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requirements for award of the degree of Population Health Sciences in the  
Faculty of Health Sciences

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# Abstract

My abstract will go here and it will be a solid abstract. Full of the things that go in abstracts. Such as numbers, acronyms, other words, and lots of punctuation.

It will have multiple paragraphs too!

## Acknowledgements

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# Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Signed

Dated

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# Abbreviations

**EWAS** - epigenome-wide association study **GWAS** - genome-wide association study  **$h^2$**  - narrow-sense heritability  **$h^2_{\text{SNP}}$**  - SNP-heritability  **$H^2$**  - broad-sense heritability **MR** - Mendelian randomization **mQTL** - methylation quantitative trait loci **SNP** - single nucleotide polymorphism

# Preface

This template is based on (and in many places copied directly from) the Reed College LaTeX template, but hopefully it will provide a nicer interface for those that have never used TeX or LaTeX before. Using *R Markdown* will also allow you to easily keep track of your analyses in **R** chunks of code, with the resulting plots and output included as well. The hope is this *R Markdown* template gets you in the habit of doing reproducible research, which benefits you long-term as a researcher, but also will greatly help anyone that is trying to reproduce or build onto your results down the road.

Hopefully, you won't have much of a learning period to go through and you will reap the benefits of a nicely formatted thesis. The use of LaTeX in combination with *Markdown* is more consistent than the output of a word processor, much less prone to corruption or crashing, and the resulting file is smaller than a Word file. While you may have never had problems using Word in the past, your thesis is likely going to be about twice as large and complex as anything you've written before, taxing Word's capabilities. After working with *Markdown* and **R** together for a few weeks, we are confident this will be your reporting style of choice going forward.

**Why use it?**

*R Markdown* creates a simple and straightforward way to interface with the beauty of LaTeX. Packages have been written in **R** to work directly with LaTeX to produce nicely formatting tables and paragraphs. In addition to creating a user friendly interface to LaTeX, *R Markdown* also allows you to read in your data, to analyze it and to visualize it using **R** functions, and also to provide the documentation and commentary on the results of your project. Further, it allows for **R** results to be passed inline to the commentary of your results. You'll see more on this later.

### **Who should use it?**

Anyone who needs to use data analysis, math, tables, a lot of figures, complex cross-references, or who just cares about the final appearance of their document should use *R Markdown*. Of particular use should be anyone in the sciences, but the user-friendly nature of *Markdown* and its ability to keep track of and easily include figures, automatically generate a table of contents, index, references, table of figures, etc. should make it of great benefit to nearly anyone writing a thesis project.

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# Chapter 1

## Introduction

DNA methylation is a dynamic, chemical modification to DNA that can occur at millions of sites across the genome. The modification is associated with changes in gene expression and genome stability. Consequently, studying how DNA methylation covaries with phenotypes within human populations has the potential to yield aetiological insights. Further, association studies can identify new biomarkers that could predict future health outcomes or help diagnose current ones. This thesis will focus on the most common contemporary study design for assessing the association between DNA methylation and complex traits in humans, epigenome-wide association studies (EWAS).

To date, there have been hundreds of EWAS performed on complex traits that have identified varying numbers of DNA methylation sites as well as varying strengths of association. The interpretability of such studies has been questioned (1) and there are properties of these studies as well as the relationship between DNA methylation and complex traits that are not well understood. For traits in which few or no associations have been identified, it is unknown whether DNA methylation, as measured for

EWAS, captures any substantial trait variation. For EWAS that have identified DNA methylation sites, these results may reflect genuine biological effects, but they also may be the product of other events, for example they could have arisen due to technical artefacts (2), genetic factors (3), or other strong confounding factors (1). Even if the associations are reliable, it is currently unclear whether identifying and characterizing these associations enhances our current biological understanding of the complex traits assessed. Ultimately, if the goal of an EWAS is to ascertain how DNA methylation influences the trait of interest or vice versa, it is also important to fully explore whether these sites are causally related to the trait.

Knowledge of how much trait variation is captured by DNA methylation arrays and whether the associations in EWAS are likely reliable or helpful in understanding complex trait biology is imperative for the interpretability of future EWAS.

In this chapter I present the historical interpretation of ‘information flow’ in human cells (the central dogma), describe DNA methylation in the context of regulatory processes that augment that information flow, discuss its potential for use in population level studies and describe the current state of EWAS research. Then I explain how we might be able to draw on the methods developed by geneticists to understand 1. what information has been gained from EWAS, 2. what information is left to gain from EWAS and 3. the causal nature of DNA methylation-trait associations identified in EWAS.

## **1.1 The central dogma of molecular biology**

To gauge how molecular mechanisms result in more observable phenotypes, it is important to understand how molecular machinery interacts. The central dogma of molecular biology was originally proposed by Francis Crick (4,5) and described how

information flowed from nucleic acids to proteins within cells (**Figure 1.1**). Crick postulated that information could flow from nucleic acids to proteins, but not from proteins to nucleic acids. By information, Crick was specifically referencing changes in polymer sequence. Although this is generally the process of information flow, it does not describe other complex interactions that impact function without changing polymer sequence. Post-translational and post-transcriptional modifications can influence the lifespan and function of proteins and RNA respectively (6–9). Proteins of the same polypeptide sequence can take on slightly different conformations when interacting with other cellular factors (10) and certain proteins (known as prions) can even alter the conformation of other polypeptides with the same sequence (11). Further, modifications to the DNA and to DNA-bound proteins may have a profound influence on the concentration of certain gene transcripts as well as the post-transcriptional splicing of transcripts (12,13). As mentioned, DNA methylation is one such modification that can occur.

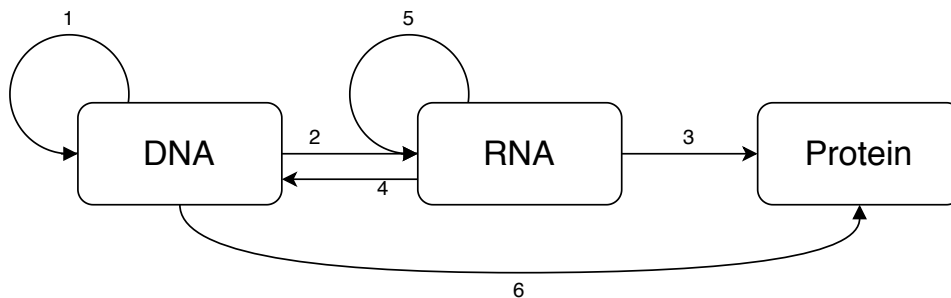


Figure 1.1: The central dogma of molecular biology. The dogma stipulates that sequence changes (that is the addition, removal or mutation of elements) can only occur in the direction of the arrows. 1. DNA replication 2. Transcription 3. Translation 4. Reverse transcription 5. RNA replication 6. Direct DNA-protein translation.

The importance of these molecular alterations to phenotypic change is exemplified in the developmental stages of human life. Humans start as a single cell and after



roughly nine months are transformed into a multicellular organism with trillions of cells, including hundreds of unique cell types. As these cells arise from a single progenitor, they must contain identical genetic sequences (bar somatic mutations which occur at a low rate – recently estimated to be roughly one mutation per cell division (14)). The process by which the body is able to create such diversely functioning cells and tissues, must come from regulation of how the genetic sequence is read and from the regulation of its products.

For reasons which I will describe in more detail later, DNA methylation is now being used widely by epidemiologists to try and understand how molecular changes, that do not involve direct sequence alterations, might result in variation amongst observable phenotypes. In order to understand how DNA methylation might influence observable phenotypes, one must first understand the role it plays within cells.

## **1.2 DNA methylation as part of the regulatory machinery**

DNA methylation is correlated with gene expression levels and has been hypothesised to contribute to gene regulation (15–18). This is the main mechanism by which it is thought DNA methylation influences variability in phenotypes. However, DNA methylation is just one of many epigenetic marks that are involved in gene regulation (**Figure 1.2**). In this section I describe epigenetics and evidence gathered about the function of different epigenetic marks.

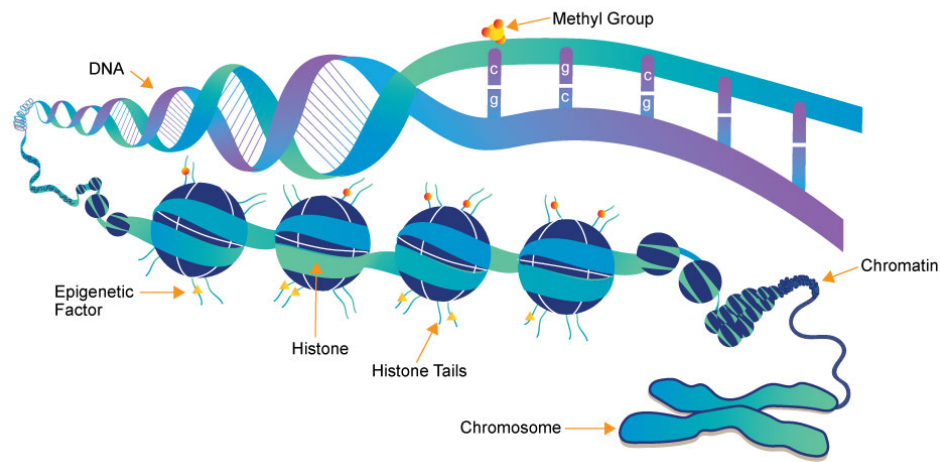


Figure 1.2: Graphic of epigenetic marks taken from (19).

### 1.2.1 Defining epigenetics

The definition of epigenetics is much debated (20). In the 1940s the ‘epigenetic landscape’ was introduced by Waddington to describe how genes influence cell fates (21). Since then the term “epigenetics” has been used in many forms, so to avoid confusion, throughout the rest of the thesis, I will define epigenetics as: “the study of mitotically (and potentially meiotically) heritable alterations in gene expression that are not caused by changes in DNA sequence” (13). In an extension to this, epigenetic marks therefore refer to chemical changes to the genome and genome-bound proteins that are mitotically heritable (i.e. changes that remain after cell division) and may influence gene expression without changing the DNA sequence.

### **1.2.2 Histone modifications**

Histones are DNA-bound proteins that when modified can influence chromatin state and transcription factor interactions with the DNA (22). These proteins are contained in nucleosomes, which consist of four histone proteins, each of which is present twice in the nucleosome. Post-translational modifications can occur to any of the histone monomers and these have been associated with both positive and negative changes in gene expression (22–24). Histone modifications are numerous and complex in nature. To briefly describe the complexity, there are at least nine types of histone modification that can occur (25), each of the histone monomers can be modified across many different sites (23–25), and for any one site multiple of the same modification can occur (23,24). It is the combination of modifications across all histones that plays a role in gene expression regulation (23,24). Furthermore, histone modifications are subject to rapid change upon environmental stimulus to help induce or repress gene expression (22). The complexity of histone modifications, and the practical difficulties in collecting or assaying samples to assess these epigenetic marks, remains a barrier to their wide-spread measurement for use in population-based analyses (1). They may become far more prominent in the future as our understanding and ability to measure the modifications in a meaningful way increases.

### **1.2.3 DNA methylation**

DNA methylation is the addition of a methyl group to DNA, which primarily occurs at the 5' cytosine of a CpG site. Little is known about the role of non-CpG site DNA methylation in humans and current EWAS tend to only measure CpG methylation. Two papers initially suggested that the function of this epigenetic mark was to repress gene expression (26,27). Since then, association with various important

intracellular processes such as X-inactivation, genomic imprinting and suppression of transposon action have been elucidated (27–29). This genomic modification is also conserved amongst a wide variety of species, including various bacteria, plants, fungi, and mammals (18,30–32). Interestingly, one of the hypothesised functions of DNA methylation – protection against ‘parasitic genomic sequences’ – is common to both human cells and bacterial cells (18,30). However, the relationship with gene expression may not be the same in prokaryotic organisms (30). Despite the abundance of research conducted in the area in the last 50 years, the role DNA methylation plays in regulating gene expression within human cells is not fully understood and research is still ongoing.

One thing research has revealed is that the location of DNA methylation is important to its relationship with gene expression. CpG sites are not randomly distributed throughout the genome but are often found in clusters (18) and it is thought that methylation and de-methylation of CpG sites in groups, is what drives their association with regulatory function (18). Clusters known as ‘CpG islands’ are found at the majority of protein coding genes and constitute small areas of the genome that are enriched for CpG sites (18,33). The location of these islands as well as other CpG sites relative to genes and other regulatory elements is also of importance. Several studies have shown that higher levels of DNA methylation at transcription start sites tends to be associated with a lower levels of gene expression (18,34,35), but gene body DNA methylation is positively correlated with expression (**Figure 1.3**) (36,37). This suggests that regulation of DNA methylation in clusters at specific sites relative to genes is important in determining observed relationships with gene expression. Supporting this, there are clear biological processes that regulate DNA methylation at nearby sites together, for example, CpGs at transcription factor binding sites can be de-methylated as a group when the transcription factor binds (38). Further,

nearby sites are often correlated (39,40). However, there is no evidence to suggest that neighbouring sites do indeed act in tandem or whether it is likely one site from the group is driving regulatory function. This is something I explore in **Chapter 5**.

