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**Integrating causal inference and epigenome-wide association studies
identifies CpG sites as putative mediators for genetic influences on
cardiovascular disease risk**

Tom G. Richardson^{1*}, Jie Zheng¹, George Davey Smith¹, Nicholas J. Timpson¹, Tom
R. Gaunt¹, Caroline L. Relton¹, Gibran Hemani¹

¹ *MRC Integrative Epidemiology Unit (IEU), School of Social and Community Medicine, University of
Bristol, Oakfield House, Oakfield Grove, Bristol, BS8 2BN, United Kingdom*

*Corresponding author: Dr. Tom G. Richardson, MRC Integrative Epidemiology Unit, School of Social
and Community Medicine, University of Bristol, Oakfield House, Oakfield Grove, Bristol BS8 2BN,
UK. Tel: +44 (0)117 3313370; E-mail: Tom.G.Richardson@bristol.ac.uk

Abstract

The extent to which genetic influences on cardiovascular disease risk are mediated by changes in DNA methylation levels has not been systematically explored. We developed an analytical framework that integrates genetic fine mapping and Mendelian randomization with epigenome-wide association studies to evaluate the causal relationships between methylation levels and 14 cardiovascular disease traits.

We identified 10 genetic loci known to influence proximal DNA methylation which were also associated with cardiovascular traits ($P < 3.83 \times 10^{-08}$). Bivariate fine mapping identified strong evidence that the individual variants responsible for the observed effects on cardiovascular traits at the *ADCY3* and *ADIPOQ* loci were potentially mediated through changes in DNA methylation, whereas the other loci require further evaluation, though we highlight that we are unable to reliably separate causality from horizontal pleiotropy. Causal effect estimates ranged between 0.109-0.992 cardiovascular trait units per standard deviation change in DNA methylation and were replicated using results from large-scale consortia.

Genetic variants and CpG sites identified in this study were enriched for histone mark peaks in relevant tissue types and gene promoter regions. Integrating our results with expression quantitative trait loci data we provide evidence that variation at these regulatory regions is likely to also influence gene expression at these loci.

Word Count: 204

Introduction

Approximately 88% of trait-associated variants detected using Genome Wide Association Studies (GWAS) reside in non-coding regions of the genome, which suggests that they may be influencing mechanisms which act through gene regulation¹. Recent studies have incorporated data on genetic variants associated with gene expression (expression quantitative trait loci (eQTL)) into results from GWAS of complex traits to help identify the putative causal variant in a genomic region, as well as provide evidence suggesting which genes may be influenced by this variant²⁻⁵. This direction of inquiry can be extended to other ‘omic’ data types to gain further insights into the mechanistic pathway between genetic variant and causally associated trait. In this study, we introduce an alternative analytical framework to integrate genetic predictors of DNA methylation levels with complex traits to evaluate bi-directional causal relationships.

DNA methylation is an epigenetic regulation mechanism which has been shown to play a key role in many biological processes and disease susceptibility⁶⁻⁸. Recent studies have had success in identifying genetic variants associated with DNA methylation (methylation quantitative trait loci (mQTL)) and report that they appear to overlap with eQTL at a large number of loci across the genome^{9; 10}. This suggests that both DNA methylation and gene expression could reside along the causal pathway between genetic variation and disease, although thus far uncovering evidence of a mediated effect between mQTL and traits has been limited in contrast to using eQTL¹¹⁻¹⁴. Identifying epigenetic markers for disease risk should prove valuable in understanding the underlying biological mechanisms for trait-associated variants¹⁵. Indeed, the value of this approach was demonstrated in a recent study that applied the SMR² method to uncover pleiotropic effects between methylation levels and a range of complex traits¹⁶.

Mendelian randomization (MR) is a method by which genetic variants robustly associated with modifiable exposures can be used as instrumental variables to infer causality amongst correlated traits^{17; 18}. If DNA methylation resides along the causal pathway between genetic variant and trait, we would expect it to be correlated with our trait of interest. However, much like other traits analysed in epidemiological studies, DNA methylation is prone to confounding and reverse causation. Using an MR framework we can investigate whether DNA methylation has a causal relationship with

a phenotypic outcome, suggesting that it may reside along the causal pathway to disease¹⁹. Effects such as this can be referred to as ‘mediation’, as DNA methylation is mediating the effect from genetic variant to phenotype along the same biological pathway. As discussed in a recent review, MR has advantages over alternative approaches in mediation analysis (such as the Causal Inference Test²⁰), as it can detect the correct direction of effect in the presence of measurement error²¹. It is important to note that all current methods are faced with the challenge of distinguishing mediation from horizontal pleiotropy, defined as effects where genetic variation influences multiple phenotypes simultaneously²² (such as DNA methylation and a complex trait) via independent biological pathways.

Recent approaches to MR have shown that the robustness of causal inference is improved if there are many instruments because one can evaluate whether the SNP effects on the causal trait are proportional to the SNP effects on the consequential trait^{17;23}. We exploit this property to evaluate the causal influence of complex traits (which typically have many instruments) on DNA methylation (i.e. bi-directional MR²⁴). Although a pitfall of evaluating the causal influence of DNA methylation on complex traits is that DNA methylation is typically instrumented by only a single cis-acting variant. Hence, an unreliable MR estimate of causality could arise due to the mQTL simply being in linkage disequilibrium with a variant that influences the cardiovascular trait through means other than the methylation level.

In this study we have developed and implemented a method to address this issue, which integrates fine mapping to evaluate the likelihood of the mQTL being the same causal variant as the SNP influencing the cardiovascular trait. There are also methods which have been devised for this purpose using intermediate traits^{2; 25; 26}. Therefore we have used the joint likelihood mapping (JLIM) method²⁷ to support findings. We have also undertaken functional informatics and incorporated eQTL data as this may support findings suggesting that DNA methylation resides on the causal pathway between variant and disease. However, a limitation of using single variant instruments in general is that it is not possible to reliably distinguish horizontal pleiotropy from mediation²⁸.

