the prediction errors to our estimation. As MESC is a special case of S-LDSC, the prediction error in the expression scores can lead to errors in *cis*-eQTL annotations. Therefore, the observed overestimation of the total heritability

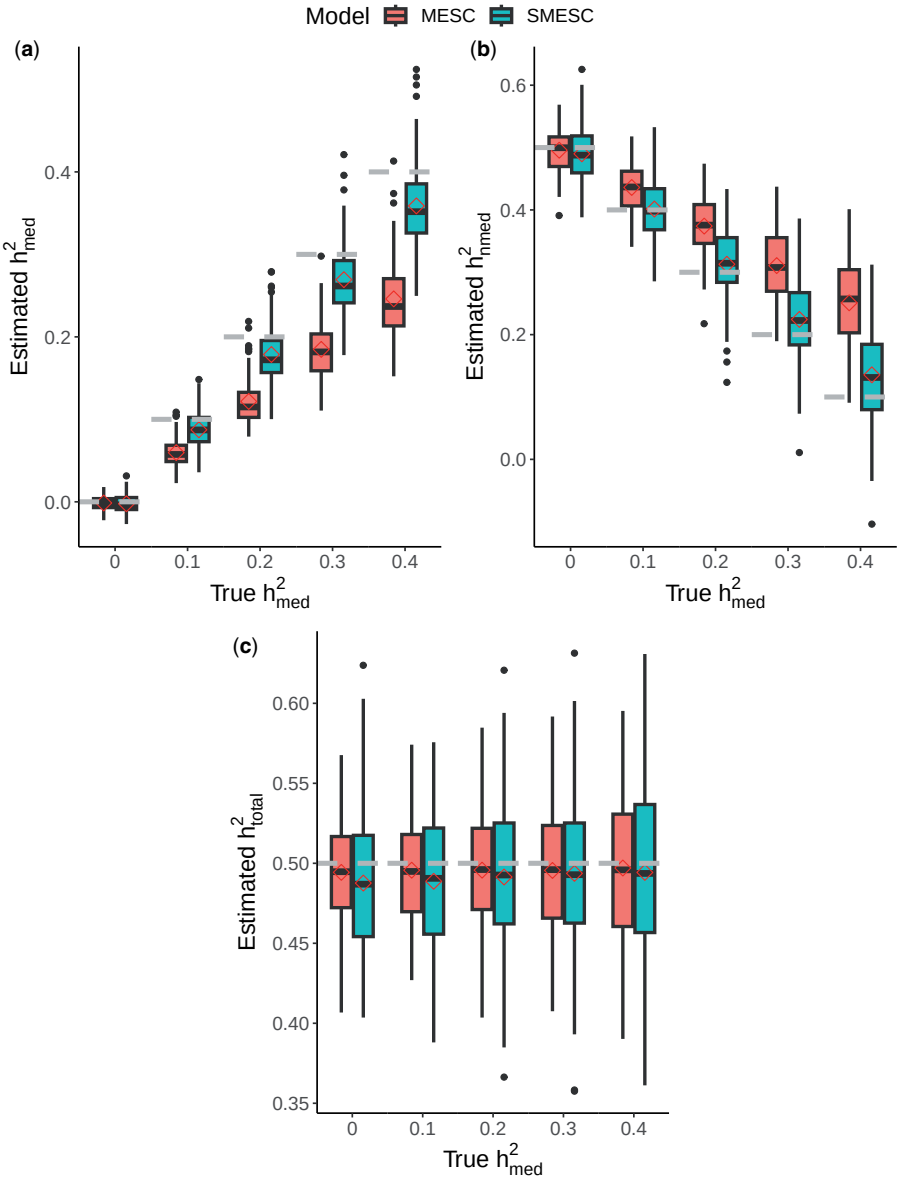

Figure 5. Comparisons of the estimation of (a) mediated heritability, (b) nonmediated heritability, and (c) total heritability between unstratified and stratified MESC with true and estimated expression scores under the simulation setting 4. The total heritability was fixed at 0.5, and the x-axis represents different simulation settings where the mediated heritability was varied from 0 to 0.4. The label "S" means stratified. The dashed gray lines represent the true values and the red rhombuses represent the means of the estimates.

may be the result of S-LDSC being biased due to inaccurate continuous annotations, whereas there is no such concern for LDSC without annotations [34].