These strong associations between DNA methylation and gene expression do not necessarily mean that the addition or removal of methyl groups will actively impact gene expression. Elucidating the causal nature of the association between DNA methylation and gene expression has been fraught with difficulties and has often provided conflicting results. One study showed an enzyme that catalyzes the addition of methyl groups to the DNA, DNA methyltransferase 3A, is required in haematopoietic stem cells for them to differentiate, suggesting gene expression changes required for differentiation were not possible without addition of methyl groups to the DNA (41). However, studies have provided evidence that DNA methylation is unlikely to initiate the ‘silencing’ of gene expression and may occur at transcription start sites of genes after they’ve already been repressed (42,43). To further complicate things, if DNA methylation does influence gene expression, the mechanism of action is unclear and may depend on the gene being examined. One study showed the presence of DNA methylation at the binding sites of the transcription factor, *MYC*, was inversely associated with its binding (44), but another study suggested the presence of DNA methylation didn’t have the same impact on the binding of the transcription factor, *SPI* (45). Although the body of work presented in this thesis does not aim to explore if and how DNA methylation influences gene expression, it is important to note the relationship between the two isn’t clear when thinking of the implications of DNA methylation-trait associations (46). This will be discussed further in the following sections.

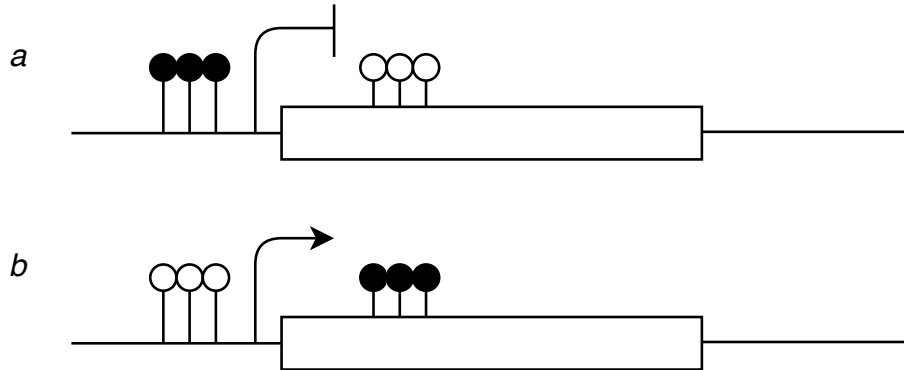


Figure 1.3: Simplified diagrams of the associations between DNA methylation and gene regulation. In *a*, CpG sites are methylated at the promoter region, but not in the gene body, which is associated with lack of gene expression. In *b*, the opposite is occurring.

### 1.3 Population-based studies of DNA methylation associations

The importance of DNA methylation in disease has already been established in rare developmental disorders caused by aberrant imprinting patterns (47,48), but the relationship between DNA methylation and complex traits is unclear and is the focus of this thesis. In this section I discuss the study of complex traits, the appeal of studying DNA methylation for public health, introduce the most common study design to assess DNA methylation-trait associations, EWAS, and overview some successes and complications of the work.

#### 1.3.1 Studying complex trait associations

Using DNA methylation in population-based studies, comprises studying its association with complex traits, which are phenotypes that are caused by a myriad of factors. The phenotypic value of any complex trait can be partitioned into its

corresponding genetic ( $G$ ) and environmental effects ( $E$ ) like so

$$z = G + E \quad (1.1)$$

Both the environmental and genotypic values can further be partitioned into various effects (49). It's important to remember that DNA methylation itself is a complex trait and thus methylation of DNA at a given CpG site, is the result of a variety of genetic and environmental factors (18). However, unless explicitly stated, when discussing complex traits, I will be referring to human health and socio-economic outcomes rather than molecular phenotypes such as DNA methylation and gene expression.

Phenotypic values for any complex trait will vary across the population as each individual has a unique genomic sequence (except monozygotic twins) and is exposed to a variety of different environments, both external and internal.

Studying the associations between complex traits and other measures across the population can help deduce the aetiology of that trait, but phenotypic values may also covary with measures that have no implication for that traits aetiology. Identifying these covariations can still be useful for phenotype prediction.

It should be noted that different fields of study may have different views on the importance of certain measures. As discussed, there is evidence that DNA methylation may be inconsequential to gene expression changes (42,43), making it an unattractive measure to study when interested in the aetiology of cellular phenotypes in molecular biology. However, for epidemiological studies, understanding how DNA methylation covaries with complex traits could help provide useful predictors and further could yet yield insights into the underlying biology of complex traits. This will be explained in more detail in the coming sections.

### **1.3.2 The potential value of DNA methylation measures to epidemiologists**

Epigenetic modifications are of potential interest to those studying any phenotype. Arguably, epigenetics could be required at some level for all phenotypic changes and, if causal, could be the difference between individuals who develop disease and those who do not (50). Further, epigenetic marks are modifiable, which means theoretically it would be possible to prevent or treat disease by altering such epigenetic patterns of individuals (51). There are large practical issues with this, which are discussed in more detail in **Section 1.3.4**. However, even if targeting epigenetic marks is not easy, as long as it is possible to observe them, they could be used as diagnostic biomarkers and predictors (1,50,52,53). Thus, the ability to measure, and the research in to understanding epigenetic mechanisms, could have broad consequences for public health.

A major difference between DNA methylation and other epigenetic marks, is that DNA methylation is more stable. Enzymes do exist that can actively de-methylate the DNA, for example the ten-eleven translocation (TET) enzymes, but cell division or excision of the nucleotide is required for full de-methylation of a DNA molecule (54,55). Biologically, this suggests DNA methylation might be involved in long-term repression of gene expression, which is thought to be the case for X-inactivation (56), and practically it makes studying the epigenetic mark easier because stability ensures the marks are more resistant to changes after collection of samples. Also, even though it isn't clear that DNA methylation precedes gene expression regulation, the regulatory processes that govern whether genes are transcribed are linked. There are known examples of how DNA methylation tends to associate with other epigenetic marks, including positive correlation with the histone modification H3K9me3 (18) and histone deacetylation (18,57). This means measuring DNA methylation may



capture regulatory information, even if addition or removal of methyl groups to the DNA would have little impact on gene expression. Recently, it has been shown that epigenetic marks can be used to predict each other with high accuracy (58), adding weight to the argument DNA methylation measurements capture far more than just DNA methylation itself.

### **1.3.3 Epigenome-wide association studies**

When considering designing a study to assess whether one trait associates with another, one should have *a priori* hypotheses or evidence that suggest studying the association would be of relevance to scientific understanding and public health. For studying the association between DNA methylation and complex traits, there is an abundance of evidence suggesting this could yield results of relevance. I've already explained why this may be: no phenotypic change is possible without some molecular change, DNA methylation is a relatively easy molecular measurement to make and it is known to highly correlate with an important component of cellular regulation – gene expression. Further, DNA methylation has the potential to improve upon the prediction of complex traits beyond what can be done with current epidemiological and clinical measures.

EWAS are the most common study design for assessing the association between DNA methylation and a complex trait. They typically involve measuring hundreds of thousands of DNA methylation sites across the genome in a case-control or cohort setting and using linear models to assess the association between DNA methylation and the trait of interest.

Ideally, in every sample used in an EWAS, DNA methylation would be measured across all sites in the genome. Unfortunately, this is not currently possible and sequencing technologies that offer something similar are often very expensive.

There are three alternatives available to DNA methylation studies. Firstly, one could sequence a small portion of the genome if this section is of particular interest, however this candidate gene approach is unlikely to be profitable unless the genes targeted already have good evidence for epigenetic variation with the trait of interest. As complex traits are polygenic (49) and we have incomplete knowledge of their underlying biology, a hypothesis-free approach, that samples from as much of the genome as possible, is preferable. Secondly, measuring DNA methylation on repeat sequences of the genome, such as long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs), can provide estimates for global DNA methylation changes (59). These measurements indicate if a trait is related to large perturbations of DNA methylation across the genome, but give little mechanistic insight into what effects these changes may be having, as methylation at functional genes is not measured. Thirdly, one could employ an array approach that covers DNA methylation genome-wide at selected sites. This last approach is the most common for population-based studies as it enables measurement of DNA methylation at hundreds of thousands of sites at a relatively cheap price per sample (60). Without capabilities to measure methylation at every site in the genome, one must decide which sites are worth measuring. Current commonly used array technologies include the Illumina Infinium HumanMethylation450 (HM450) Beadchip and the Illumina Infinium HumanMethylationEPIC (HMEPIC) Beadchip, which measure DNA methylation at over 450,000 sites and over 800,000 sites respectively. They cover roughly 1.5-4% of CpG sites in the genome (61). In order to capture what was thought to be the most relevant DNA methylation sites in relation to complex traits, the probes were chosen to map to 99% of RefSeq genes (62). One reason for which this might have been done, is to help improve interpretation of EWAS findings. Identifying methylation changes at a specific gene suggests investigating the relationship of that gene and the complex trait further may

yield interesting results, whereas interpreting complex trait associations with DNA methylation at a site in a relatively uncharacterized region of the genome would be more difficult for obvious reasons.

To measure DNA methylation, these array technologies, as well as sequencing techniques, often begin with bisulphite conversion of the DNA (63). This converts any un-methylated cytosine base to uracil and leaves methylated cytosines unchanged (64). The DNA samples can then be distributed amongst the array and the probes on the array will bind cytosines present at the regions their sequence corresponds to (64). If a probe binds its target cytosine, then it will fluoresce, and this can fluorescence can be quantified to give ‘beta values’. Beta values range between zero and one, with zero corresponding to no methylation across all DNA samples at the target cytosine and one corresponding to methylation across all DNA samples at the target cytosine. DNA methylation is by nature a binary measurement, but mixtures of DNA samples (i.e. multiple cells) mean that a continuous variable is generated unless single cell procedures are adopted.

This study design has been widely adopted over the past ten years and the relationship between a plethora of traits, from smoking to anthropometric measures to childhood adversities, and DNA methylation has now been studied (65–68). There are also large consortia that are pooling samples to gain power for these studies, for example the Pregnancy and Childhood Epigenetics (PACE) consortium (69). A few traits have been identified as being associated with large variations in DNA methylation, one of which is smoking, where strong associations across thousands of sites have been identified and many replicated in EWAS of smoking (65). It has been revealed that the association at some sites is driven by smoking causing changes in DNA methylation and over time these DNA methylation changes may be (mostly) reversible by giving up smoking (70). Also, DNA methylation can be used

to predict smoking status, and one study has provided evidence that DNA methylation of a single locus can predict smoking status with high accuracy (71). Another measure shown to relate to large variation in DNA methylation across the genome is age. Similarly to smoking, DNA methylation makes a highly accurate predictor for age and is thought to be able to establish whether rate of ‘biological’ aging is different from chronological aging (72–74). These studies have shown that large perturbations in the DNA methylome can be related to complex traits and highlight the potential for EWAS to identify accurate predictors for these traits.

#### **1.3.4 Problems for EWAS**

Despite the promise of understanding the underlying biological processes related to traits, studying the relationship between DNA methylation and complex traits provides many practical difficulties that often make the results of EWAS hard to interpret (1).

##### **Confounding**

One issue that plagues all observational epidemiology, including EWAS, is confounding. This is where the traits of interest share a common cause, which can generate effects and bias effect estimates, hindering correct interpretation of the association between traits. Complex traits are strongly correlated with each other, often in clusters, which can lead to large amounts of measured and unmeasured confounding being present in EWAS. Of course, in order to produce therapies to prevent or treat disease by altering DNA methylation or other parts of the epigenome, causality must be established. Therefore, problems of confounding must be overcome in EWAS to use these results to start developing methods of targeting DNA methylation changes, this is discussed more in **Section 1.4.4**.