Together, the causal relationships between DNA methylation and cardiovascular traits are delineated into four potential categories (Figure 1):

1. The genetic variant has an effect on the phenotype, mediated by DNA methylation.
2. The genetic variant has an effect on the phenotype by alternative biological mechanisms, which then has a downstream effect on DNA methylation at this locus.
3. The genetic variant which influences DNA methylation is simply in linkage disequilibrium (LD) with another variant which is influencing the associated trait.
4. The genetic variant is influencing both DNA methylation and phenotype by two independent biological pathways (also known as horizontal pleiotropy).

We have developed a framework to systematically navigate through these scenarios and have applied it to analyse 14 different cardiovascular traits. In our discovery analysis we used genotype and DNA methylation data from prepubertal individuals to discover causal pathways on early childhood phenotypes. Replication was then undertaken using GWAS summary statistics from large-scale consortia.

Materials and Methods

The Avon Longitudinal Study of Parents and Children (ALSPAC)

ALSPAC is a population-based cohort study investigating genetic and environmental factors that affect the health and development of children. The study methods are described in detail elsewhere^{29; 30}. Briefly, 14,541 pregnant women residents in the former region of Avon, UK, with an expected delivery date between 1st April 1991 and 31st December 1992, were eligible to take part in ALSPAC. Detailed information and biosamples have been collected on these women and their offspring at regular intervals, which are available through a searchable data dictionary.

Written informed consent was obtained for all study participants. Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees.

Accessible Resource for Integrative Epigenomic Studies project (ARIES)

Samples: Blood samples were obtained for 1,018 ALSPAC mother-offspring pairs (mothers at two timepoints and their offspring at three timepoints) as part of the Accessible Resource for Integrative Epigenomic Studies project (ARIES)³¹. The Illumina HumanMethylation450 (450K) BeadChip array was used to measure DNA methylation at over 480,000 sites across the epigenome.

Methylation assays: DNA samples were bisulfite treated using the Zymo EZ DNA MethylationTM kit (Zymo, Irvine, CA). The Illumina HumanMethylation450 BeadChip (HM450k) was used to measure methylation across the genome and the following arrays were scanned using Illumina iScan, along with an initial quality review using GenomeStudio. A purpose-built laboratory information management system (LIMS) was responsible for generating batch variables during data generation. LIMS also reported quality control (QC) metrics for the standard probes on the HM450k for all samples and excluded those which failed QC. Data points with a read count of 0 or with low signal:noise ratio (based on a p-value > 0.01) were also excluded based on the QC report from Illumina to maintain the integrity of probe measurements. Methylation measurements were then compared across timepoints for the same individual and with SNP-chip data (HM450k probes clustered using k-means) to identify and remove sample mismatches. All remaining data from probes was normalised with the

Touleimat and Tost³² algorithms using R with the *wateRmelon* package³³. This was followed by rank-normalising the data to remove outliers. Potential batch effect were removed by regressing data points on all covariates. These included the bisulfite-converted DNA (BCD) plate batch and white blood cell count which was adjusted for using the *estimateCellCounts* function in the *minfi* Bioconductor package³⁴.

Genotyping assays: Genotype data were available for all ALSPAC individuals enrolled in the ARIES project, which had previously undergone quality control, cleaning and imputation at the cohort level. ALSPAC offspring selected for this project had previously been genotyped using the Illumina HumanHap550 quad genome-wide SNP genotyping platform (Illumina Inc, San Diego, USA) by the Wellcome Trust Sanger Institute (WTSI, Cambridge, UK) and the Laboratory Corporation of America (LCA, Burlington, NC, USA). Samples were excluded based on incorrect sex assignment; abnormal heterozygosity (<0.320 or >0.345 for WTSI data; <0.310 or >0.330 for LCA data); high missingness (>3%); cryptic relatedness (>10% identity by descent) and non-European ancestry (detected by multidimensional scaling analysis). After QC, 500,527 SNP loci were available for the directly genotype dataset. Following QC the final directly genotyped dataset contained 526,688 SNP loci.

Imputation: Imputation was performed using a joint reference panel using variants discovered through whole genome sequencing (WGS) in the UK10K project³⁵ along with known variants taken from the 1000 genomes reference panel. Additional functionality was developed in IMPUTE2³⁶ to use each reference panel to impute missing variants in their counterparts before ultimately combining them together. Following Gaunt et al (2016), prior to imputation we performed strict filtering using Hardy-Weinberg equilibrium $P > 5 \times 10^{-7}$ & MAF > 0.01. After imputation we converted the dosages to bestguess genotypes and filtered to only keep variants with an imputation quality score ≥ 0.8 .

Phenotypes: The 14 phenotypes analysed in this study are highlighted in bold. At the age 7 ALSPAC clinic (mean age: 7.5, range: 7.1 – 8.8), height was measured to the nearest 0.1cm using a Harpenden stadiometer (Holtain Crosswell, Dyfed, UK) and weight was measured to the nearest 0.1kg using Tanita electronic scales. Body Mass Index (BMI) was calculated as $(\text{weight (kg)})/(\text{height (m)})^2$. Blood Pressure was measured with a Dinamap 9301 vital monitor completed by trained staff using the appropriate cuff size. Two readings of both systolic and

diastolic blood pressure (SBP & DBP respectively) were taken when the study participants were at rest and the mean of each were used as a measurement in our analysis.