Simulation results for MESC under violations of the independence assumptions

We next investigated the performance of MESC under different kinds of violations of the independence assumptions. We chose to only present the estimation results with true expression scores to avoid the confounding effects from the prediction errors in the following discussion.

In the first simulation setting where all independence assumptions were satisfied, we found both stratified and unstratified MESC hit the target of h^2_{med}, h^2_{nmed}, and h^2 (Fig. 1). The standard deviations of stratified MESC were larger than the unstratified ones because of a larger number of predictors, which is not related to any specific simulation setting and expected to persist in most other settings as well.

In the second simulation setting where the independence assumptions of *cis*-eQTL effect sizes and gene effect sizes were weakly violated, we found that unstratified MESC underestimated h^2_{med} and overestimated h^2_{nmed} in the presence of mediating effects, whereas the stratified version tended to be approximately unbiased (Fig. 3). Both versions had approximately unbiased estimation of the total heritability. This scenario was used in Yao *et al.* [20] to illustrate that stratified MESC can mitigate the accuracy loss due to the correlation between *cis*-eQTL effect sizes and gene effect sizes. However, this scenario only weakly violates the assumptions for two reasons: (1) the variances of the gene effects in each gene category did not vary much as they were set to be proportional to the reciprocal of the *cis*-heritability and the genes were categorized by their *cis*-heritability and (2) the values