### **Cell type heterogeneity**

As discussed, epigenetic factors guide differentiation of a single pluripotent cell to hundreds of cell types in human development. As these cell types can have large differences in morphology and function, it is clear that epigenetic marks, including DNA methylation, will vary between cell types (75,76). This poses two distinct problems for EWAS. Firstly, when collecting samples to measure DNA methylation, unless cells are purified, then a pool of cell types will be present in the samples, each with their own distinct DNA methylation patterns. This can lead to issues of confounding by cell type. For example, in a case control study, cases may be more likely to have increased numbers of CD4+ Th2 immune cells and these cells may on average have a higher level of DNA methylation at site X. In this scenario if one were to take blood cells, measure DNA methylation, and assess the association between DNA methylation and the trait of interest, one might find an association between DNA methylation at site X and the trait, but this may just be function of the increased number of CD4+ Th2 cells present in cases and site X may have no causal relationship with the trait itself. There have been efforts to try and account for cell type heterogeneity in EWAS (75,77,78), but to completely prevent its confounding effects, cells should be collected from a homogenous tissue or purified. In addition to, generating false positives, this confounding could mask true effects found within specific cell types. The second problem arising from cell-type specific patterns of DNA methylation is the uncertainty that the cell type being studied is one in which DNA methylation covaries with the trait of interest. Non-invasive cells to collect, such as blood, skin, and saliva, are common amongst epidemiological studies, but it is unclear whether EWAS in these studies are relevant to a large proportion of complex traits. This is studied, with regards to blood, in **Chapter 5**. Studies have actually shown high levels of correlation between DNA methylation across

cell types at many CpGs (79), but it is unknown whether the correlated sites are important to trait variation. Further, this correlation may complicate interpretation of EWAS findings with regards to translational potential. Some organs, such as the brain, are difficult to target with therapies so even if a promising epigenetic target is identified, it may be almost impossible to translate this into something clinically useful.

### **Measuring DNA methylation**

DNA methylation arrays face certain technical issues. Some probes map to SNPs, which can lead to incorrect detection of DNA methylation, others map to multiple sites across the genome (i.e. are non-specific) and others may cross-hybridise. Batch effects can also substantially bias results in EWAS (2). Considerable effort has been made to characterise the arrays to identify potentially faulty probes (80,81) and methods developed (some originally for use in RNA-based studies) to help correct for batch effects (82–84). In **Chapter 4** I explore the extent to which batch effects tend to be removed in current EWAS and whether EWAS results are enriched for potentially faulty probes not removed by study authors.

### **Complexity of regulatory mechanisms**

EWAS identify single sites in the genome for which DNA methylation variation is associated with a trait of interest. As discussed, DNA methylation at a single site will likely be correlated with DNA methylation at neighbouring sites and other nearby epigenetic marks. This makes inferring mechanism of action very difficult. Differentially methylated region (DMR) analysis is often employed, which aims to determine if multiple neighbouring sites share an association with the trait of interest with the same direction of effect (40,76). These give evidence as to whether

the sites covary similarly with the trait of interest, but do not provide evidence that the sites are acting independently or not. There are ways to circumvent the issues of biological complexity, but without additional gene expression data these often involve assuming the genes immediately adjacent to DNA methylation changes are of importance to the trait. However, no systematic evaluation of whether this assumption holds true for the majority of cases has been conducted.

## **Treatments**

Currently there are therapies used in the clinic that target enzymes responsible for epigenetic alterations, for example DNA methyltransferase inhibitors and histone deacetylase inhibitors (85,86). They are primarily used to treat cancers, but as with many cancer treatments, are highly toxic. These therapies impact the epigenome globally and do not target any specific regions of the genome. This makes them highly undesirable for most diseases and as of yet there are no epigenetic therapies targeting specific regions of the genome. Methods, such as adapting CRISPR-cas9 enzymes, are being used in laboratories to alter DNA methylation at specific sites (87), and some have even achieved *in vivo* targeted epigenetic modulation in mice (88). However, it is unclear whether these techniques can be scaled up for clinical use in humans and how long it may take to overcome the various complications.

In summary, there is great potential for EWAS to identify sites in the genome that could be targeted for treatment, but there are several challenges still to overcome. A great importance should be placed on using the data available to inform future designs of EWAS to maximise the potential of these studies.

## **1.4 Using methods from genetics to help inform future EWAS**

In order to remedy some of the problems EWAS face and to help understand whether the “experiment” of measuring DNA methylation across many epidemiological cohorts and studies has been successful, we can borrow ideas and methods developed in genetics and genetic epidemiology.

Genetics is concerned with ascertaining the connection between traits and genetic variation. Germline genetic variants, the units of measurement for genome-wide association studies (GWAS), are fixed from conception and the association between these variants and complex traits tends to be unconfounded (89,90). Therefore, the properties of these variants and DNA methylation are different and one would expect the genetic and epigenetic architectures of complex traits to differ. However, genetic epidemiologists have had to overcome problems to help interpret GWAS, which are also pertinent to EWAS. These include understanding how much trait variation is captured by all the variants used in the study and how to infer function from genetic variation. Further, cataloging genetic associations has proven an invaluable resource for the research community (91) and these variants can be used as tools to augment the understanding of DNA methylation-trait associations. In this section I briefly describe some examples of these efforts and explain how they might be adapted to help inform future EWAS.

### **1.4.1 Catalogues of genome-wide associations**

Cataloging genome-wide associations has a broad range of applications for researchers, from replication of GWAS, to identifying overlapping GWAS signals between traits, to pooling the data to try and understand the genetic architecture of



complex traits as a whole. There are multiple databases now available to the genetic epidemiologist community that have catalogued these associations. These include manually curated databases of publicly available GWAS data, The GWAS Catalog, (91) and the IEU Open GWAS Project (92). A corollary database for EWAS is likely to also provide value for epigenetic epidemiologists. At the very least it would provide an easy tool to assess whether results replicate. Catalogues such as EWASdb (93) and the EWAS Atlas (94) are currently available but fall short of some key researcher requirements including ease of use and access to full summary statistics. The development of a new database, The EWAS Catalog, is the focus of **Chapter 3**.

### **1.4.2 Total variance captured by all sites measured genome-wide**

In addition to cataloguing the information gained, efforts also need to be made in understanding the epigenetic architecture of complex traits to enable interpretation of these data.

As discussed, the phenotypic value of any trait can be partitioned into genetic effects and environmental effects. Thus, the variation of phenotypic values are the function of the genetic variance ( $\sigma_G^2$ ) and environmental variance ( $\sigma_E^2$ ),

$$\sigma_z^2 = \sigma_G^2 + \sigma_E^2 \quad (1.2)$$

The environmental effects could be split into an infinite number of different factors, most of which would negligibly influence the phenotypic variance. Knowing the extent to which each factor contributes to phenotypic variation is important for two reasons. Firstly, if a factor substantially influenced phenotypic variation then by

modifying that factor one could modify the phenotypic values across a population. Secondly, one can identify which factors may best predict phenotypic values within a population. Currently, it is unknown how much variation in complex traits is attributable to DNA methylation changes. As discussed, assessing whether DNA methylation effects complex traits is difficult, but understanding whether they covary can still help quantify its total predictive capacity. Further, understanding how DNA methylation, as measured in contemporary EWAS, covaries with complex traits can help give insight on the validity of current study designs.

Many EWAS have been conducted and few have identified strong associations that capture substantial complex trait variation. Tissue types used and sites measured may partially explain this. It is a possibility that DNA methylation might not covary with many complex traits, or it could be that the associations between DNA methylation at individual sites are numerous, but the associations at each site are too small to detect with current sample sizes.

By combining information across all sites measured, one could quantify the total variation captured by DNA methylation for a complex trait of interest and so could properly assess the utility of association studies using the tissue types and sites measured.

Methods have already been developed to assess the total contribution of genetic variants measured in GWAS to complex trait variation (95,96) and in **Chapter 5**, I repurpose these methods developed to estimate the proportion of phenotypic variance correlated with DNA methylation across a range of phenotypes.

### 1.4.3 Inferring biology from signals

As discussed, the complexity of cellular processes makes it difficult to infer what consequences a change in methylation at a specific site may have. Similarly, for the majority of SNPs identified in GWAS, the functional change that relates to an association between genetic variation at that site and the trait of interest is unclear. Complex traits themselves are the result of a large number of complicated biological pathways that are determined by potentially thousands of gene products. It is often assumed that the signal from GWAS highlight genomic regions of importance to the trait and thus as a step to investigate the nature of the signal, sites identified are mapped to nearby genes. These genes can then be mapped to pathways and gene set enrichment analysis performed to assess whether the genes identified are present in any particular pathways more than expected by chance. This method has been adopted by epigenetic epidemiologists for use to examine EWAS signal (97). Given the DNA methylation probes on contemporary arrays were chosen specifically based on their proximity to protein coding genes, this gene mapping technique may actually be more valid for CpG sites.

Establishing causality from DNA methylation signal is difficult (See **Sections 1.3 1.4.4**). Thus, when applying gene set enrichment analyses to identify prominent pathways in EWAS signals, the pathways identified may be downstream consequences of one or many confounders rather than of aetiological relevance to the trait of interest. Further, the EWAS signals, and therefore pathways they might influence, may be a consequence of trait variation. This is important to remember when interpreting the results of such an analysis, but it doesn't render the results useless. There is a huge body of work that characterises gene action and relationships of this gene action with various traits. By mapping EWAS signals to genes and pathways, a path between the trait (or a confounder) and changes in DNA methylation might become

clearer. One example of this comes with EWAS of smoking, that have consistently identified DNA methylation at the *AHRR* gene (65,98–101). This gene is known to play a role in handling toxic substances found in tobacco smoke (102,103). Thus, large changes in DNA methylation related to this gene points towards epigenetic changes at that locus influencing the cellular response to smoking. This shows, that despite difficulties in interpreting EWAS findings and subsequent pathway analysis, EWAS can actually add to the pool of information about underlying trait biology when used in conjuncture with other evidence.

Understanding both the causes and consequences of complex traits are pertinent to intervening on health outcomes. As EWAS has the potential to identify both, it could identify important facets of trait biology missed by GWAS studies; however, it is unclear as to whether the analogous gene set enrichment design adopted by EWAS is currently adding to the information discovered by GWAS. In **Chapter 6** I compare overlap of GWAS and EWAS signals in this context and discuss the use of both study designs in elucidating underlying trait biology.

#### **1.4.4 Establishing causality**

##### **Mendelian randomization**

As discussed, population-based studies of DNA methylation suffer from the same limitations as any observational epidemiology study, namely confounding and reverse causation. One method that aims to mitigate confounding is Mendelian randomization (MR) (89,90,104), which uses genetic variants as proxies for the exposure of interest in an instrumental variable framework (illustrated in **Figure 1.4**). Using genetic variants as instruments has the advantage that the direction of effect will always be from instrument to exposure and not vice versa, making interpretation of the studies simpler. Furthermore, unlike environmental phenotypes, that tend to

be highly correlated and clustered into groups, genetic variants associated with a trait tend to be unconfounded (89,90). In the absence of assortative mating, genetic variants should be distributed randomly across the population, so in effect those grouped by genotype should exhibit differences in exposure, but confounding factors should not differ between genotype groups (90). Assortative mating has been reliably shown to occur with some traits such as social behaviours and anthropometric measures (105–107). Assortment tends to occur on visible social factors and so intentional assortment based on DNA methylation profiles is very unlikely. However, DNA methylation may associate with factors that are assorted on, for example alcohol consumption (108,109), which may lead to unintentional assortment on DNA methylation profiles. The impact this may have on MR studies using DNA methylation has not been explored, and this analysis is beyond the scope of this thesis but is something that should be noted when assessing the reliability of such MR studies.

### **Availability of data for MR**

Another advantage of MR is the data it uses. Thousands of GWAS have been conducted giving researchers ample instruments for a wide variety of traits and many of these instruments are easily accessible through databases such as the GWAS Catalog (91) and IEU Open GWAS Project (92). Furthermore, it isn't necessary to use individual-level data to conduct MR studies; summary statistics from GWAS are all that is needed to provide data in a two-sample MR framework (110,111). This is especially valuable to conducting MR studies using DNA methylation data as DNA methylation is not widely measured across cohorts and case-control studies. Thus, without a method to combine summary data from both GWAS of DNA methylation and GWAS of other complex traits, well-powered MR studies would

not be possible to assess the potential effect of DNA methylation on complex traits (and *vice versa*).

### **Assumptions of MR**

In order for MR analyses to be valid, they must satisfy three instrumental variable assumptions, these are illustrated in **Figure 1.4**. Testing assumption one, the instruments associate with the exposure of interest, is simple, but the other two assumptions cannot technically be proven to be true. Horizontal pleiotropy, where genetic variants associate with more variables than just the exposure of interest, can lead to violations in assumptions two and three. Ideally, MR would be performed in the context where the genetic effect on the exposure had been characterised such that the mechanism of action was understood clearly. This would help give evidence against assumptions two and three being broken. Unfortunately, this is rarely possible. However, a plethora of methods have now been developed to test for pleiotropic effects, given the exposure of interest has multiple independent genetic variants reliably associated with it.