Non-fasting blood samples were taken from participants who attended the age 10 clinic (mean age: 9.9, range: 8.9–11.5). Plasma lipid concentrations (total cholesterol (**TC**), triglycerides (**TG**) and high density lipoprotein cholesterol (**HDL**)) were measured by modification of the standard Lipid Research Clinics Protocol with enzymatic reagents for lipid determination (Myers et al., 2000). Low density lipoprotein cholesterol (**LDL**) concentration was subsequently calculated using the Friedwald equation (Warnick, 1990):

$$LDLc = TC - (HDLc + TG \times 0.45)$$

Very low density lipoprotein cholesterol (**VLDL**) concentration was calculated as:

$$VLDLc = TC - (HDLc + LDLc)$$

apolipoprotein A (**Apo A1**) and apolipoprotein b (**Apo B**) were measured by immunoturbidimetric assays (Roche UK, Welwyn Garden City, UK). Interleukin 6 (**IL-6**) and **adiponectin** were measured by enzyme-linked immunosorbent assay (R&D Systems, Abingdon, UK). High-sensitivity C-reactive protein (**CRP**) was measured by an automated particle-enhanced immunoturbidimetric assay (Roche UK, Welwyn Garden City, UK). **Leptin** was measured in-house by a linked immunosorbent assay which had been validated against commercial methods (Wallace et al., 2001). All assay coefficients of variation were < 5%.

Statistical Analysis

We undertook an mQTL-wide association study (MWAS) to evaluate the association between all eligible mQTL and each trait in turn. This was decided over a conventional epigenome-wide association study (EWAS) (i.e. evaluating the association between methylation levels at CpG sites and traits) due to a larger proportion of individuals in ALSPAC having genotype data rather than 450K data after merging on phenotypes.

All variants with previous evidence of genetic association with DNA methylation in ARIES (referred to hereafter as mQTL) were used in this analysis⁸, and the methods have been described in detail previously. Briefly, to discover mQTLs, Gaunt et al⁸ used a linear regression model adjusted for age, sex, bisulphite conversion batch, the top ten ancestry principal components and cell counts to evaluate the associations of 8,074,398 imputed genetic variants against each of the 395,625 eligible methylation probes. Methylation probes were filtered for exclusion based on evaluations by Naeem et al³⁷, using their criteria of overlapping SNPs at CpG probes, probes which map to multiple locations and repeats on the 450K array. A conservative multiple testing correction was applied to define mQTL ($P < 1.0 \times 10^{-14}$). This threshold was selected as it equates to a false positive rate of 0.2% after applying a Bonferroni correction accounting for the number of tests undertaken previously in ARIES. Furthermore, this strict threshold reduces the risk of MR analyses suffering from weak instrument bias. Full details on the mQTL analysis can be found in the study by Gaunt et al⁸.

The mQTL discovery study used the COJO-slet routine in GCTA to identify independent mQTLs, which was important to ensure variants used as instruments were independent for downstream MR analyses. We excluded mQTL associated with a CpG site which was over 1Mb distance away (known as trans-mQTL), therefore leaving mQTL which were only associated with a nearby CpG site (known as cis-mQTL). This was to reduce the possibility of pleiotropy in our analysis as variants which associated with methylation at multiple CpG sites across the epigenome may be influencing independent biological pathways simultaneously. This left 37,812 independent mQTL eligible for analysis.

Excluding trans-effects was an important consideration in our study design as we anticipated that single instrument MR analyses may be necessary at a later stage when evaluating causal effects, which therefore restricts our ability to investigate pleiotropy using multiple valid instruments. mQTL were analysed in turn with each trait using linear regression with adjustment for age and sex. A sensitivity analysis also adjusting for the first 10 principal components was undertaken to evaluate whether population stratification was influencing our results in this analysis, although we did not anticipate this based on previous evaluations of population structure in the ALSPAC cohort³⁸. Results were plotted using a Manhattan plot using code derived from the qqman R package³⁹. Scripts to generate this plot are available at the location specified in the web resources section.

Mendelian randomization analysis

Observed associations between genotype and traits which survived a stringent multiple testing threshold (i.e. $P < 0.05/\text{number of tests undertaken}$) were then analysed using Mendelian randomization (MR). This analysis was undertaken to estimate the potential causal effect of DNA methylation on cardiovascular traits, as we anticipated observing evidence of association having already undertaken an MWAS. MR was undertaken using two stage least squares (2SLS) regression with DNA methylation as our exposure, phenotypic trait as our outcome and using the relevant mQTL as our instrumental variable. Measures of DNA methylation were initially taken from the childhood time point in ARIES (mean age: 7.5, standard deviation: 0.15) as this was the closest time point to phenotype measurements. Follow-up analyses were also undertaken using methylation data from the birth time point (using cord blood) and the adolescent time point (mean age: 17.1, standard deviation: 1.01). The R package ‘systemfit’⁴⁰ was used to obtain causal effect estimates using 2SLS.

We replicated observed effects by undertaking a two-sample MR analysis (2SMR)⁴¹ using estimated effects between genetic variants and associated traits obtained from published studies. Moreover, a two-sample framework removes any potential bias encountered in the discovery analysis due to effects on both methylation and trait being obtained in the same sample. When observed effects for sentinel mQTL were not available from published studies we used variants in LD with these SNPs instead ($r^2 > 0.8$).

Figure 1 illustrates the 4 possible explanations investigated where evidence of a causal effect was observed using MR. Figure 2 provides an overview of our approach to investigate these explanations. To robustly test explanation ii) we performed the reverse MR analysis, evaluating whether the cardiovascular trait influenced DNA methylation levels at the CpG site of interest. Instruments for this analysis were identified using the NHGRI-EBI GWAS catalog⁴². Relevant GWAS for interleukin-6 were not available at the time of analysis and so we identified instruments based on findings from Naitza et al⁴³ ($P < 5.0 \times 10^{-08}$). A p-value great than 0.05 indicated that explanation ii) was unlikely in each instance.