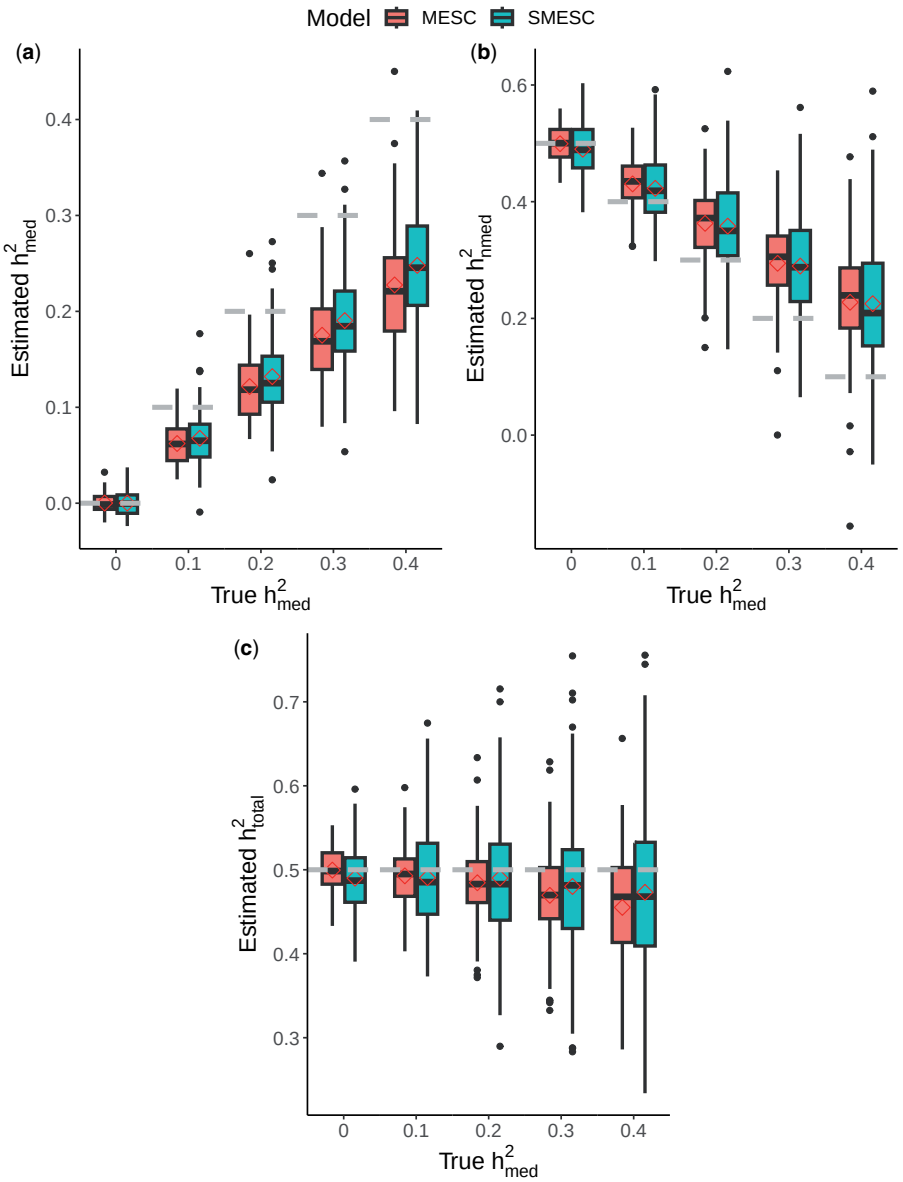

Figure 6. Comparisons of the estimation of (a) mediated heritability, (b) nonmediated heritability, and (c) total heritability between unstratified and stratified MESC with true expression scores under the simulation setting 5. The total heritability was fixed at 0.5, and the x-axis represents different simulation settings where the mediated heritability was varied from 0 to 0.4. The label "S" means stratified. The dashed gray lines represent the true values and the red rhombuses represent the means of the estimates.

of the *cis*-heritability and the gene effect variances happened to meet Equation (3), where the mean of h^2_{cis} times the mean of var(α) approximates the mean of the product of h^2_{cis} and var(α) in each gene category. However, we may not have this equality in real data, which means that the good performance of stratified MESC under this specific setting may not translate to robust performance for real data.

Our third simulation setting considered some mild violations of the independence assumptions of *cis*-eQTL effect sizes and gene effect sizes, where we found that both stratified and unstratified MESC underestimated h^2_{med} and overestimated h^2_{nmed} in the presence of mediating effects, while they both had an approximately unbiased estimation of the total heritability (Fig. 4). Unstratified MESC performed worse in partitioning the mediated and nonmediated heritability than stratified MESC. The biases were caused by the negative correlation between the *cis*-eQTL effect sizes and gene effect sizes, and the equality in Equation (3) no longer holds. However, after categorizing genes by their *cis*-heritability, stratified MESC had the same advantage over unstratified MESC as in the second setting that the variances of the gene effects in each gene category did not vary too much, so the stratification led to better (but still biased) performance.

In the fourth simulation setting, even the *cis*-eQTL effect sizes and gene effect sizes were uncorrelated (cor = −0.049), they did not satisfy Equation (3). We found that both stratified and unstratified MESC underestimated h^2_{med} and h^2 in the presence of mediating effects, while they only exhibited slight overestimation of the h^2_{nmed} (Fig. 5). These results suggest that solely relying on the uncorrelation assumption might not be sufficient for ensuring the robustness of MESC. Additional assumptions, such as those outlined in Equation (3), need to be identified.