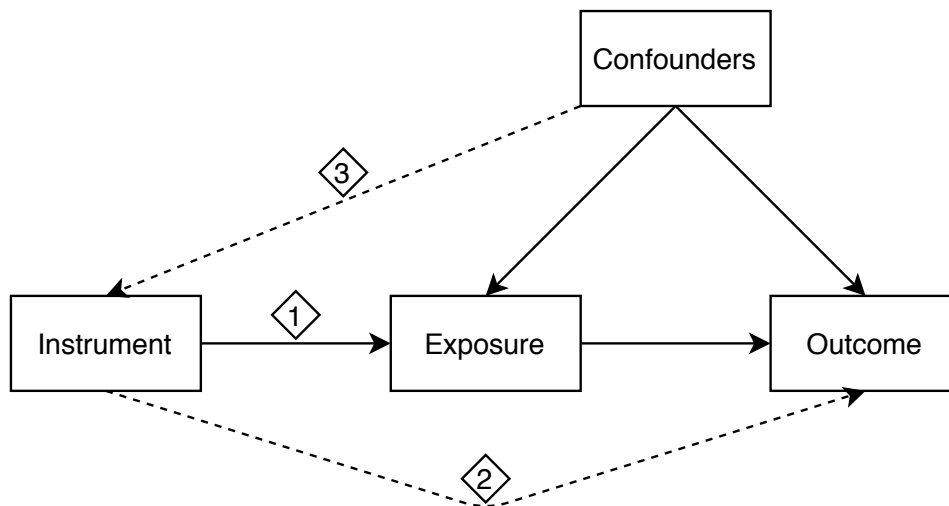


Figure 1.4: Mendelian randomization. Mendelian randomization can be used to test the causal nature of exposure-outcome relationships provided the assumptions are met. Assumption 1. There is an association between the instrument and the exposure. Assumption 2. There are no associations between the instrument and outcome, except through the exposure. Assumption 3. The instrument is not associated with any factors that confound the exposure-outcome relationship.

### Applying MR in a DNA methylation context

MR can be applied to studies of DNA methylation by using methylation quantitative trait loci (mQTL), genetic variants associated with changes in DNA methylation levels, as proxies for DNA methylation variation (50,112,113). As mentioned previously, using a two-sample MR framework is especially useful to help increase power for these studies (50). For each DNA methylation site few independent mQTLs have been identified (3), which prevents the use of various tests to examine whether the instruments are likely to be pleiotropic. Further, without reliable associations between SNPs and DNA methylation at some sites, MR cannot be conducted. Both cis-mQTLs (mQTLs within 1Mb of the DNA methylation site) and trans-mQTLs (mQTLs over 1Mb away from the DNA methylation site) have

been identified in GWAS of DNA methylation variation. As genetic architecture of DNA methylation changes is also not well understood, the mechanism of action for each mQTL can only be speculated at present. Cis-mQTLs are thought to be less likely to be pleiotropic than trans-mQTLs as the mechanism of action seems more likely to be related to the binding of regulatory machinery that may influence DNA methylation levels (112,114). For example, a genetic variant may decrease the affinity of a transcription factor for that region and so the transcription factor will bind less frequently and/or for a shorter period, this would lead to increased methylation at that site (115). On the contrary, the mechanism of trans-mQTL action, especially those on separate chromosomes to the DNA methylation site of interest, is more likely to be pleiotropic (114), for example a trans-mQTL could influence gene expression of a transcription factor that binds many sites and alters their DNA methylation (116), this would make the trans-mQTL associate with multiple DNA methylation sites. Therefore, if one limits mQTLs to those in cis, this gives greater confidence that horizontal pleiotropy isn't biasing results.

Due to the complexity of cellular regulatory mechanisms (**Section 1.2**), it may be impossible to identify the exact cause of changes in complex traits, even with replicated MR results that give strong evidence of an effect of DNA methylation on a complex trait.

As mentioned, DNA methylation varies both temporally and across cell types. If the instruments used to represent DNA methylation are viewed as influencing life-time differences in DNA methylation between individuals, then temporal variation can be ignored. However, cell type-specific effects are likely to occur for some mQTLs. One could imagine a scenario in which blood cells require a specific gene (gene A) to be expressed that is completely useless in adipose cells, which could mean all adipose cells have 100% DNA methylation at the promoter region of gene A. Thus,



any genetic variants that associate with DNA methylation variation in blood would not also associate with variation in adipose cells.

With all this in mind, it is important to maintain the idea that making strong conclusions of causality in the context of DNA methylation is difficult, but triangulating evidence from multiple sources could be key to understanding the role of DNA methylation in underlying trait biology (117).

Such evidence can come from different sources of statistical methodology that can be used to assess causality in observational studies (118–120). One that has been applied in EWAS includes taking advantage of temporal measurements (121). If DNA methylation is measured before the trait of interest, then chances of reverse causation are greatly diminished, although confounding may still be an issue. DNA methylation associations may also be tested across different, relevant tissue types and molecular biology can be used to augment epidemiological evidence. Tools exist to experimentally manipulate DNA methylation at specific regions of the genome in cultured cells or in model organisms (87,88). Using a negative control design, one could follow up any findings from an epidemiological study like an EWAS, in the laboratory by assessing if changes in the DNA methylation sites identified have any intracellular impact. This could be done for a variety of tissue types of interest.

To truly provide strong evidence that changes in DNA methylation is causally related to a trait, one must take a cross-disciplinary approach.

## **1.5 Overview of thesis aims**

DNA methylation has great potential for use in an epidemiological sense and as samples with DNA methylation data continue to grow it is important to understand

the limitations of EWAS and how to maximise it's potential. My thesis aims to address this by exploring what information has been gained from EWAS (**Chapters 3 and 4**), what information is still to gain from EWAS (**Chapter 5**), whether EWAS might add to our biological understanding of complex traits above GWAS (**Chapter 6**) and by applying MR in a particular case, the potential for confounding in EWAS (**Chapter 7**). See the flowchart in **Figure 1.5** for a graphical depiction.

In **Chapter 3** a database of published EWAS is curated and made publicly available, which will be used in later chapters. The aim of **Chapter 4** is to analyse the results present in this database jointly to allow the discovery of commonalities across methylome-trait associations and provide a platform to explore what is driving these commonalities. Further, the chapter explores the extent to which published results are reliable by assessing replication rate and whether sites measured by unreliable probes are prominent.

After exploring the information already gained from EWAS, **Chapter 5** investigates the information still to gain from EWAS. The aim of the chapter is to apply methods developed to assess SNP-heritability to estimate the proportion of complex trait variation that is associated with sites commonly measured in EWAS.

**Chapter 6** will then aim to assess whether the discoveries of EWAS may provide extra biological insight for traits of interest on top of those from GWAS. Tests will be applied to assess whether there is more overlap between the sites, genes or pathways identified by some large EWAS ( $N > 4500$ ) and their corresponding GWAS than expected by chance.

Finally, **Chapter 7** will apply MR to explore the causal nature of associations between DNA methylation and lung cancer. This application case-study will compare and contrast findings to conventional EWAS estimates to give an example of the

potential residual confounding that can be present in EWAS.

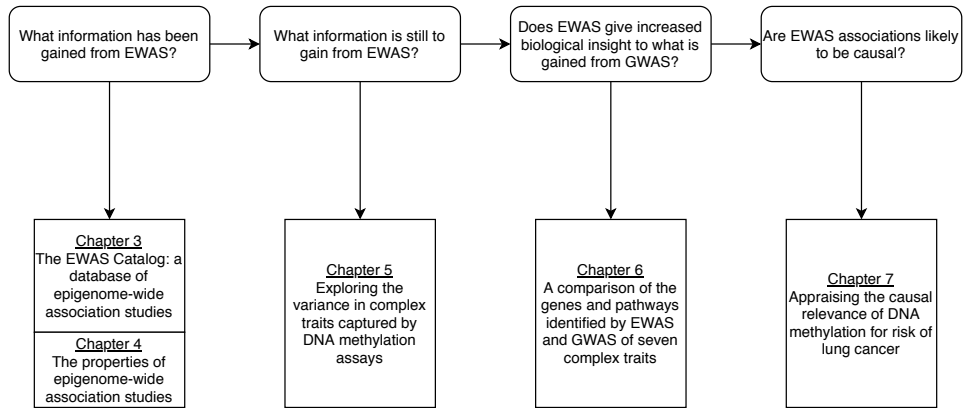


Figure 1.5: Flowchart showing questions being asked in the thesis and the corresponding chapters attempting to help answer them.

## **Chapter 2**

### **Methods**

## **Chapter 3**

# **The EWAS Catalog: a database of epigenome-wide association studies**

### **3.1 Abstract**

Epigenome-wide association studies (EWAS) seek to understand the link between patterns of DNA methylation, the addition of a methyl group to a DNA molecule that may change how the molecule interacts with other cellular factors, at thousands or millions of sites across the genome to various traits and exposures. In recent years, the increase in availability of DNA methylation measures in population-based cohorts and case-control studies has resulted in a dramatic increase in the number of EWAS being performed and published. To make this rich source of molecular data more accessible, a manually curated database has been made containing CpG-trait associations (at  $P < 1 \times 10^{-4}$ ) from published EWAS, each assaying over 100,000

CpGs in at least 100 individuals. The database currently contains these associations from 178 published EWAS as well as full summary statistics for over 180 million association tests of 427 EWAS in the Avon Longitudinal Study of Parents and Children (ALSPAC) and the Gene Expression Omnibus (GEO). It is accompanied by a web-based tool and R package that allow these associations to be easily queried. This database will give researchers the opportunity to quickly and easily query EWAS associations to gain insight into the molecular underpinnings of disease as well as the impact of traits and exposures on the DNA methylome. The EWAS Catalog is available at: <http://www.ewascatalog.org>.

## **3.2 Introduction**

Epigenome-wide association studies (EWAS) aim to assess the associations between phenotypes of interest and DNA methylation across the genome (50,63,122). These associations may then be used for disease diagnosis or prediction (50,63,122). Also, unlike genetic variants, changes in DNA methylation are responsive to the environment and so may be targeted for treatment. EWAS of smoking (65), body mass index (BMI) (67) and aging (72) have shown that various exposures are related to large perturbations in DNA methylation across the genome. Furthermore, a paper recently estimated that over 60% of the total proportion of BMI variation was captured by DNA methylation at about 150 CpG sites (123). In recent years, there has been a dramatic increase in the number of EWAS being performed and published due to technological advancements making it possible to measure DNA methylation at hundreds of thousands of CpG sites cheaply and effectively. Giving researchers easy access to EWAS outputs will help them gain insight into the molecular underpinnings of disease as well as the impact of traits and exposures on the DNA methylome. Furthermore, current collections of summary statistics have

already proven useful to various fields, for example the GWAS Catalog (91) has been cited over 2000 times in papers contributing to new methods and exploring the genetic architecture of a plethora of traits.

At the time of making the database, to our knowledge, there were no databases that had collected well-curated EWAS on all traits (no just diseases) in an online database accessible to researchers. During production one database fulfilled those metrics: EWAS Atlas (94). Other databases are available but are limited to certain diseases (e.g. MethHC (124)). The EWAS Atlas provides a simple-to-use website with annotated CpG sites and information on traits. Ideally a database of EWAS results will provide summary statistics, including betas, standard errors and p-values where provided from publications, in an easily accessible manner, this enables researchers to explore various aspects of the published data without having to retrieve the published article. For example, researchers might compare effect estimates between studies in the database or check to see if their results are replicated in another published study. At the time of writing the EWAS atlas platform did not enable users to download effect estimates and standard errors. Another caveat is that there is currently only published data on the platform, not full summary statistics from EWAS.

The EWAS Catalog aims to improve upon current databases to 1) allow easy and programmatic access to summary statistics for downstream analyses by researchers and 2) provide full summary statistics from a range of EWAS conducted in multiple cohorts. To this end we have produced The EWAS Catalog, a manually curated database of currently published EWAS, 387 EWAS performed in the Avon Longitudinal Study of Parents and Children (ALSPAC) (125,126) and 40 EWAS performed from data from the Gene Expression Omnibus (GEO) database. The process and data inclusion are summarised in **Figure 3.1**.