Bivariate fine mapping

Bivariate fine mapping was undertaken using FINEMAP⁴⁴ at each locus detected in the previous analysis. FINEMAP generates a Bayes factor for each variant at a locus which reflects the likelihood that it is the underlying causal variant at this region. Bivariate fine mapping requires all variants at a locus to be fine mapped using two different effect estimates 1) observed effects between SNPs and DNA methylation and 2) observed effects between SNPs and outcome phenotypes. As all mQTL effects were initially pruned to identify independent loci, we only included variants which were in high LD ($r^2 \geq 0.8$) with the sentinel SNP for each association signal before applying FINEMAP using the default settings. Interpretation of these results is therefore based on at least one underlying causal variant at each loci, as follow-up analyses are necessary to evaluate whether multiple causal variants may be contributing to observed effects. Posterior probabilities to reflect the likelihood of multiple causal variants were calculated using FINEMAP.

This analysis was undertaken to evaluate explanation iii), that the mQTL analysed may simply be in LD with the putative causal variant for the phenotypic trait. This was necessary as when evaluating the relationship between DNA methylation at a CpG site and outcome trait there may likely only be one valid instrumental variable (i.e. the mQTL at this region). Bivariate fine mapping in this instance therefore evaluates whether the causal mQTL at a locus is likely the same causal variant for the observed effect on the outcome trait. However, it does not rule out the possibility of a single variant influencing DNA methylation and outcome trait through independent biological pathways (i.e. explanation iv).

Concordance between the top SNPs for the two sets of fine mapping analyses would suggest that explanation i) may be responsible for the observed effect and that DNA methylation resides on the causal pathway between variant and phenotypic trait. Bivariate fine mapping using effect estimates for both methylation and cardiovascular traits was advantageous in this study as we were able to obtain estimates for all SNPs in our data set without having to rely on summary statistics. The concordance rate was defined as the same variant from both analyses identified as being causal after accounting for chance. This was achieved by identifying the rank of the top variant from the methylation based analysis in the list of variants from the cardiovascular trait analysis and then dividing that rank by the total number of variants in the region. A concordance rate < 0.05 suggested that explanation iii) was unlikely. To further evaluate explanation iii), we also used the joint likelihood mapping (JLIM) approach²⁷. Although JLIM doesn't specify the likely causal variant at a region, it can be used to examine whether the underlying causal variation is responsible for observed effects on both methylation and cardiovascular trait in a two-sample framework. Prior probabilities were not integrated into these analyses using FINEMAP which allowed for a more direct comparison with results of the JLIM method.

Impact of mQTL on gene expression and histone modification

We applied 2SMR to evaluate the relationship between methylation and expression using observed effects between SNPs and expression in relevant tissue types from the GTEx consortium⁴⁵. When observed effects for sentinel mQTL were not available from GTEx we identified a surrogate SNP instead ($r^2 > 0.8$).

We also assessed whether any mQTL were in LD ($r^2 > 0.8$) with any previously reported histone quantitative trait loci (hQTL)⁴⁶. When this was true, we applied 2SMR to evaluate the causal relationship between methylation and histone modification at these loci. This analysis was for exploratory purposes as there are aspects of the relationship between DNA methylation and histone modification which remain unexplored, despite progress by recent studies^{47; 48}.

Functional informatics

The variant effect predictor (VEP)⁴⁹ was applied to the top ranked mQTL from the bivariate fine mapping analysis to calculate their predicted consequence. Enhancer

annotations were obtained from the 450K annotation file from Illumina and additional regulatory data was obtained from Ensembl⁵⁰ to evaluate whether mQTL and CpG sites were located within regulatory regions of the genome. As we were interested in cardiovascular and lipid traits in this study, tissue specific data from the Roadmap Epigenomics Project⁵¹ was used to infer whether the potential causal variants and CpG sites at each locus resided within histone mark peaks and regions of DNase hypersensitivity. These tissues were adipose derived mesenchymal stem cells, adipose nuclei, aorta, fetal heart, left ventricle, right atrium and right ventricle, which were selected due to their biological relevance in cardiovascular aetiology.

Enrichment analysis was undertaken to test whether lead SNPs and associated CpG sites were located in regulatory regions more than can be accounted for by chance. To calibrate background expectations, we obtained matched SNPs using snpSNAP⁵² and identified matched CpG sites by randomly sampling probes from the 450K array which were in similar regions across the genome (i.e. within CpG islands/1st Exons etc.). Enrichment was investigated using the hypergeometric test and multiple testing was accounted for by randomly sampling controls SNPs/probes and re-running analyses for 10,000 iterations.

Results

Mining for putative causal influences of methylation on cardiovascular traits

We undertook 529,368 tests to evaluate the association between previously identified mQTL in ARIES with each trait in turn (37,812 unique variants x 14 traits). We identified 10 independent association signals which, after multiple testing correction, provided strong evidence of association ($P < 9.45 \times 10^{-08}$ (i.e. $0.05/529,368$)) and can be found in Table 1. Two of these effects were observed at the same CpG site near *ADIPOQ*, although they were identified using two independent mQTL ($r^2 = 0.02$).

The 10 sentinel mQTL identified in this analysis were only strongly associated with DNA methylation at a proximal CpG site and not any other CpG sites in the epigenome based on our findings in ARIES. A summary of these mQTL can be found in Table S1. We repeated our analysis with adjustment for the first 10 principal components, although results did not suggest that population stratification was an issue in this analysis (Table S2).