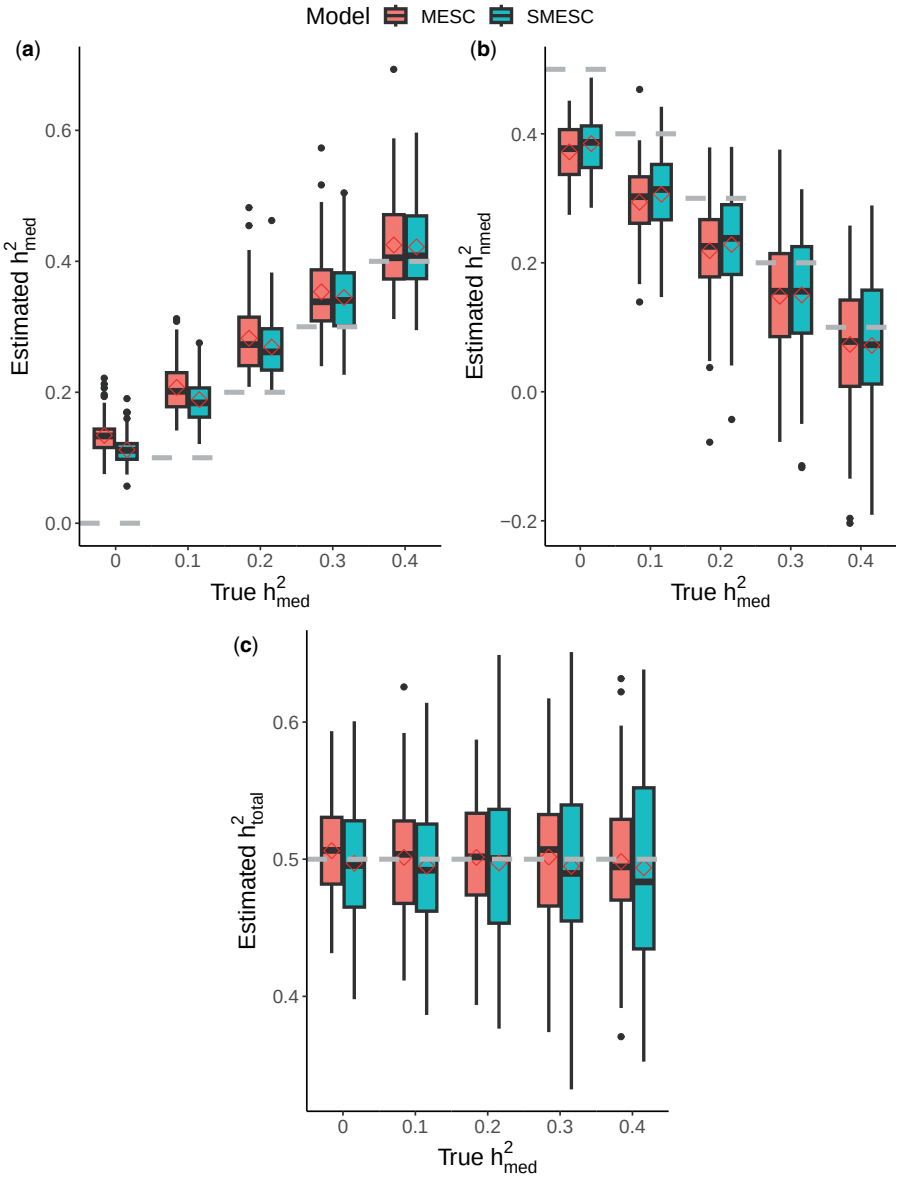

Figure 7. Comparisons of the estimation of (a) mediated heritability, (b) nonmediated heritability, and (c) total heritability between unstratified and stratified MESC with true expression scores under the simulation setting 6. The total heritability was fixed at 0.5, and the x-axis represents different simulation settings where the mediated heritability was varied from 0 to 0.4. The label "S" means stratified. The dashed gray lines represent the true values and the red rhombuses represent the means of the estimates.

Table 2. Summary table for the simulation results of the accuracy of MESC and stratified MESC (S-MESC) under six kinds of assumptions violations.