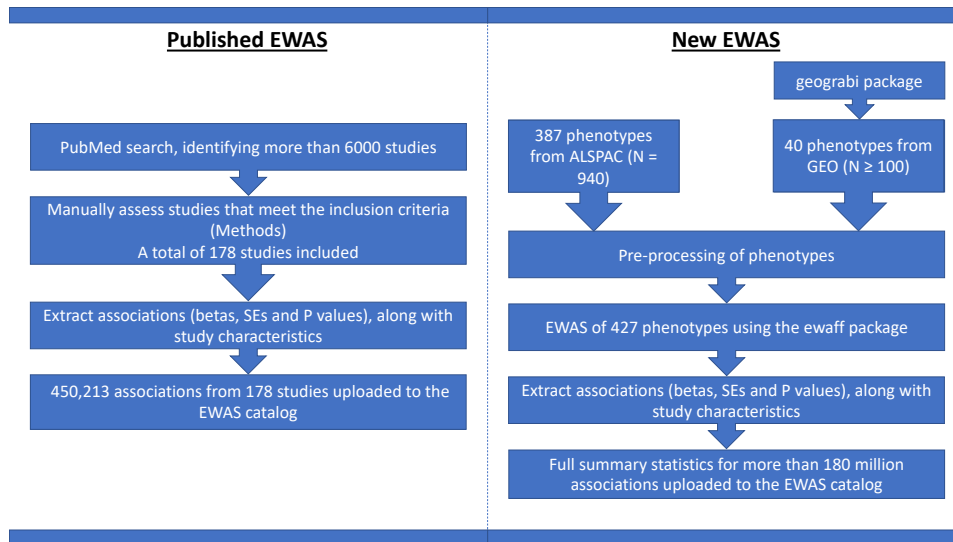


Figure 3.1: **Project flowchart.** On the left is a brief description of how the CpG-phenotype associations were assembled from published works and on the right is a brief description of the EWAS performed using individual level data.

In this chapter, Dr James Staley built the original website, Dr Matthew Suderman has been key in development and maintenance of the website and there was a team to help gather and input the data. I helped develop and maintain the website, gather and input the data, run the EWAS within the ALSPAC cohort and on data from the GEO database. The team, led by myself, is continuing to develop and maintain the database. Full acknowledgements to the team can be found on the website: <http://www.ewascatalog.org/about/>.

## 3.3 Methods

### 3.3.1 Implementation

The EWAS Catalog web app was built using the Django Python package (<https://djangoproject.com>). The data is stored in a combination of MySQL



databases and fast random access files (64) and can be queried via the web app or the R package ([www.github.com/ewascatalog/ewascatalog-r/](https://www.github.com/ewascatalog/ewascatalog-r/)).

### **3.3.2 Overview of publication data extraction**

To identify publications, periodic literature searches are performed in PubMed using the search terms: “epigenome-wide” OR “epigenome wide” OR “EWAS” OR “genome-wide AND methylation” OR “genome wide AND methylation”.

Our criteria for inclusion of a study into The EWAS Catalog are as follows:

1. The EWAS performed must contain over 100 humans
2. The analysis must contain over 100,000 CpG sites
3. The DNA methylation data must be genome-wide
4. The study must include previously unpublished EWAS summary statistics

CpG-phenotype associations are extracted from studies at  $P < 1 \times 10^{-4}$ . All these criteria along with the variables extracted are documented on the website ([www.ewascatalog.org/documentation](http://www.ewascatalog.org/documentation)). Experimental factor ontology (EFO) terms were mapped to traits to unify representation of these traits. These EFO terms were manually entered after looking up the trait in the European Bioinformatics Institute database ([www.ebi.ac.uk/efo](http://www.ebi.ac.uk/efo)).

Based on these criteria, from 2020-08-27, The EWAS Catalog contained 450213 associations from 605 studies.

### **3.3.3 Overview of GEO data extraction**

To recruit additional datasets suitable for new EWAS analysis, the *geograbi* R package (<https://github.com/yousefi138/geograbi>) was used to both query GEO for experiments matching The EWAS Catalog inclusion criteria (de-

scribed above) and extract relevant DNA methylation and phenotype information. The query was performed on 20 March 2019 and identified 148 such experiments with 32,845 samples where DNA methylation and phenotype information could be successfully extracted. From these, the aim was to repeat the analyses performed in the publications linked by PubMed IDs to each GEO record. Thus, I looked up the corresponding full texts for each dataset and identified the main variables of interest. Of the 148 putative GEO studies, only 34 (23%) contained sufficient information to replicate the original analysis.

### **3.3.4 EWAS methods**

#### **Avon Longitudinal Study of Parents and Children (ALSPAC)**

EWAS were conducted for 387 continuous and binary traits in peripheral blood DNA methylation of ALSPAC mothers in middle age ( $N = 940$ ), generated as part of the Accessible Resource for Integrated Epigenomics Studies (ARIES) project (127).

ALSPAC recruited pregnant women in the Bristol and Avon area, United Kingdom, with an expected delivery date between April 1991 and December 1992 (<http://www.bris.ac.uk/alspac/>). Over 14,000 pregnancies have been followed up (both children and parents) throughout the life-course. Full details of the cohort have been published previously (125,126). The EWAS performed for the EWAS catalog were done so using phenotypic and DNA methylation data from the mothers ( $N = 940$ ). All continuous and binary phenotypes were extracted from the same timepoint that blood was drawn for DNA methylation assays.

Ethical approval for ALSPAC was obtained from the ALSPAC Ethics and Law Committee and from the UK National Health Service Local Research Ethics Committees.

Written informed consent was obtained from both the parent/guardian and, after the age of 16, children provided written assent. The study website contains details of all the data that is available through a fully searchable data dictionary (<http://www.bristol.ac.uk/alspac/researchers/access/>).

Study data were collected and managed using REDCap electronic data capture tools hosted at ALSPAC (128,129). REDCap (Research Electronic Data Capture) is a secure, web-based software platform designed to support data capture for research studies, providing 1) an intuitive interface for validated data capture; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for data integration and interoperability with external sources.

Ancestry principal components were generated within ALSPAC mothers using PLINK (v1.9). Before analysis, genetic data went through quality control and were imputed as follows.

Mothers were genotyped using the Illumina human660W-quad genome-wide single nucleotide polymorphism (SNP) genotyping platform (Illumina Inc., San Diego, CA, USA) at the Centre National de Génomique (CNG; Paris, France). SNPs were removed if they displayed more than 5% missingness or a Hardy-Weinberg equilibrium P value of less than  $1.0 \times 10^{-6}$ . Additionally, SNPs with a minor allele frequency of less than 1% were removed. Samples were excluded if they displayed more than 5% missingness, had indeterminate X chromosome heterozygosity or extreme autosomal heterozygosity. Samples showing evidence of population stratification were identified by multidimensional scaling of genome-wide identity by state pairwise distances using the four HapMap populations as a reference, and then excluded. Cryptic relatedness was assessed using a IBD estimate of more than 0.125 which is expected to correspond to roughly 12.5% alleles shared IBD or a

relatedness at the first cousin level. Related subjects that passed all other quality control thresholds were retained during subsequent phasing and imputation.

Imputation of mother's genotype data in ALSPAC was done with ALSPAC children's data. So, genotypes in common between the sample of mothers and sample of children were combined. SNPs with genotype missingness above 1% due to poor quality were removed along with subjects due to potential ID mismatches. We estimated haplotypes using ShapeIT (v2.r644) which utilises relatedness during phasing. We obtained a phased version of the 1000 genomes reference panel (Phase 1, Version 3) from the Impute2 reference data repository (phased using ShapeIt v2.r644, haplotype release date Dec 2013). Imputation of the target data was performed using Impute V2.2.2 against the reference panel (all polymorphic SNPs excluding singletons), using all 2186 reference haplotypes (including non-Europeans).

After quality control and imputation, independent SNPs ( $r^2 < 0.01$ ) were used to calculate the top 10 ancestry principal components.

For all traits, linear regression models were fitted with DNA methylation at each site as the outcome and the phenotype as the exposure. DNA methylation was coded as beta values between 0 and 1. For a particular site, a beta value of 0 represents no methylation being detected in all cells measured and a value of 1 represents all cells being methylated at that site. Covariates included age, the top 10 ancestry principal components, and 20 surrogate variables.

### **Transforming phenotypic data**

Values of continuous phenotypes were defined as outliers and set to missing, then all phenotypes with 100 or more non-missing values were kept for further analysis. To ensure all phenotypes were approximately normal, each of their distributions

were examined and then transformed. If a variable was deemed right-skewed, it was log-transformed then its distribution was re-assessed by eye. Square-roots and cube-roots were used to try and approximate normality if log-transformation did not work. If a variable was deemed left-skewed, it was squared, and the distribution re-assessed by eye.

### **EWAS statistical models**

For all traits, linear regression models were fitted with DNA methylation as the outcome and the phenotype as the exposure as for the ARIES data. Twenty surrogate variables were included as covariates. Other covariates were considered, but surrogate variables only were used for two reasons: 1) to help automate the process and 2) because covariates used in the original EWAS were not included with many of the GEO datasets.

Statistical analyses were conducted in R (Version 3.3.3). The `smartsva` package (130) was used to create surrogate variables and the `ewaff` R package (<https://github.com/perishky/ewaff>) was used to conduct the EWAS, all p-values are two-sided.

## **3.4 Results**

### **3.4.1 Database interface and use**

There are two ways to access this large, curated database: through the main website [www.ewascatalog.org](http://www.ewascatalog.org) or by using the R package “`ewascatalog`”. The website provides a simple user interface, which resembles that of the GWAS catalog (91), whereby there is a single search bar to explore the database and links to tabs that contain documentation on the contents and how to cite its use (Figure 1). Users may

enter a CpG, gene, genome position or trait into the search bar and it will rapidly return detail for relevant EWAS associations, including CpG, trait, sample size, publication and association (effect and P value) (Figure 1). This information along with additional information such as ancestry, outcome, exposure units, and tissue analysed are available for download as a tab-separated value (tsv) text file. Unlike other EWAS databases, we provide the option of downloading summary results for both the user's search and for the entire database.

The screenshot displays the EWAS Catalog website interface. At the top is a navigation bar with links: EWAS Catalog, Search, About, Documentation, and Download. The main header features the EWAS Catalog logo and the text 'The MRC-IEU catalog of epigenome-wide association studies'.

Below the header, there are three search examples:

- Search CpG sites:** A search bar containing 'cg00029284' with a magnifying glass icon.
- Search Genes:** A search bar containing 'FTO' with a magnifying glass icon.
- Search Traits:** A search bar containing 'Depression' with a magnifying glass icon.

A large blue button labeled 'Search' is positioned below the first search example.

The results section is titled 'Results' and shows a table of search results for 'Depression'. The table has columns: Author, PMID, Outcome, Exposure, Analysis, N, CpG, Location, Gene, Beta, and P. There are 3 entries displayed.

Author	PMID	Outcome	Exposure	Analysis	N	CpG	Location	Gene	Beta	P
Bostrom AE	28618313	DNA methylation	Depression	-	93	cg13227621	chr1:45671361	ZSWIM5	-0.22	8.9E-09
Bostrom AE	28618313	DNA methylation	Depression	-	93	cg04102384	chr6:31668622	BAT5	-0.59	1.1E-08
Weder N	24655651	Depression	DNA methylation	-	190	cg03535461	chr1:23885568	ID3	NA	5.4E-08

Below the table, it says 'Showing 1 to 3 of 3 entries'. There are 'Previous' and 'Next' buttons. A 'Download' button with a download icon is circled in red.

Figure 3.2: Using the EWAS catalog. At the top of the figures is the home page URL, ewascatalog.org. Below that are examples of three types of searches possible: 1. CpG sites, 2. genes and 3. traits. Finally, the results are displayed after searching the catalog for “Depression”. Circled in red is the download button, this button enables the user to download the results of their search as a tab-separated value file. This file will contain the information shown on the website as well as additional analysis information.

The R package, along with installation instructions and examples are available at <https://github.com/ewascatalog/ewascatalog-r/>. Once installed, the database can be queried directly in R using the “ewascatalog()” function similar to the website: simply supply the function with a CpG site, gene, genome position or trait and the function returns the same output as is downloadable from the website.

### 3.5 Discussion

In this chapter, a database of previously published EWAS and the full summary statistics of 427 newly performed EWAS within ALSPAC and GEO has been established. This is freely available for all researchers to use and provides a platform to explore what information has been gained from EWAS as well as a platform that can be used to pool all existing data to gain new insights into both the EWAS study itself and how DNA methylation associates with traits. Despite the fact The EWAS Atlas has similar aims to The EWAS Catalog, the latter provides full summary statistics, extra information and a user-friendly platform to enable more downstream analyses.