Inferring putative causal relationships

Putative causal effect estimates between methylation and cardiovascular traits were obtained at each locus in the MR analysis using mQTL as our instrumental variables (Table 2). Effect estimates suggested a direct relationship between methylation and cardiovascular traits at the *IL6R* [MIM: 147880], *APOB* [MIM: 107730], *SORT1* [MIM: 602458] and *ADCY3* [MIM: 600291] loci (i.e. increased methylation results in an observed increase in the cardiovascular trait), whereas an inverse relationship was observed at the *ADIPOQ* [MIM: 612556], *ABO* [MIM: 110300], *LEPR* [MIM: 601007], *APOA1* [MIM: 107680] and *FADS1* [MIM: 606148] loci (i.e. increased methylation causes a decrease in cardiovascular trait levels). Due to the two independent mQTL contributing to methylation at the *ADIPOQ*, we undertook multivariate MR which provided strong evidence of an inverse relationship between methylation and adiponectin at this locus (beta = -0.548, standard error = 0.107, $P = 3.79 \times 10^{-7}$).

Taking these putative associations forward, we evaluated the potential for reverse causal relationships by performing MR of the cardiovascular traits against the DNA methylation levels using SNPs from GWAS as our instruments. There was no evidence to suggest that the putative associations were due to the cardiovascular traits influencing the methylation levels (Tables S3) and therefore suggests that these effects are unlikely to be attributed to explanation ii).

Using methylation data from 2 other time points across the life course (at birth and adolescence (mean age: 17.1)) we observed consistent directions of effect as was observed using data from the childhood time point (mean age: 7.5) (Tables S4 & S5). Evidence of association was observed at each locus in this analysis except for the *ABO* and *IL6R* loci when using the cord data. We reproduced similar effects for 9 of the 10 mQTLs on cardiovascular traits using effect estimates from published studies (Table 3). The only locus we were not able to find a replication effect estimate for was the mQTL at *IL6R* as it was not in LD ($r^2 > 0.8$) with any previously published findings for interleukin 6.

Evaluating putative causal variants to infer mediated effects

There was concordance amongst the top SNPs in the bivariate fine mapping analyses at the *ABO*, *ADCY3* and *ADIPOQ* (common signal) loci, as the variant with the largest Bayes factor was the same for the effect on DNA methylation and outcome trait (Tables S6). These results lend support to the hypothesis that DNA methylation resides on the causal pathway between genetic variant and outcome trait (i.e. explanation i). There was a lack of concordance for the results at the *ADIPOQ* (low frequency signal), *SORT1*, *FADS1* and *LEPR* loci, suggesting that the mQTL may be in LD with the putative causal variant for the phenotypic trait (i.e. explanation iii). Results of the JLIM method supported evidence at the *ADIPOQ* & *ADCY3* loci, although we were unable to further evaluate signals at the *ABO* & *IL6R* regions due to unavailable GWAS summary results for interleukin-6 (Table S7). Posterior probabilities from FINEMAP suggested that there was likely only a single variant influencing trait variation for each observed effect (Table S7).

Investigating the role of DNA methylation with gene expression and histone modification

To further dissect the relationship between DNA methylation and complex traits we sought to evaluate the influence of the methylation levels on local gene expression. We observed evidence of a causal relationship between methylation and expression at 8 of the 10 loci using data from the GTEx consortium (Table 4). Effect estimates suggest an inverse relationship (i.e. increased methylation results in decreased gene expression) at the *ADIPOQ* (low frequency signal) and *APOA1* loci, whereas a direct relationship was observed at the other 6 loci (i.e. increased methylation results in increased gene expression). We were unable to identify a surrogate variant ($r^2 > 0.8$) to obtain a suitable effect estimate at the *LEPR* and *ADIPOQ* (common signal) loci.

mQTL at the *APOA1* and *IL6R* loci were also in high LD with previously reported histone quantitative trait loci (hQTL) based on findings by Grubert et al⁴⁶. Results from our 2SMR analyses to evaluate the influence of methylation levels on histone modification provided strong evidence of a causal effect as well as an inverse relationship in each instance (Table S8).

Functional informatics

To better understand the functional role underlying these putative causal associations, variants and CpG sites were evaluated to discern whether they reside within regulatory regions across the genome. An overview of the regulatory data used can be found in Table S9. The lead variants based on the bivariate fine mapping analysis (using effect estimates on DNA methylation) were used in this analysis and the VEP was used to predict their functional consequences (Table S10).

Every associated CpG site identified in this study resides within multiple histone mark peaks based on tissue data from the Roadmap Epigenomics Project (Table S11). All sites also reside in either enhancer or promoter/promoter flanking regions with the exception of the CpG site near *ADIPOQ*. There was strong evidence of enrichment for regulatory annotations for both SNPs and CpG sites which supports previous evidence that they are likely to have a causal downstream effect on phenotypic variation (Table S12).

Discussion

We have designed a framework to evaluate the putative causal influences of DNA methylation on complex traits and disease using Mendelian randomization. For observed effects on cardiovascular traits that appear to be caused by methylation, we used bivariate fine mapping and joint likelihood mapping to evaluate if the putative causal variant influencing methylation was the same causal variant responsible for influencing the trait. The bivariate fine mapping suggested that cardiovascular traits may be influenced by altered DNA methylation levels at the *ABO*, *ADCY3*, *ADIPOQ*, *APOA1*, *APOB* and *IL6R* gene regions. However, joint likelihood mapping only supported findings at the *ADCY3* and *ADIPOQ* loci. This provides compelling evidence DNA methylation may play a mediatory role for the effects at these loci, whereas the remaining candidate loci require further evaluation. Furthermore, two sample MR analyses provided evidence that DNA methylation levels influenced gene expression at these loci, suggesting that predicted functional effects for the causal variants indicate a coordinated system of effects that are consistent with causality. This was important to demonstrate, as single valid instruments meant that we were unable to robustly show that variants were not influencing methylation and traits independently. This is a current limitation also encountered by an alternative approach to evaluate the relationship between DNA methylation and complex traits¹⁶. This type of approach is particularly attractive for therapeutic evaluation of drug targets as it can provide valuable insight into the underlying mechanisms between genetic variants and disease.