	MESC			S-MESC		
Violations	h^2_{med}	h^2_{nmed}	h^2	h^2_{med}	h^2_{nmed}	h^2
None	=	=	=	=	=	=
Negative correlation between $h^2_{cis,i}$ and var(α_i) but Equation (3) holds	↓	↑	=	=	=	=
Negative correlation between $h^2_{cis,i}$ and var(α_i) but similar var(α_i) in the same gene category	↓	↑	=	↓	↑	=
$h^2_{cis,i}$ and var(α_i) uncorrelated but varied var(α_i) in the same gene category	↓	↑	↓	↓	↑	↓
Negative correlation between $h^2_{cis,i}$ and var(α_i) in each gene category	↓	↑	↓	↓	↑	↓
Positive correlation between $h^2_{cis,i}$ and var(γ_j)	↑	↓	=	↑	↓	=

Notation: "=" represents unbiased, "↑" represents overestimated, and "↓" represents underestimated. $h^2_{cis,i}$: *cis*-heritabilities; var(α_i): the variances of the gene effect sizes; var(γ_j): nonmediated SNP effect sizes. The subscript i is the index for gene i and the subscript j is the index for SNP j. h^2_{med}: the mediated heritability; h^2_{nmed}: the nonmediated heritability; and h^2: the total heritability.

Table 3. Summary table of 20 complex traits in this study.

Trait	Abbreviation	Sample size	PubMed ID
Attention-Deficit/Hyperactivity Disorder	ADHD	53,293	30478444 [44]
Anorexia Nervosa	AN	72,517	31308545 [45]
Autism Spectrum Disorder	ASD	46,351	30804558 [46]
Asthma	Asthma	385,822	31427789 [47]
Anxiety Disorder	AXD	31,880	31116379 [48]
Breast Cancer	BC	247,173	32424353 [49]
Body Mass Index	BMI	806,834	30239722 [50]
Cognitive Performance	CP	257,828	30038396 [51]
Crohn's Disease	Crohn	40,266	28067908 [52]
Educational Attainment	EA	765,283	35361970 [53]
Height	Height	385,748	31427789 [47]
Heart Rate	HR	361,411	31427789 [47]
Hypertension	Hypertension	298,307	31427789 [47]
Inflammatory Bowel Disease	IBD	59,957	28067908 [52]
Lung Cancer	LC	85,716	28604730 [54]
Major Depressive Disorder	MDD	500,199	30718901 [55]
Myasthenia Gravis	Mg	51,453	Nealelab [56]
Schizophrenia	SCZ	130,644	35396580 [57]
Type 2 Diabetes	T2D	933,970	35551307 [58]
Ulcerative Colitis	UC	45,975	28067908 [52]

In the fifth simulation setting, the independence assumptions of *cis*-eQTL effect sizes and gene effect sizes were strongly violated. We found that both stratified and unstratified MESC underestimated h^2_{med} and overestimated h^2_{nmed} in the presence of mediating effects (Fig. 6). Different from the third setting, they both underestimated h^2 as well. Although unstratified MESC still performed worse than stratified MESC, we did not see substantial difference between them as in the third setting, because the gene categories did not capture the underlying structure of the gene effects, so it failed to mitigate the differences of the variances of the gene effects. Additionally, from the fourth and fifth simulations, we note that the model misspecification of the SNPs effects with continuous annotations may compromise the consistency of S-LDSC.

In the sixth simulation setting where the independence assumptions of *cis*-eQTL effect sizes and nonmediated SNP effect sizes were violated, we found that both stratified and unstratified MESC overestimated h^2_{med} and underestimated h^2_{nmed}, although stratified MESC seemingly performed better than unstratified MESC (Fig. 7). They had an approximately unbiased estimation of the total heritability. There were biases because the SNP categories from the baselineLD model did not have a SNP category of all *cis*-eQTLs, thus failing to capture the underlying structure of the nonmediated SNP effects. In fact, Yao *et al.* [20] considered a scenario where only SNPs in the coding regions had nonmediated SNP effect sizes to illustrate that stratified MESC could mitigate the accuracy loss due to the correlation between *cis*-eQTL effect sizes and nonmediated SNP effect sizes. However, there was no violation of independence assumptions, as the baselineLD model correctly annotates the coding regions so that the nonmediated SNP effect sizes were independent of *cis*-eQTL effect sizes in each SNP category.

