Beyond the CpG: an integrative approach to decoding DNA methylation in immunometabolic health

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CHAPTER ONE

Introduction

Healthy Ageing

Age is the leading risk factor for most common, chronic diseases, but the ageing process itself is highly heterogenous[1,2]. While some individuals suffer from decades of multimorbidity and functional decline, others enjoy a comparatively healthy older age with lower levels of frailty and limited disease development. Modifiable lifestyle factors, such as diet and physical activity, can influence these trajectories and targeted interventions have successfully improved health in older adults[3,4]. Yet, key challenges remain to identify the molecular determinants of age-related phenotypes and uncover the biological roots driving intervention effects[5].

Markers of health offer valuable insights into the processes and pathways underlying ageing. They encompass a broad spectrum of measures, from functional tests and anthropometry to clinical chemistry and cellular biomarkers, each with distinct strengths and limitations[6]. While physiological metrics are typically easier to observe and interpret in the clinic, they emerge following a series of subtler shifts in health[7]. Early detection of such inter-individual variation is where biochemical and molecular markers shine[8]. Interleukin-6 (IL-6) levels can predict cardiovascular disease and all-cause mortality years prior to clinical onset[9,10], nutrient-sensing genes are robustly linked to longevity and represent promising therapeutic targets[11–13], and epigenetic clocks integrating DNA methylation (DNAm) at sensitive sites can accurately estimate chronological age[14,15]. These examples offer a glimpse into the promise of molecular biology in ageing research, but the links between markers and the mechanics of their regulation await full elucidation.

Immunometabolism

Profound shifts in both immunity and metabolism accompany ageing, and these systems are intimately engaged in a bidirectional crosstalk termed *immunometabolism*[16,17]. This dynamic communication shapes physiological function and dysfunction throughout the lifecourse, providing a compelling framework for studying the biology of ageing, particularly in tissues, such as blood, adipose, and muscle, which sit at the crossroads between immune surveillance and metabolic regulation[18,19].

Among these interconnected systems, age-related changes in the immune compartment have been especially well characterized[20,21]. Leukocyte populations undergo extensive remodelling with age, culminating in a distinct immunological landscape known as *immunosenescence*[22]. This state is marked by a decline in naïve T-cell populations relative to terminally differentiated memory cells, inversion of CD4+/CD8+ T-cell ratios, and reduced T-cell receptor diversity[23,24]. Such qualitative and quantitative immunological alterations have consequences for the health of older adults, contributing to impaired tumour surveillance, increased susceptibility to autoimmunity, and

higher risk of chronic, non-communicable disease (**Fig. 1a**)[25–27].

Paradoxically, even as adaptive immunity declines, a persistent, low-grade inflammation referred to as *inflammaging* emerges[28]. Sustained in part by chronic stimulation from misplaced self-molecules and cellular debris, affected leukocytes remain in a state of prolonged activation[29]. These exhausted immune cells, no longer finely attuned to specific threats, mount maladaptive responses and exhibit a heightened propensity to differentiate into pro-inflammatory subtypes, such as Th17 effectors (**Fig. 1b**)[30,31]. Inflammaging is characterized by elevated serum levels of molecular mediators, including IL-6 and TNF-α[32,33]. The physiological relevance of these changes is underscored by studies in centenarians, where genetic variants in the *IL6* locus strongly correlate with longer lifespans[34], and by growing evidence that dampening inflammation may be central to attaining exceptional longevity[35,36].