The *ABO* locus identified in this study has been associated with many different traits and diseases by previous studies^{25; 53; 54}, as well as evidence implicating expression quantitative trait loci as putative causal SNPs for this effect⁵⁵. Here we provide evidence that DNA methylation may reside along the causal pathway to these observed effects (MR effect estimate: 0.29 (Standard error=0.06) change in trait per standard deviation change in methylation). A deletion (rs200533593) was found to be the putative causal variant for both the observed effect on DNA methylation and phenotypic variation.

The observed effect of genetic variation at *ADCY3* on body mass index is a relatively recent finding⁵⁶⁻⁵⁸. In this study, our bivariate fine mapping analysis suggests that an intergenic variant (rs6737082) may be responsible for the observed signal which is mediated through DNA methylation at this locus (MR effect estimate: 0.11 (0.05)). Furthermore, a variant in LD with

rs6737082 (rs713586, $r^2=0.80$) has been previously reported to regulate DNA methylation at this location in adipose tissue⁷.

There were two independent effects detected in our study near the *ADIPOQ* gene which were associated with adiponectin. The common variant signal was located upstream of *ADIPOQ* within the *RFC4* gene but associated with DNA methylation levels proximal to *ADIPOQ*, which can help explain this variant's observed effect on adiponectin (MR effect estimate: -0.36 (0.12)). Concordance in the bivariate fine mapping analysis suggested a non-coding transcript variant (rs169109) was responsible. The lead SNP from the ADIPOGen consortium⁵⁹ at this locus (rs6810075) is neither an mQTL nor in high LD with rs169109 ($r^2 = 0.20$), suggesting that these two association signals are influencing adiponectin levels by alternative biological mechanisms. The low frequency variant signal was previously detected by the UK10K project³⁵, although bivariate fine mapping results at this locus suggest that the causal mQTL was in linkage disequilibrium with the trait-associated variant.

The CpG site associated with the mQTL at this locus resides between the *APOA1* gene and *APOA1-antisense* (*APOA1-AS*), a negative transcriptional regulatory of *APOA1* which has been shown to increase *APOA1* expression both in vitro and in vivo⁶⁰. The highest ranked mQTL based on our bivariate fine mapping using estimates with DNA methylation is in a promoter region upstream of *APOA1*, suggesting that it may be more likely influencing *APOA1* rather than *APOA1-AS*. There are previously reported GWAS association signals at this locus with lipid traits^{61: 62}. However, given the evidence in this study of a causal effect with DNA methylation (MR effect estimate: -0.30 (0.08)) it is likely that these are downstream effects of the observed effect on Apo A1 variation. Furthermore, it is more biologically plausible this is the case as this gene is responsible for the protein synthesis of Apo A1.

The signal at the *IL6R* locus has been previously associated with a range of traits related to respiratory and cardiovascular health⁶³⁻⁶⁵. Our results suggest that genetic variation at *IL6R* influences DNA methylation at this region, which in turn will have a downstream effect on interleukin-6 and subsequently other traits and diseases (MR effect estimate: 0.47 (0.18)). Furthermore, this association signal was not in LD with a previously reported missense variant at this locus (rs2228145, $r^2 = 0.47$ in ALSPAC) which was also supported by findings from an in-depth functional study of this variant⁶⁶.

Evidence from the GTEx consortium suggests that mQTL at all eight of the loci with available expression data overlap with eQTL effects, which serves as an independent replication of the

relationships discovered through DNA methylation levels. It is biologically plausible that a variant's impact of DNA methylation levels may have a downstream effect on gene expression along the causal pathway to disease^{67; 68}, which may help explain these observations. Effects at four loci in particular appear to be biologically plausible in this regard, as the likely genes influenced by these variants are involved in the protein synthesis of the associated trait (i.e. *ADIPOQ* with adiponectin, *APOB* with Apo B, *APOA-I* with Apo A1 and *IL6R* with interleukin-6). Furthermore, each CpG site identified in this study resides within histone mark peaks in adiposity tissue according to data from the Roadmap Epigenomics project where we observed enrichment compared to matched CpG sites in similar regions of the genome.

As with any study which applies MR using a single instrument to investigate causal relationships in epidemiology, an important limitation is the inability to disentangle potential pleiotropic effects where the same causal variant influences both exposure (i.e. DNA methylation) and outcome (i.e. cardiovascular trait) through independent pathways. To reduce the possibility of this we selected mQTL in our study that were only influencing proximal CpG sites and not elsewhere in the epigenome, as such instruments would be more likely to influencing traits via alternative biological mechanisms. Although in ARIES there are CpG sites which have 2 or 3 independent instruments (such as the CpG site at *ADIPOQ* in this study), distinguishing mediation from pleiotropy at these loci remains a challenging endeavour. Future studies which continue to uncover mQTL across the genome (as well as across various tissue types) should facilitate analyses which are able to robustly address concerns over pleiotropy by using methods such as MR-Egger⁶⁹. These findings should also facilitate analyses which model the joint effects of multiple causal mQTL at loci across the genome, as opposed to evaluating mQTL effects independently of each other as we have in this study.