The EWAS catalog team will continue to collate and upload newly published EWAS and further increase the number of full summary statistics on the website by performing additional EWAS on available datasets and by inviting EWAS authors to provide full summary statistics. Currently work is ongoing to include additional functionality to allow users to easily and systematically compare their EWAS findings to EWAS in the database. With this full summary data, it is possible to make greater strides into discovering the epigenetic architecture of traits.

Therefore, despite the fact no extra information about EWAS was presented in this



chapter, a platform has been made that enables us to explore 1) what information has been gained from EWAS and 2) what could explain EWAS associations. This will be explored in the next chapter.

## **Chapter 4**

# **Properties of EWAS**

Here is a reference to Caroline's paper: (50)

## **Chapter 5**

**h2ewas**

## **Chapter 6**

# **EWAS-GWAS comparison**

## **Chapter 7**

# **Appraising the causal relevance of DNA methylation for risk of lung cancer**

### **7.1 Abstract**

#### **7.1.1 Background**

DNA methylation changes in peripheral blood have recently been identified in relation to lung cancer risk. Some of these changes have been suggested to mediate part of the effect of smoking on lung cancer. However, limitations with conventional mediation analyses mean that the causal nature of these methylation changes has yet to be fully elucidated.

### **7.1.2 Methods**

We first performed a meta-analysis of four epigenome-wide association studies (EWAS) of lung cancer (918 cases, 918 controls). Next, we conducted a two-sample Mendelian randomization analysis, using genetic instruments for methylation at CpG sites identified in the EWAS meta-analysis, and 29,863 cases and 55,586 controls from the TRICL-ILCCO lung cancer consortium, to appraise the possible causal role of methylation at these sites on lung cancer.

### **7.1.3 Results**

16 CpG sites were identified from the EWAS meta-analysis ( $FDR < 0.05$ ), 14 of which we could identify genetic instruments for. Mendelian randomization provided little evidence that DNA methylation in peripheral blood at the 14 CpG sites play a causal role in lung cancer development ( $FDR > 0.05$ ), including for cg05575921-*AHRR* where methylation is strongly associated with both smoke exposure and lung cancer risk.

### **7.1.4 Conclusions**

The results contrast with previous observational and mediation analysis, which have made strong claims regarding the causal role of DNA methylation. Thus, previous suggestions of a mediating role of methylation at sites identified in peripheral blood, such as cg05575921-*AHRR*, could be unfounded. However, this study does not preclude the possibility that differential DNA methylation at other sites is causally involved in lung cancer development, especially within lung tissue.

## 7.2 Introduction

Lung cancer is the most common cause of cancer-related death worldwide (131). Several DNA methylation changes have been recently identified in relation to lung cancer risk (121,132,133). Given the plasticity of epigenetic markers, any DNA methylation changes that are causally linked to lung cancer are potentially appealing targets for intervention (134,135). However, these epigenetic markers are sensitive to reverse causation, being affected by cancer processes (135), and are also prone to confounding, for example by socio-economic and lifestyle factors [(136); Elliott2014].

One CpG site, cg05575921 within the aryl hydrocarbon receptor repressor (*AHRR*) gene, has been consistently replicated in relation to both smoking (65) and lung cancer (101,121,132) and functional evidence suggests that this region could be causally involved in lung cancer (137). However, the observed association between methylation and lung cancer might simply reflect separate effects of smoking on lung cancer and DNA methylation, i.e. the association may be a result of confounding (104), including residual confounding after adjustment for self-reported smoking behaviour (138,139). Furthermore, recent epigenome-wide association studies (EWAS) for lung cancer have revealed additional CpG sites which may be causally implicated in development of the disease (121,132).

Mendelian randomization (MR) uses genetic variants associated with modifiable factors as instruments to infer causality between the modifiable factor and outcome, overcoming most unmeasured or residual confounding and reverse causation (89,90). In order to infer causality, three core assumptions of MR should be met: 1) The instrument is associated with the exposure, 2) The instrument is not associated with any confounders, 3) The instrument is associated with the outcome only through the

exposure. MR may be adapted to the setting of DNA methylation (112,127,140) with the use of single nucleotide polymorphisms (SNPs) that correlate with methylation of CpG sites, known as methylation quantitative trait loci (mQTLs) (3).

In this study, we performed a meta-analysis of four lung cancer EWAS (918 case-control pairs) from prospective cohort studies to identify CpG sites associated with lung cancer risk and applied MR to investigate whether the observed DNA methylation changes at these sites are causally linked to lung cancer.

In this chapter, Dr Rebecca Richmond performed analysis in the CCHS cohort (see section Potential causal effect of *AHRR* methylation on lung cancer risk: one sample MR of the results) and contributed to writing the introduction and discussion. I completed all the analysis and wrote each section except the methods and results sections for which I did not complete the analysis.

## **7.3 Methods**

### **7.3.1 EWAS study details**

A meta-analysis of four lung cancer case-control EWAS was conducted to identify DNA methylation sites associated with lung cancer. DNA methylation in each EWAS was assessed using the Illumina Infinium® HumanMethylation450 Bead-Chip. All EWAS are nested within prospective cohorts that measured DNA methylation in peripheral blood samples before diagnosis: EPIC-Italy (185 case-control pairs), Melbourne Collaborative Cohort Study (MCCS) (367 case-control pairs), Norwegian Women and Cancer (NOWAC) (132 case-control pairs) and the Northern Sweden Health and Disease Study (NSHDS) (234 case-control pairs). Study populations, laboratory methods, data pre-processing and quality control methods have been described in detail elsewhere (121) 3 and are outlined below.



At the various laboratory sites, samples were distributed into 96-well plates and processed in chips of 12 arrays (8 chips per plate) with case-control pairs arranged randomly on the same chip. Methylation data were pre-processed and normalized in each study, and probe filtering was performed as previously described (121), leaving 465,886 CpGs suitable for the analysis in EPIC-Italy, 485,330 CpGs in MCCS, 450,890 CpGs in NOWAC and 482,867 CpGs in NSHDS.

### **European Prospective Investigation into Cancer and Nutrition-Italy (EPIC-Italy)**

EPIC-Italy includes 47,749 volunteers (32,579 women) aged 35–70 years at the time of recruitment (1992–1998). Anthropometric measurements and lifestyle variables including detailed information on smoking history were collected at recruitment through standardized questionnaires, together with a blood sample. Within EPIC-Italy we conducted a nested case-control study utilizing incident cases diagnosed within follow-up and healthy controls individually matched to cases by gender, date of birth ( $\pm 5$  years), date of inclusion in the study and study centre. Analysis was performed for 185 incident cases diagnosed within follow-up and matched controls. Laboratory procedures were carried out at the Human Genetics Foundation (Turin, Italy) and DNA extracted from buffy coats as previously described (121). All participants signed an informed consent form, and the ethical review boards of the International Agency for Research on Cancer and of each local participating centre approved the study protocol.

### **Melbourne Collaborative Cohort Study (MCCS)**

The MCCS is a prospective cohort study of 41,514 volunteers (24,469 women) aged between 27 and 76 years at baseline (1990-1994). At baseline attendance,

participants completed questionnaires that measured demographic characteristics and lifestyle factors. Height and weight were directly measured, and a blood sample was collected and stored. Incident cases of lung cancer were identified through linkage with the State and National Cancer Registries during follow-up up to the end of 2011. The MCCS sample included 367 cases and 367 matched controls selected from MCCS participants who were lung cancer free at the age of diagnosis of the matching case (density sampling). Matching variables included gender, date of blood collection (within 6 months), date of birth (within 1 year), country of birth (Australia and UK versus Southern Europe), type of biospecimen (lymphocyte, buffy coat and dried blood spot) and smoking status (never smokers; short-term former smokers: quitting smoking less than 10 years before blood draw; long-term former smokers: quitting smoking 10 years or more before blood draw; current light smokers: less than 15 cigarettes per day at blood draw; and current heavy smokers: 15 cigarettes or more at blood draw). For the MCCS, laboratory procedures were carried out at the Genetic Epidemiology Laboratory, the University of Melbourne according to manufacturers' protocols. DNA extraction from lymphocytes and buffy coats was performed as previously described (121). The Cancer Council Victoria's Human Research Ethics Committee approved the study protocol. Subjects gave written consent to participate and for the investigators to obtain access to their medical records.

### **Norwegian Women and Cancer (NOWAC)**

The biobank of the NOWAC cohort was established in the years 2003-2006. Those who filled in an eight-page questionnaire and accepted the invitation to donate blood were sent blood drawing equipment together with a two-page epidemiological questionnaire. Around 50 000 women returned two tubes of blood to the Institute of

Community Medicine at UiT The Arctic University of Norway and data linkage to the National Cancer Registry of Norway was performed. During follow-up to the end of 2011, 132 eligible cases of lung cancer were identified and were used for the EWAS. For each case, one control with an available blood sample was selected and matched on time since blood sampling and year of birth in order to control for effects of storage time and ageing. The cases and the controls were processed together for all laboratory procedures in order to reduce any batch effect. Laboratory procedures were carried out at the Human Genetics Foundation (Turin, Italy). DNA extraction from buffy coats was performed as previously described (121). All participants gave informed consent. The study was approved by the Regional Committee for Medical and Health Research Ethics in North Norway. Data storage and linkage was approved by the Norwegian Data Inspectorate.

#### **Northern Sweden Health and Disease Study (NSHDS)**

NSHDS is an ongoing prospective cohort and intervention study intended for health promotion of the population of Västerbotten County in northern Sweden. All residents were invited to participate by attending a health check-up at their local health care centre at 40, 50 and 60 years of age. At the health check-up, participants were asked to complete a self-administered questionnaire covering various factors such as education, smoking habits, physical activity and diet. In addition, height and weight were measured and participants were asked to donate a blood sample. Incident lung cancer cases were identified through linkage to the regional cancer registry. One control was chosen at random for each lung cancer case from appropriate risk sets consisting of all cohort members alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of the index case. Matching criteria were the same as for the MCCS except there was

no matching for type of biospecimens as DNA was extracted from whole blood for all samples. After quality control, a total of 234 incident lung cancer cases and 234 individually matched controls were available for this analysis. Laboratory procedures for NSHDS were carried out at two sites. DNA extraction from the buffy coat was conducted at Umeå University, Sweden, as previously described. Illumina Infinium HumanMethylation450 BeadChip analysis was conducted at the ALSPAC/IEU Laboratory at the University of Bristol. All study subjects provided written informed consent at time of the recruitment into the NSHDS.

### **7.3.2 EWAS Meta-analysis**

To quantify the association between the methylation level at each CpG and the risk of lung cancer conditional logistic regression models were fitted for beta values of methylation (which ranges from 0 (no cytosines methylated) to 1 (all cytosines methylated)) on lung cancer status for the four studies. The cases and controls in each study were matched, details can be found above. Surrogate variables were computed in the four studies using the SVA R package (141) and the proportion of CD8+ and CD4+ T cells, B cells, monocytes, natural killer cells and granulocytes within whole blood were derived from DNA methylation (75). The following EWAS models were included in the meta-analysis: Model 1 – unadjusted; Model 2 – adjusted for 10 surrogate variables (SVs); Model 3 – adjusted for 10 SVs and derived cell proportions. EWAS stratified by smoking status was also conducted (never (N=304), former (N=648) and current smoking (N=857)). For Model 1 and Model 2, the case-control studies not matched on smoking status (EPIC-Italy and NOWAC) were adjusted for smoking.

An inverse-variance weighted fixed effects meta-analysis was performed of the EWAS (918 case-control pairs) using the METAL software. Direction of effect,

effect estimates and the  $I^2$  statistic were used to assess heterogeneity across the studies in addition to effect estimates across smoking strata (never, former and current). All sites identified at a false discovery rate (FDR)  $< 0.05$  in Model 2 and 3 were also present in the sites identified in Model 1. The effect size differences between models for all sites identified in Model 1 were assessed by a Kruskal-Wallis test and a post-hoc Dunn's test. There was little evidence for a difference ( $P > 0.1$ ), so to maximize inclusion into the MR analyses we took forward the sites identified in the unadjusted model (Model 1).

### 7.3.3 Mendelian randomization

Two-sample MR was used to establish potential causal effect of differential methylation on lung cancer risk (110,111).

#### **Sample 1: Accessible Resource for Integrated Epigenomic Studies (ARIES)**

In the first sample, mQTL-methylation effect estimates ( $\beta_{GP}$ ) for each CpG site of interest were identified in an mQTL database from the Accessible Resource for Integrated Epigenomic Studies (ARIES) (<http://www.mqtldb.org>). Details on the methylation pre-processing, genotyping and quality control (QC) pipelines are outlined below.