In summary, we found that stratified MESC performed quite well when the SNP and gene categories satisfy the independence assumptions. However, as expected, violations of the independence assumptions lead to biased estimations (Table 2). Positive correlation between *cis*-eQTL effect sizes and nonmediated SNP effect sizes tends to cause overestimation of the mediated heritability, whereas negative correlation between *cis*-eQTL effect sizes and gene effect sizes tends to lead to underestimation. Moreover, when the independence assumptions are strongly violated, even the total heritability estimation may be biased. Overall, despite an increase in standard deviations, stratified MESC had lower biases than unstratified MESC under model misspecifications, making it a better choice when we do not know the underlying correlation structures.

Applying MESC on complex traits with functional annotations

We applied MESC to estimate the mediated heritability of 20 complex traits from publicly available GWAS summary statistics datasets (summarized in Table 3). Our objective was to investigate whether incorporating gene functional annotations could improve the default MESC results. In our analysis, we considered only autosomal SNPs with MAF >1% and excluded the major histocompatibility complex (MHC) block on chromosome 6 (30–31 Mb). The expression scores meta-analyzed over all tissues from GTEx v8 [5] were obtained from the MESC website (see the "Data availability" section). The default method in MESC stratifies the genes by their *cis*-heritability. In addition to this approach, we used five gene lists as functional annotations, which include: all coding genes, genes near significant GWAS peaks [37], genes essential in mice [38–40], genes essential in cultured cell lines [41], genes with any disease association in ClinVar [42] and FDA-approved drug targets [43] from the Macarthur laboratory GitHub page (see the "Data availability" section).

Our findings revealed that the incorporation of gene functional annotations yielded higher average estimates of the proportion of heritability mediated by *cis*-eQTL (mean = 18.7%) than the default method (mean = 16.3%) (Fig. 8). In addition, we observed a strong correlation between the estimates from both methods [cor = 0.982, 95% CI = (0.953–0.993)]. These results suggest that gene functional annotations might improve the performance of MESC. However, further investigations may be necessary to assess the impact of specific functional annotations and potential sources of bias in the results.

Discussion

We have performed a comprehensive investigation of how MESC is impacted by its strict independence assumptions and the imperfect prediction of expression scores through both analytical and simulation studies. We found that MESC yields unbiased