Weak instrumental variables and reverse causation are other factors which can bias MR analyses. Our analysis is unlikely to have suffered from the former as each mQTL had a large effect on DNA methylation in cis ($P < 1.0 \times 10^{-14}$) and were robustly associated with traits which we were able to replicate using results from studies with large population samples. We conducted analyses to evaluate whether reverse causation was an issue in our study (i.e. trait variation was causal to changes in DNA methylation at each locus), although results suggested that this was not the case.

In this study we have demonstrated the value of two-sample Mendelian randomization (2SMR) to undertake MR analyses using summary statistics^{41; 70}. This allowed us to provide evidence

of replication for the observed effects in our study as well as investigate the relationship between DNA methylation and expression along the causal pathway to disease. This approach has the attractive advantage of enabling the potential epigenetic-complex trait interplay to be interrogated on a much wider scale, foregoing the requirement that ‘omic’ data and phenotypes are measured in the same sample.

Description of Supplemental Data

Supplemental data includes Tables S1-S12.

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Web Resources

ALSPAC - (<http://www.bristol.ac.uk/alspac>)

ALSPAC data dictionary - (<http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/>)

ARIES explorer - www.ariesepigenomics.org.uk/

GWAS catalog - <https://www.ebi.ac.uk/gwas/>

GTEEx - www.gtexportal.org/

Ensembl - www.ensembl.org/

Epigenomics Roadmap Project - www.roadmapepigenomics.org/

[Multicoloured manhattan plot script - https://github.com/MRCIEU/qqman_multiple_colours.](https://github.com/MRCIEU/qqman_multiple_colours)

OMIM - <https://www.omim.org/>

snpSNAP - <https://data.broadinstitute.org/mpg/snpsnap/>

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Figures

Figure 1: Explanations evaluated to explain observed associations between methylation quantitative trait loci and trait outcomes

i) The genetic variant has an effect on the phenotype, mediated through DNA methylation. ii) The genetic variant has an effect on the phenotype by alternative biological mechanisms, which then has a downstream effect on DNA methylation at this locus. iii) The genetic variant which influences DNA methylation is simply in linkage disequilibrium with another variant which is influencing the associated trait. iv) The genetic variant is influencing both DNA methylation and phenotype by two independent biological pathways (also known as horizontal pleiotropy).

Figure 2: Analysis pipeline to evaluate explanations for observed associations between methylation quantitative trait loci and trait outcomes

This flowchart provides an overview of the analysis plan in this study to evaluate 4 different explanations which may explain trait-associated methylation quantitative trait loci (mQTL). LD – linkage disequilibrium, GWAS – Genome-wide association study.

Figure 3: Manhattan plot illustrating observed association between methylation quantitative trait loci and cardiovascular traits

Manhattan plot illustrating the observed association between methylation quantitative trait loci (mQTL) and various cardiovascular traits. Points represent $-\log_{10}$ p-values (y-axis) for genetic variants according to their genomic location (x-axis). Effects that survive the multiple testing threshold in our analysis ($P < 9.45 \times 10^{-08}$ – represented by the red horizontal line) are coloured according to their associated trait and annotated according to the likely impacted gene.

Tables

Table 1: Results of linear regression analysis between genetic variants and traits

SNP	Gene	CpG	Trait	Sample Size	Beta	SE	P-value	% explained
rs266772	<i>ADIPOQ</i>	cg05578595	Adiponectin	4248	-0.992	0.070	1.72×10^{-44}	4.51%
rs687621	<i>ABO</i>	cg21160290	Interleukin-6	4241	-0.265	0.022	1.15×10^{-31}	3.05%
rs13375019	<i>LEPR</i>	cg04111102	C-reactive protein	4251	-0.213	0.022	2.65×10^{-22}	2.20%
rs7549250	<i>IL6R</i>	cg02856953	Interleukin-6	4241	-0.176	0.022	9.71×10^{-16}	1.40%
rs169109	<i>ADIPOQ</i>	cg05578595	Adiponectin	4248	-0.167	0.022	1.44×10^{-14}	1.34%
rs541041	<i>APOB</i>	cg25035485	Apo B	4251	-0.209	0.028	3.76×10^{-14}	1.32%
rs7528419	<i>SORT1</i>	cg00908766	Apo B	4251	-0.196	0.026	4.63×10^{-14}	1.30%
rs625145	<i>APOA1</i>	cg04087571	Apo A1	4251	0.200	0.027	9.78×10^{-14}	0.94%
rs174544	<i>FADS1</i>	cg19610905	Cholesterol	4250	-0.143	0.023	8.61×10^{-10}	0.86%
rs6749422	<i>ADCY3</i>	cg01884057	Body mass index	6076	0.109	0.018	1.28×10^{-9}	0.55%

- SNP – Single Nucleotide Polymorphism, Gene – likely implicated gene, CpG – 450K probe ID, Trait – Associated Trait, Sample Size – sample size for this effect, Beta – Observed effect size (units in standard deviations), SE – Standard Error of the effect size, P-value – P-value for observed effect, % explained – proportion of variance in trait explained by mQTL.