**DNA methylation data** Samples were drawn from the Avon Longitudinal Study of Parents and Children [(126); Fraser2013]. Blood from 1,018 mother-child pairs were selected for analysis as part of the Accessible Resource for Integrative Epigenomic Studies (ARIES, <http://www.ariesepigenomics.org.uk/>) (127). There are three timepoints in children and two in their mothers, the timepoints with mean ages (in brackets) in ARIES are as follows for children: birth,

childhood (7.5), adolescence (17.1) and for mothers: during pregnancy (28.7), and at middle age (46.9). Following DNA extraction, samples were bisulphite converted using the Zymo EZ DNA Methylation™ kit (Zymo, Irvine, CA, USA). Following conversion, genome-wide methylation was measured using the Illumina Infinium HumanMethylation450 (HM450) BeadChip. Methylation data were normalised in R with the watermelon package (142) using the Touleimat and Tost (143) algorithm to reduce the non-biological differences between probes. Methylation data in ARIES were rank-normalised to remove outliers, and then Matrix eQTL software (3) was used to perform preliminary association analysis of SNPs with all CpG sites in the Illumina Infinium HM450 array with the exception of those failing QC, and those reported to map to more than one location (n=19,834) or to contain a genetic variant at the CpG site (n=74,182) (81).

**Genetic data** Children were 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). Individuals were excluded on the basis of incorrect gender 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). Following QC the final directly genotyped dataset contained 500,527 SNP loci.

Mothers were genotyped using the Illumina Human660W-quad genome-wide SNP genotyping platform (Illumina Inc., San Diego, USA) at the Centre National de Génotypage (CNG, Paris, France). Individuals were excluded based on non-European ancestry, missingness, relatedness, gender mismatches and heterozygosity. PLINK (v1.07) (144) was used to carry out quality control measures on an initial

set of 10,015 subjects and 557,124 directly genotyped SNPs. Following QC the final directly genotyped dataset contained 526,688 SNP loci.

Imputation was performed to increase the SNP density for all genotyped mothers and children combined. Genotypes were phased together using ShapeIt, and then imputed against the 1000 genomes reference panel (phase 1 version 3, phased using ShapeIt version 2, December 2013, using all populations) using Impute (version 2.2.2). Genotypes were filtered to have Hardy-Weinberg equilibrium  $P > 5 \times 10^{-7}$ , MAF  $> 1\%$  and imputation info score  $> 0.8$ . Best guess genotypes were used for subsequent analysis. The final imputed dataset used for the analyses presented here contained 8,074,398 loci.

Written informed consent has been obtained from all ALSPAC participants. Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and Local Research Ethics Committees. Please note that the study website contains details of all the data that is available through a fully searchable data dictionary: <http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/>

#### **7.3.4 Sample 2: Transdisciplinary Research in Cancer of the Lung and The International Lung Cancer Consortium**

In the second sample, summary data was extracted from a GWAS meta-analysis of lung cancer risk conducted by the Transdisciplinary Research in Cancer of the Lung and The International Lung Cancer Consortium (TRICL-ILCCO) (29,863 cases, 55,586 controls) for individuals genotyped using the Illumina Infinium OncoArray-500K BeadChip (Illumina Inc. San Diego, CA) and independent samples for which prior genotyping was performed (145). They were used to obtain mQTL-lung cancer estimates ( $\beta_{GD}$ ).

For each independent mQTL ( $r^2 < 0.01$ ), we calculated the log odds ratio (OR) per SD unit increase in methylation by the formula  $\frac{\beta_{GD}}{\beta_{GP}}$  (Wald ratio). Standard errors were approximated by the delta method (146). Where multiple independent mQTLs were available for one CpG site, these were combined in a fixed effects meta-analysis after weighting each ratio estimate by the inverse variance of their associations with the outcome. Heterogeneity in Wald ratios across mQTLs was estimated using Cochran's Q test, which can be used to indicate horizontal pleiotropy (147). Differences between the observational and MR estimates were assessed using a Z-test for difference.

If there was evidence for an mQTL-CpG site association in ARIES in at least one time-point, it was assessed whether the mQTL replicated across time points in ARIES (FDR < 0.05, same direction of effect). Further, this association was re-analysed using linear regression of methylation on each genotyped SNP available in an independent cohort (NSHDS), using *rvtests* (148). The same NSHDS samples on which DNA methylation was measured were genotyped using the Illumina Infinium OncoArray-500k BeadChip (Illumina Inc. San Diego, CA) and quality control parameters were applied under the recently published TRICL-ILCCO GWAS study on lung cancer (145). Genetic imputation was performed on these samples using the Haplotype Reference Consortium (HRC) Panel (release 1) (133) through the Michigan Imputation Server (149). Replicated mQTLs were included where possible to reduce the effect of winner's curse using effect estimates from ARIES. We assessed the instrument strength of the mQTLs by investigating the variance explained in methylation by each mQTL ( $r^2$ ) as well as the F-statistic in ARIES **Table 7.1**. The power to detect the observational effect estimates in the two-sample MR analysis was assessed a priori, based on an alpha of 0.05, sample size of 29,863 cases and 55,586 controls (from TRICL-ILCCO) and calculated variance explained



( $r^2$ ).

Table 7.1: Instrument strength in ARIES

SNP	CpG	Beta	SE	P	N	F	R2
rs1048691	cg23387569	0.35	0.053	3.9e-11	834	45	0.05
rs1939110	cg11660018	-0.40	0.048	2.6e-16	834	70	0.08
rs13087163	cg01901332	-0.19	0.035	5.8e-08	834	30	0.03
rs7927381	cg01901332	0.38	0.069	3.9e-08	834	31	0.04
rs878481	cg05951221	-0.32	0.042	5.9e-14	834	58	0.07
rs1048691	cg16823042	0.32	0.053	2.8e-09	834	36	0.04
rs734568	cg03636183	0.28	0.043	6.7e-11	834	44	0.05
rs72967500	cg23771366	-0.63	0.062	1.3e-22	834	102	0.11
rs3748971	cg21566642	-0.50	0.080	5.3e-10	834	39	0.05
rs9643220	cg25305703	0.34	0.049	7.1e-12	834	48	0.05
rs77433148	cg08709672	-0.80	0.137	6.3e-09	834	34	0.04
rs17518433	cg09935388	-0.33	0.046	1.8e-12	834	51	0.06
rs463924	cg26963277	-0.39	0.045	6.8e-18	834	78	0.09
rs56080708	cg27241845	0.72	0.070	2.4e-23	834	105	0.11
rs11744553	cg05575921	0.22	0.040	7.2e-08	834	30	0.03
rs11746538	cg05575921	-0.37	0.058	3.0e-10	834	41	0.05

SE = standard error, P = P value, N = sample size, F = F statistic

MR analyses were also performed to investigate the impact of methylation on lung cancer subtypes in TRICL-ILCCO: adenocarcinoma (11,245 cases, 54,619 controls), small cell carcinoma (2791 cases, 20,580 controls), and squamous cell carcinoma

(7704 cases, 54,763 controls). We also assessed the association in never smokers (2303 cases, 6995 controls) and ever smokers (23,848 cases, 16,605 controls) (145) 25. Differences between the smoking subgroups were assessed using a Z-test for difference.

We next investigated the extent to which the mQTLs at cancer-related CpGs were associated with four smoking behaviour traits which could confound the methylation-lung cancer association: number of cigarettes per day, smoking cessation rate, smoking initiation and age of smoking initiation using GWAS data from the Tobacco and Genetics (TAG) consortium (N=74,053) (150) 29.

### **7.3.5 Supplementary analyses**

#### **Assessing the potential causal effect of *AHRR* methylation: one sample MR**

Given previous findings implicating methylation at *AHRR* in relation to lung cancer (121,132) 2, 3, we performed a one-sample MR analysis (151) 30 of *AHRR* methylation on lung cancer incidence using individual-level data from the Copenhagen City Heart Study (CCHS) (357 incident cases, 8401 remaining free of lung cancer). Copenhagen City Heart Study is a prospective study of the general population (152). Copenhagen residents were invited to complete a questionnaire and undergo a physical examination and are followed through a unique person identifier in the Danish health registries. All participants gave written informed consent, and a Danish ethics committee approved the study (KF100.2039/91).

**Phenotypic data** Participants were asked whether they smoked at the day of attendance or previously. If they answered affirmative to either of these questions, they were asked about their current and former smoking behaviour, including age

of smoking initiation, age of smoking cessation, and number of daily consumed cigarettes, cheroots, cigars, and weekly grams of pipe tobacco. Based on these answers, participants were categorized as never, former, and current smokers. In addition, participants reported on alcohol consumption, occupational exposure to dust and/or welding fumes, exposure to passive smoking, education, and familial cases of lung cancer. The answers were reviewed together with an examiner at the day of attendance. Body mass index was calculated as measured weight in kilograms divided by measured height (in meters) squared.

**Methylation data** At the physical examination, blood samples were drawn for DNA from which *AHRR* methylation extent was measured (101). The *AHRR* cg05575921 methylation extent was measured in duplicate samples of bisulphite treated DNA from peripheral blood from 9,234 individuals. We used a Taqman assay developed in our own laboratory, and included standard curves, as well as internal controls in each 384-well plate. Coefficients of variation at the methylation level of 71% varied from 5.0 to 6.7%. Laboratory technicians were blinded to smoking and disease status of the individuals. Results were validated with pyrosequencing on a subset of samples.

**Genetic data** Genotypes from the iCOGs array (153) and prospective data on lung cancer incidence were also available for these participants. Of the 9234 individuals, genotype data from iCOGS on 8778 were available. In short, DNA isolated from leukocytes was genotyped with a custom Illumina iSelect genotyping array, designed to test genetic variants related to breast, ovary and prostate cancer, comprising roughly 211,000 SNPs after rigorous quality control (153).

**Lung cancer data** For lung cancer (ICD7, codes 1624 or 4624 until 1977, and ICD10, code C34 from 1978 and onwards), the date of first diagnosis was taken from the national Danish Cancer Registry from 1943 to December 2012.

**Identification of mQTLs for CCHS one-sample MR** mQTLs located within 1Mb of cg05575921 *AHRR* were identified in ARIES (FDR<0.05). Of those mQTLs which replicated within the CCHS, we performed an LD pruning step using a less stringent  $r^2$  threshold of 0.2 and generated an unweighted allele score, calculated by coding and then summing the alleles to reflect the average number of methylation-increasing alleles carried by an individual. Associations between the allele score and several potential confounding factors (sex, alcohol consumption, smoking status, occupational exposure to dust and/or welding fumes, passive smoking) were investigated. Then MR analyses were performed using two-stage Cox regression, with adjustment for age and sex, and further stratified by smoking status.

### **Tumour and adjacent normal methylation patterns**

DNA methylation data from lung cancer tissue and matched normal adjacent tissue (N=40 squamous cell carcinoma and N=29 adenocarcinoma), profiled as part of The Cancer Genome Atlas (TCGA), were used to assess tissue-specific DNA methylation changes across sites identified in the meta-analysis of EWAS, as outlined previously (154) 31.

### **mQTL association with gene expression**

For the genes annotated to CpG sites identified in the lung cancer EWAS, we examined gene expression in whole blood and lung tissue using data from the gene-tissue expression (GTEx) consortium (155) 32.

Analyses were conducted in Stata (version 14) and R (version 3.2.2). For the two-sample MR analysis we used the MR-Base R package TwoSampleMR (156) 33. An adjusted P value that limited the FDR was calculated using the Benjamini-Hochberg method (157) 34. All statistical tests were two-sided.

## 7.4 Results

A flowchart representing our study design along with a summary of our results at each step is displayed in Figure 1.

(Figure 1 here)

### 7.4.1 EWAS meta-analysis

The basic meta-analysis adjusted for study-specific covariates identified 16 CpG sites which were hypomethylated in relation to lung cancer ( $FDR < 0.05$ , Model 1, Figure 2). Adjusting for 10 surrogate variables (Model 2) and derived cell counts (Model 3) gave similar results (Table 1). The direction of effect at the 16 sites did not vary between studies (median  $I^2 = 38.6$ ) (Supplementary Table 2), but there was evidence for heterogeneity of effect estimates at some sites when stratifying individuals by smoking status (Table 1).

(Figure 2 here)

(Table 1 here)

### 7.4.2 Mendelian randomization

We identified 15 independent mQTLs ( $r^2 < 0.01$ ) associated with methylation at 14 of 16 CpGs. Ten mQTLs replicated at  $FDR < 0.05$  in NSHDS (Supplementary Table 3). MR power analyses indicated  $>99\%$  power to detect ORs for lung cancer of the same magnitude as those in the meta-analysis of EWAS.