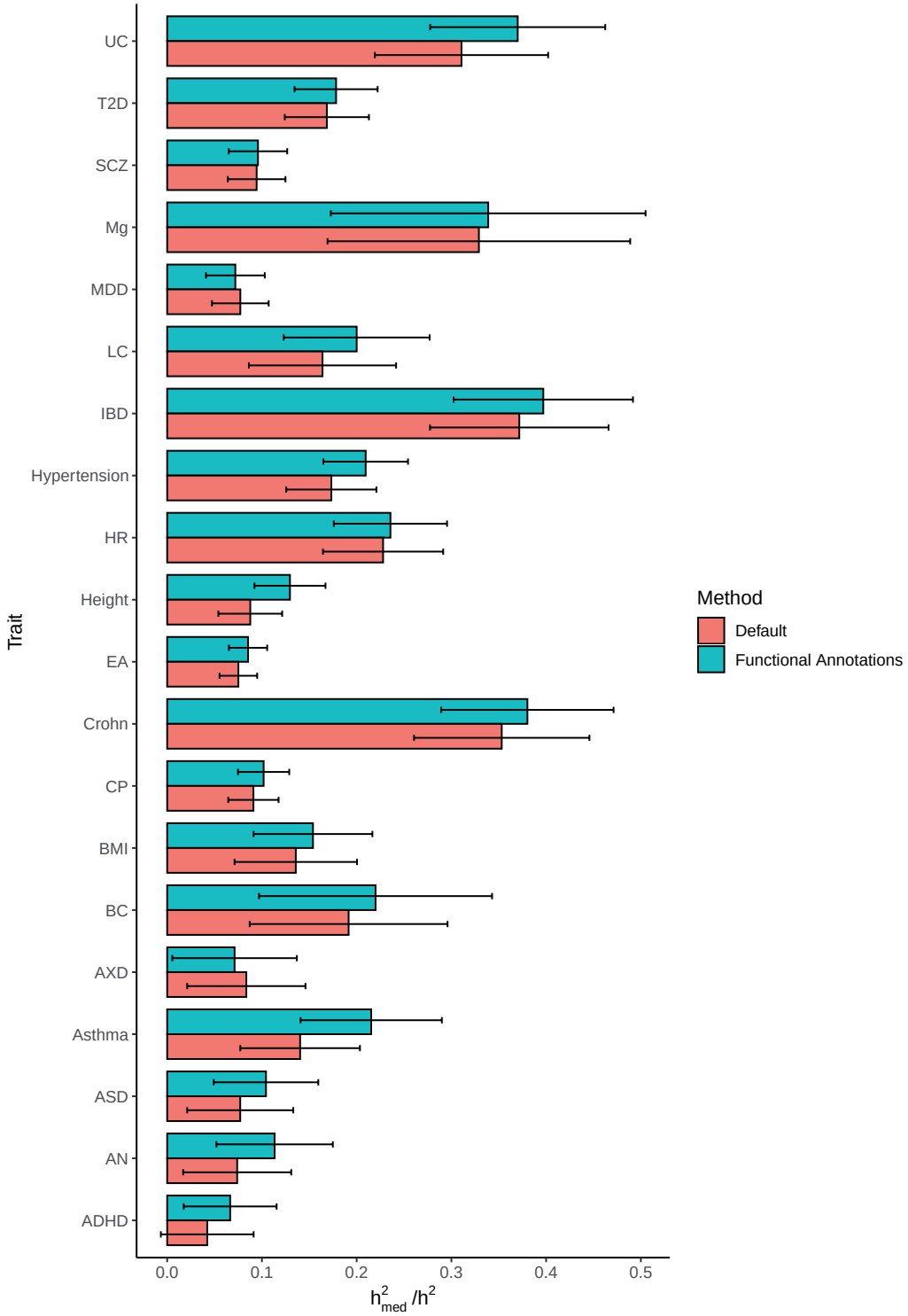

Figure 8. Comparison of the proportion of heritability mediated by *cis*-eQTL estimated by MESC using two different gene stratification methods. "Default" represents stratifying genes by their *cis*-heritability, while "Functional Annotation" represents additionally stratifying genes by functional annotations. Error bars represent jackknife standard errors.

estimates of mediated heritability when the assumptions are satisfied or weakly violated, where the gene effect sizes or the nonmediated SNP effect sizes are similar in each gene or SNP category and the prediction of the expression scores is accurate. However, if the independence assumptions are strongly violated, which means that the effect sizes varied much in some gene or SNP categories, MESC will no longer be unbiased. Thus, it is crucial to identify gene and SNP categories that likely satisfy the independence assumptions to the greatest extent before performing MESC. However, the gene effect sizes and the nonmediated SNP effect sizes underlying complex biological mechanisms are usually difficult to estimate. The gene effect sizes are based on GReX which are unobservable and the nonmediated SNP effect sizes are hard to separate from mediated SNP effect sizes in

real data. The large number of genes and SNPs also make it challenging to estimate the per-gene and per-SNP effect sizes. The choice of gene and SNP categories would therefore be highly based on the scientists' experiences. MESC provides a default choice of gene categories based on the *cis* expression heritabilities and SNP categories based on baselineLD model [28, 31]. The baselineLD model may be more promising because SNPs are partitioned by their functional annotations, although it is still unknown whether these annotations fit the independence assumptions. But the gene categories seem to be an *ad hoc* choice as there is no evidence showing that genes with similar *cis*-expression heritabilities will have similar effects on traits. The MESC software provides options for users to self-define the gene and SNP categories in addition to the default categories. We recommend that users carefully categorize genes or SNPs with similar effect sizes based on their functional annotations from the literature.