Table 2: Results of Mendelian randomization analysis between DNA methylation and traits

SNP	Gene	CpG	Trait	Sample Size	Beta	SE	P-value
rs266772	<i>ADIPOQ</i>	cg05578595	Adiponectin	646	-0.846	0.168	5.93 x 10 ⁻⁷
rs687621	<i>ABO</i>	cg21160290	Interleukin-6	646	-0.293	0.061	1.77 x 10 ⁻⁶
rs13375019	<i>LEPR</i>	cg04111102	C-reactive protein	646	-0.265	0.076	0.001
rs7549250	<i>IL6R</i>	cg02856953	Interleukin-6	646	0.468	0.175	0.008
rs169109	<i>ADIPOQ</i>	cg05578595	Adiponectin	646	-0.363	0.121	0.003
rs541041	<i>APOB</i>	cg25035485	Apo B	646	0.298	0.114	0.009
rs7528419	<i>SORT1</i>	cg00908766	Apo B	646	0.271	0.064	2.74 x 10 ⁻⁵
rs625145	<i>APOA1</i>	cg04087571	Apo A1	646	-0.301	0.082	2.68 x 10 ⁻⁴
rs174544	<i>FADS1</i>	cg19610905	Cholesterol	646	-0.363	0.121	0.003
rs6749422	<i>ADCY3</i>	cg01884057	Body mass index	846	0.106	0.048	0.028

- SNP – Single Nucleotide Polymorphism, Gene – likely implicated gene, CpG – 450K probe ID, Trait – Associated Trait, Sample Size – sample size for this effect, Beta – Observed effect size (units in standard deviations), SE – Standard Error of the effect size, P-value – P-value for observed effect

Table 3: Results of replication analysis using two-sample Mendelian randomization

SNP	Gene	CpG	CpG effect	Trait effect	2SMR effect	P-value	Study
rs266772	<i>ADIPOQ</i>	cg05578595	0.982 (0.103)	-0.629 (0.143)	-0.641 (0.160)	6.50 x 10 ⁻⁵	UK10K Consortium (TwinsUK individuals only) (2015)
rs687621	<i>ABO</i>	cg21160290	0.912 (0.036)	-0.245 (0.026)	-0.269 (0.03)	9.16 x 10 ⁻¹⁹	Naitza et al (2012)
rs2211651*	<i>LEPR</i>	cg04111102	0.682 (0.036)	-0.170 (0.022)	-0.249 (0.035)	3.09 x 10 ⁻¹³	Reiner et al (2012)
rs541041	<i>APOB</i>	cg25035485	0.627 (0.053)	0.098 (0.013)	0.156 (0.025)	2.05 x 10 ⁻¹⁰	Kettunen et al (2016)
rs169109	<i>ADIPOQ</i>	cg05578595	0.383 (0.036)	-0.052 (0.005)	-0.136 (0.017)	2.58 x 10 ⁻¹⁵	Dastani et al (2013)
rs7528419	<i>SORT1</i>	cg00908766	-0.980 (0.037)	-0.089 (0.012)	0.091 (0.013)	9.20 x 10 ⁻¹³	Kettunen et al (2016)
rs625145	<i>APOA1</i>	cg04087571	-0.884 (0.044)	0.057 (0.013)	-0.064 (0.015)	1.84 x 10 ⁻⁵	Kettunen et al (2016)
rs174544	<i>FADS1</i>	cg19610905	-0.655 (0.031)	0.047 (0.004)	-0.072 (0.007)	9.73 x 10 ⁻²⁵	Global Lipids Genetics Consortium (2013)
rs6749422	<i>ADCY3</i>	cg01884057	0.908 (0.026)	0.068 (0.007)	0.075 (0.008)	8.05 x 10 ⁻²¹	Felix et al (2016)

- * indicates surrogate variant used ($r^2 > 0.8$), SNP – Single Nucleotide Polymorphism, Gene – likely implicated gene, CpG – 450K probe ID, CpG effect – effect estimate of SNP on methylation, Trait effect – effect estimate of SNP on trait, 2SMR effect – effect estimates from 2-Sample MR analysis, P-value – P-value for observed effect, Study – published study where effect estimates for traits were obtained.

Table 4: Results of analysis investigating causal relationship between methylation and expression using two-sample Mendelian randomization

SNP	Gene	CpG	CpG effect	eQTL effect	eQTL P-value	eQTL tissue	2SMR	P-value
rs116552240*	<i>ABO</i>	cg21160290	0.912 (0.036)	0.548 (0.069)	1.316×10^{-13}	Adipose	0.601 (0.079)	3.28×10^{-14}
rs6737082	<i>ADCY3</i>	cg01884057	0.908 (0.026)	0.208 (0.047)	1.456×10^{-5}	Adipose	0.229 (0.052)	1.13×10^{-5}
rs266772	<i>ADIPOQ</i>	cg05578595	0.982 (0.103)	-0.339 (0.078)	1.893×10^{-5}	Adipose	-0.345 (0.087)	7.67×10^{-5}
rs688456	<i>APOA1</i>	cg04087571	-0.884 (0.044)	0.420 (0.095)	1.789×10^{-5}	Heart	-0.475 (0.11)	1.58×10^{-5}
rs541041	<i>APOB</i>	cg25035485	-0.627 (0.053)	-0.370 (0.066)	6.326×10^{-8}	Heart	0.590 (0.116)	4.06×10^{-7}
rs646776	<i>SORT1</i>	cg00908766	-0.980 (0.037)	-1.240 (0.105)	1.556×10^{-20}	Liver	1.265 (0.117)	4.01×10^{-27}
rs174559	<i>FADS1</i>	cg19610905	-0.655 (0.031)	-0.707 (0.089)	5.629×10^{-13}	Pancreas	1.079 (0.145)	1.04×10^{-13}
rs10908837	<i>IL6R</i>	cg02856953	-0.303 (0.039)	-0.120 (0.020)	4.171×10^{-09}	Whole Blood	0.396 (0.083)	2.05×10^{-6}

* indicates surrogate variant used ($r^2 > 0.8$), SNP – Single Nucleotide Polymorphism, Gene – likely implicated gene, CpG – 450K probe ID, CpG effect – effect estimate of SNP on methylation, eQTL effect – effect estimate of SNP on expression based on GTEx data, eQTL P – P-value for eQTL from GTEx, eQTL tissue – tissue type for observed effect according to GTEx, 2SMR effect – effect estimates from 2-Sample MR analysis, P-value – P-value for observed 2SMR effect