There was little evidence for an effect of methylation at these 14 sites on lung cancer ( $FDR > 0.05$ , Supplementary Table 4). For nine of 14 CpG sites the point estimates from the MR analysis were in the same direction as in the EWAS, but of a much

smaller magnitude (Z-test for difference,  $P < 0.001$ ) (Figure 3).

For 9 of out the 16 mQTL-CpG associations, there was strong replication across time points (Supplementary Table 5) and 10 out of 16 mQTL-CpG associations replicated at  $FDR < 0.05$  in an independent adult cohort (NSHDS). Using mQTL effect estimates from NSHDS for the 10 CpG sites that replicated ( $FDR < 0.05$ ), findings were consistent with limited evidence for a causal effect of peripheral blood-derived DNA methylation on lung cancer (Supplementary Figure 1).

(Figure 3 here)

There was little evidence of different effect estimates between ever and never smokers at individual CpG sites (Supplementary Figure 2, Z-test for difference,  $P > 0.5$ ). There was some evidence for a possible effect of methylation at cg21566642-*ALPPL2* and cg23771366-*PRSS23* on squamous cell lung cancer ( $OR = 0.85$  [95% confidence interval (CI) = 0.75, 0.97] and 0.91 [95% CI = 0.84, 1.00] per SD [14.4% and 5.8%] increase, respectively) as well as methylation at cg23387569-*AGAP2*, cg16823042-*AGAP2*, and cg01901332-*ARRB1* on lung adenocarcinoma ( $OR = 0.86$  [95% CI = 0.77, 0.96], 0.84 [95% CI = 0.74, 0.95], and 0.89 [95% CI = 0.80, 1.00] per SD [9.47%, 8.35%, and 8.91%] increase, respectively). However, none of the results withstood multiple testing correction ( $FDR < 0.05$ ) (Supplementary Figure 3). For those CpGs where multiple mQTLs were used as instruments (cg05575921-*AHRR* and cg01901332-*ARRB1*), there was limited evidence for heterogeneity in MR effect estimates (Q-test,  $P > 0.05$ , Supplementary Table 6).

Single mQTLs for cg05575921-*AHRR*, cg27241845-*ALPPL2*, and cg26963277-*KCNQ1* showed some evidence of association with smoking cessation (former vs. current smokers), although these associations were not below the  $FDR < 0.05$  threshold (Supplementary Figure 4).

### **Potential causal effect of *AHRR* methylation on lung cancer risk: one sample MR**

In the CCHS, a per (average methylation-increasing) allele change in a four-mQTL allele score was associated with a 0.73% [95% CI=0.56,0.90] increase in methylation ( $P < 1 \times 10^{-10}$ ) and explained 0.8% of the variance in cg05575921-*AHRR* methylation (F-statistic=74.2). Confounding factors were not strongly associated with the genotypes in this cohort ( $P \geq 0.11$ ) (Supplementary Table 7). Results provided some evidence for an effect of cg05575921 methylation on total lung cancer risk (HR=0.30 [95% CI=0.10,1.00] per SD (9.2%) increase) (Supplementary Table 8). The effect estimate did not change substantively when stratified by smoking status (Supplementary Table 8).

Given contrasting findings with the main MR analysis, where cg05575921-*AHRR* methylation was not causally implicated in lung cancer, and the lower power in the one-sample analysis to detect an effect of equivalent size to the observational results (power = 19% at  $\alpha = 0.05$ ), we performed further two-sample MR based on the four mQTLs using data from both CCHS (sample one) and the TRICL-ILCCO consortium (sample two). Results showed no strong evidence for a causal effect of DNA methylation on total lung cancer risk (OR=1.00 [95% CI=0.83,1.10] per SD increase) (Supplementary Figure 5). There was also limited evidence for an effect of cg05575921-*AHRR* methylation when stratified by cancer subtype and smoking status (Supplementary Figure 5) and no strong evidence for heterogeneity of the mQTL effects (Supplementary Table 9). Conclusions were consistent when MR-Egger (27) was applied (Supplementary Figure 5) and when accounting for correlation structure between the mQTLs (Supplementary Table 9).

### **7.4.3 Tumour and adjacent normal lung tissue methylation patterns**

For cg05575921-*AHRR*, there was no strong evidence for differential methylation between adenocarcinoma tissue and adjacent healthy tissue ( $P=0.963$ ), and weak evidence for hypermethylation in squamous cell carcinoma tissue ( $P=0.035$ ) (Figure 4, Supplementary Table 10). For the other CpG sites there was evidence for a difference in DNA methylation between tumour and healthy adjacent tissue at several sites in both adenocarcinoma and squamous cell carcinoma, with consistent differences for CpG sites in *ALPPL2* (cg2156642, cg05951221 and cg01940273), as well as cg23771366-*PRSS23*, cg26963277-*KCNQ1*, cg09935388-*GFI1*, cg0101332-*ARRB1*, cg08709672-*AVPR1B* and cg25305703-*CASC21*. However, hypermethylation in tumour tissue was found for the majority of these sites, which is opposite to what was observed in the EWAS analysis.

(Figure 4 here)

### **7.4.4 Gene expression associated with mQTLs in blood and lung tissue**

Of the 10 genes annotated to the 14 CpG sites, eight genes were expressed sufficiently to be detected in lung (*AVPR1B* and *CASC21* were not) and seven in blood (*AVPR1B*, *CASC21* and *ALPPL2* were not). Of these, gene expression of *ARRB1* could not be investigated as the mQTLs in that region were not present in the GTEx data. rs3748971 and rs878481, mQTLs for cg2156642 and cg05951221 respectively, were associated with increased expression of *ALPPL2* ( $P=0.002$  and  $P=0.0001$ ). No other mQTLs were associated with expression of the annotated gene at a Bonferroni corrected P value threshold ( $P<0.05/19=0.0026$ ) (Supplementary Table 11).



## 7.5 Discussion

In this study, we identified 16 CpG sites associated with lung cancer, of which 14 have been previously identified in relation to smoke exposure (65) 9 and six were highlighted in a previous study as being associated with lung cancer (121) 3. This previous study used the same data from the four cohorts investigated here, but in a discovery and replication, rather than meta-analysis framework. Overall, using MR we found limited evidence supporting a potential causal effect of methylation at the CpG sites identified in peripheral blood on lung cancer. These findings are in contrast to previous analyses suggesting that methylation at two CpG sites investigated (in *AHRR* and *F2RL3*) mediated > 30% of the effect of smoking on lung cancer risk (132) 2. This previous study used methods which are sensitive to residual confounding and measurement error that may have biased results [(104); Hemani2017] 12, 35. These limitations are largely overcome using MR (104) 12. While there was some evidence for an effect of methylation at some of the other CpG sites on risk of subtypes of lung cancer, these effects were not robust to multiple testing correction and were not validated in the analysis of tumour and adjacent normal lung tissue methylation nor in gene expression analysis.

A major strength of the study was the use of two-sample MR to integrate an extensive epigenetic resource and summary data from a large lung cancer GWAS to appraise causality of observational associations with >99% power. Evidence against the observational findings were also acquired through tissue-specific DNA methylation and gene expression analyses.

Limitations include potential “winner’s curse” which may bias causal estimates in a two-sample MR analysis towards the null if the discovery sample for identifying genetic instruments is used as the first sample, as was done for our main MR

analysis using data from ARIES (158) 36. However, findings were similar when using replicated mQTLs in NSHDS, indicating the potential impact of this bias was minimal (Supplementary Figure 1). Another limitation relates to the potential issue of consistency and validity of the instruments across the two samples. For a minority of the mQTL-CpG associations (4 out of 16), there was limited replication across time points and in particular, 6 mQTLs were not strongly associated with DNA methylation in adults. Further, our primary data used for the first sample in the two-sample MR was ARIES, which contains no male adults. If the mQTLs identified vary by sex and time, then this could bias our results. However, our replication cohort NSHDS contains adult males. Therefore, the 10 mQTLs that replicated in NSHDS are unlikely to be biased by the sex discordance. Also, we replicated the findings for cg05575921 *AHRR* in CCHS, which contains both adult males and females, in a two-sample MR analysis, suggesting these results are also not influenced by sex discordance. Caution is therefore warranted when interpreting the null results for the two-sample MR estimates for the CpG sites for which mQTLs were not replicated, which could be the result of weak-instrument bias.

The lack of independent mQTLs for each CpG site did not allow us to properly appraise horizontal pleiotropy in our MR analyses. Where possible we only included cis-acting mQTLs to minimise pleiotropy and investigated heterogeneity where there were multiple independent mQTLs. Three mQTLs were nominally associated with smoking phenotypes, but not to the extent that this would bias our MR results substantially. Some of the mQTLs used influence multiple CpGs in the same region, suggesting genomic control of methylation at a regional rather than single CpG level. This was untested, but methods to detect differentially methylated regions (DMRs) and identify genetic variants which proxy for them may be fruitful in probing the effect of methylation across gene regions.

A further limitation relates to the inconsistency in effect estimates between the one- and two-sample MR analysis to appraise the causal role of *AHRR* methylation. While findings in CCHS were supportive of a causal effect of *AHRR* methylation on lung cancer (HR=0.30 [95% CI=0.10,1.00] per SD), in two-sample MR this site was not causally implicated (OR=1.00 [95% CI=0.83,1.10] per SD increase). We verified that this was not due to differences in the genetic instruments used, nor due to issues of weak instrument bias. Given the CCHS one-sample MR had little power (19% at  $\alpha = 0.05$ ) to detect a causal effect with a size equivalent to that of the observational analysis, we have more confidence in the results from the two-sample approach.

Peripheral blood may not be the ideal tissue to assess the association between DNA methylation and lung cancer. While a high degree of concordance in mQTLs has been observed across lung tissue, skin and peripheral blood DNA (159) 37, we were unable to directly evaluate this here. A possible explanation for a lack of causal effect at *AHRR* is due to the limitation of tissue specificity as we found that the mQTLs used to instrument cg05575921 were not strongly related to expression of *AHRR* in lung tissue. However, findings from MR analysis were corroborated by the lack of evidence for differential methylation at *AHRR* between lung adenocarcinoma tissue and adjacent healthy tissue, and weak evidence for hypermethylation (opposite to the expected direction) in squamous cell lung cancer tissue. This result may be interesting in itself as smoking is hypothesized to influence squamous cell carcinoma more than adenocarcinoma. However, the result conflicts with that found in the MR analysis. Furthermore, another study investigating tumorous lung tissue (N=511) found only weak evidence for an association between smoking and cg05575921 *AHRR* methylation, that did not survive multiple testing correction ( $P=0.02$ ) (160) 38. However, our results do not fully exclude *AHRR* from involvement in the disease

process. *AHRR* and *AHR* form a regulatory feedback loop, which means that the actual effect of differential methylation or differential expression of *AHR/AHRR* on pathway activity is complex (130) 39. In addition, some of the CpG sites identified in the EWAS were found to be differentially methylated in the tumour and adjacent normal lung tissue comparison. While this could represent a false negative result of the MR analysis, it is of interest that differential methylation in the tissue comparison analysis was typically in the opposite direction to that observed in the EWAS. Furthermore, while this method can be used to minimize confounding, it does not fully eliminate the possibility of bias due to reverse causation (whereby cancer induces changes in DNA methylation) or intra-individual confounding e.g. by gene expression. Therefore, it doesn't give conclusive evidence that DNA methylation changes at these sites are not relevant to the development of lung cancer.

While DNA methylation in peripheral blood may be predictive of lung cancer risk, according to the present analysis it is unlikely to play a causal role in lung carcinogenesis at the CpG sites investigated. Findings from this study issue caution over the use of traditional mediation analyses to implicate intermediate biomarkers (such as DNA methylation) in pathways linking an exposure with disease, given the potential for residual confounding in this context (104) 12. However, the findings of this study do not preclude the possibility that other DNA methylation changes are causally related to lung cancer (or other smoking-associated disease) (161) 40.

# Conclusion

If we don't want Conclusion to have a chapter number next to it, we can add the `{ - }` attribute.

## More info

And here's some other random info: the first paragraph after a chapter title or section head *shouldn't be* indented, because indents are to tell the reader that you're starting a new paragraph. Since that's obvious after a chapter or section title, proper typesetting doesn't add an indent there.

## Appendix A

# The First Appendix

This first appendix includes all of the R chunks of code that were hidden throughout the document (using the `include = FALSE` chunk tag) to help with readability and/or setup.

**In the main Rmd file**

**In Chapter ??:**

## **Appendix B**

### **The Second Appendix, for Fun**

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