Even if the gene and SNP categories are carefully chosen, we may still misspecify the model for MESC. In the past, methods for quantifying total genetic heritability like GCTA [30] and LDSC [25] share similar assumptions that all SNP effect sizes have the same variances. Previous studies have established consistency and convergence properties for those two methods under weaker assumptions [34, 59]. Although we found that MESC would lose its consistency when the assumptions are violated, there are opportunities to find less strict conditions for its unbiasedness. For example, Equation (3) in the "Results" section could be such a condition, but it is computation-based and unverifiable. Further work is needed to understand the consistency of MESC.

Like most other eQTL methods, MESC highly relies on the accuracy of the gene expression data. However, the gene expression data are tissue- and context-specific, which means our estimation of the mediated heritability would only be tissue- or context-specific. We need to identify the causal tissues or contexts for complex traits before using MESC. Moreover, the expression level data may be contaminated [60], which further weakens the eQTL effect estimation results. Even if we have data collected from a well-designed eQTL study, the inevitable prediction errors of the expression scores likely lead to an underestimation of the mediated heritability. Therefore, we recommend the use of eQTL effect estimates from a large cohort to reduce prediction errors and increase the accuracy of mediated heritability estimation.

Finally, the terminology used by MESC may be confusing. Although the estimators are named as "mediated" and "nonmediated" heritabilities, only *cis*-eQTLs are used for the calculation of expression scores. To obtain statistically significant estimators for "*trans*-mediated" heritabilities, a very large eQTL study is needed, which is unavailable for current datasets [20]. As a result, the heritability mediated by GReX will be underestimated as the "*trans*-mediated" part is currently missing, and we recommend users to carefully interpret the results of mediated heritability.

Despite these challenges, MESC still has great potential to help scientists understand the role of gene expressions in the association between genetic variants and complex traits. It provides a framework with explicit modeling assumptions and efficient implementations and offers an opportunity to only use GWAS and eQTL summary statistics to infer how much the SNP effects are mediated through gene expression regulations. The definition of expression scores borrows ideas from "imputed" GReX [9], which enables distinguishing mediated effects from pleiotropic and linkage effects. If the genetic heritability mediated by *cis*-eQTLs is low, scientists may aim to collect data from other -omics platforms instead of putting more resources to eQTL studies to understand the molecular mechanism. It is also promising to find disease-relevant gene sets by the gene enrichment analysis based on their mediated heritability enrichment.

In future work, there are several possible extensions for MESC. First, as LDSC, which is used to estimate the total genetic heritability, can be extended to estimate the genetic correlation between complex traits, MESC can also be extended to estimate the genetic correlation mediated by GReX. Second, as some eQTL methods can be extended for other QTL data, MESC may be extended to analyze other omics data for the mediated effects quantification to uncover the biological processes of complex traits from other mechanisms.

Author contributions

C.L., W.L. and H.Z. conceived the study and designed the simulation settings. C.L. conducted simulation studies and real analysis. C.L. and W.J. did the analytical work. H.Z. advised on statistical and genetic analysis. C.L. wrote the manuscript. All authors contributed to manuscript editing and approved the manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

Funding

This work was supported in part by the National Institutes of Health [R01 GM134005] and the National Science Foundation grant [DMS1902903]. We conducted the research using the UKBB resource under approved data requests (access ref: 29900).

Data availability

The genotype data used for simulation studies are available via the UK Biobank data access process (see http://www.ukbiobank.ac.uk/register-apply). The eQTL data are available via GTEx data access (see https://www.gtexportal.org/home/datasets). The sources of GWAS summary statistics are summarized in Table 2. Gene sets can be found from the Macarthur laboratory (see https://github.com/macarthur-lab/gene_lists. The expression scores meta-analyzed over all tissues from GTEx v8 are available in MESC software website (see https://github.com/douglasyao/mesc/wiki/Download-expression-scores#gtex-v8-all-tissue-gene-sets). Analysis code is available at https://github.com/leaffur/Robustness-of-MESC.

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