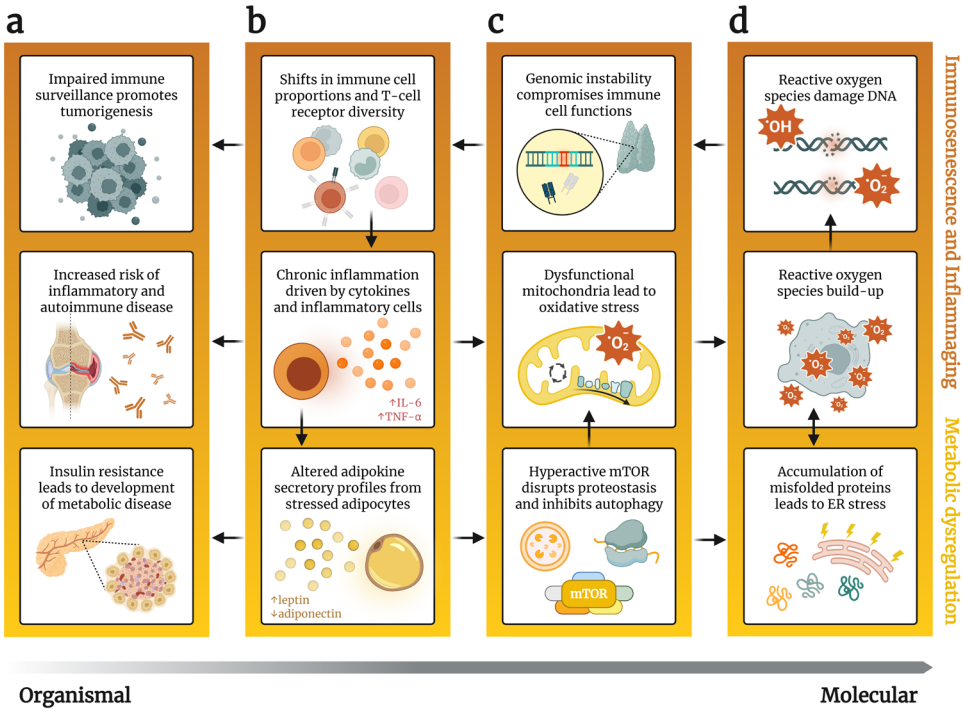

Figure 1 | Immune, inflammatory, and metabolic components of ageing across biological scales. a) Organismal-level alterations increase incidence of cancer, autoimmune, and metabolic diseases, including type 2 diabetes. **b)** Loss of naïve T-cells impairs adaptive immunity, cytokine production promotes chronic inflammation, and stressed adipocytes adjust their secretory profiles. **c)** Cellular effects include genomic disruptions of immune cell function, mitochondrial dysfunction, and hyperactive mTOR signalling. **d)** Molecular changes propagate endoplasmic reticulum (ER) and oxidative stress, fuelling DNA damage and genomic instability. *Figure created in BioRender.*

At the cellular level, age-associated changes in immune function both rely on and shape shifts in metabolism[37]. Pro-inflammatory cytokines, such as IL-6, trigger stress pathways that impair mitochondrial function, disrupt proteostasis, and contribute to genomic instability (**Fig. 1c,d**)[38,39]. Additionally, immune cells depend on metabolic reprogramming to meet the energetic and biosynthetic demands of activation[40]. While oxidative phosphorylation is largely sufficient to support the modest energy requirements of naïve T-cells, activating cells rapidly shift to glycolysis for ATP generation. Indeed, glucose deprivation alone can suppress T-cell expansion and cytokine production even when alternative metabolic substrates are available, highlighting the critical role of metabolic flexibility in ageing and the immune response[41,42].

This interplay between immunity and metabolism extends beyond the circulation as well. Chronic inflammation infiltrates other tissues, including adipose and muscle, where activation of cytokine receptors on resident cells both impairs glucose homeostasis and promotes insulin resistance[43]. Yet, adipocytes and myocytes are not mere passive targets of inflammaging; they also secrete pro- and anti-inflammatory mediators, including IL-6, adiponectin, and leptin, thereby modulating immune responses and influencing organismal energy balance[44,45]. This bidirectional crosstalk systemically propagates age-related dysfunction, underpinning a constellation of immune, inflammatory, and metabolic alterations[46]. Taken together, these processes demonstrate the pivotal role of distinct cellular phenotypes and their regulation for shaping health trajectories in later life, and position immunometabolism as a unifying framework for understanding ageing and health in the population at large.

Gene regulation and epigenetics

Cellular phenotypes are fundamentally determined by gene expression and protein production patterns[47]. This is exemplified by effector T-cell subtypes, whose distinct transcriptional and translational signatures enable their transformation from naïve cells into specialized lineages[48]. The classical markers that distinguish these subsets underscores the vital role of genomic regulation in shaping their fates (**Fig. 2a**). Lineage-defining *transcription factors* (TFs) such as T-bet drive Th1 identity by promoting expression of IFN-γ, *receptors* including CD25 detect IL-2 in the extracellular milieu and support regulatory T-cell (Treg) survival and immunosuppressive functions, and IL-17A *secretions* enables Th17 subtypes to amplify local inflammation and recruit neutrophils to sites of tissue damage[49].

The execution of these differential programs depends on a complex and tightly regulated network of molecular interactions. TFs act in concert with co-factors and the surrounding chromatin landscape to activate or repress target genes[50,51]. Epigenetic alterations, including DNAm and histone modifications, modulate chromatin accessibility, thereby determining which genomic regions are transcriptionally permissive, and which are

not[52]. Through the integration of these regulatory layers of control, cells establish the expression profiles that define their phenotypes and ultimately enable their precise functional specialization[53].

DNAm is one such epigenetic modification, involving the addition of a methyl group to the 5' position of cytosine residues, primarily at CpG dinucleotides (**Fig. 2b**). By influencing local chromatin structure and TF binding potential, DNAm can modulate expression without altering underlying genetics[54]. While traditionally regarded as a repressive mark, particularly when located in promoters or enhancers, the regulatory effects of DNAm are now recognized as strongly context dependent (**Fig. 2c**)[55,56].

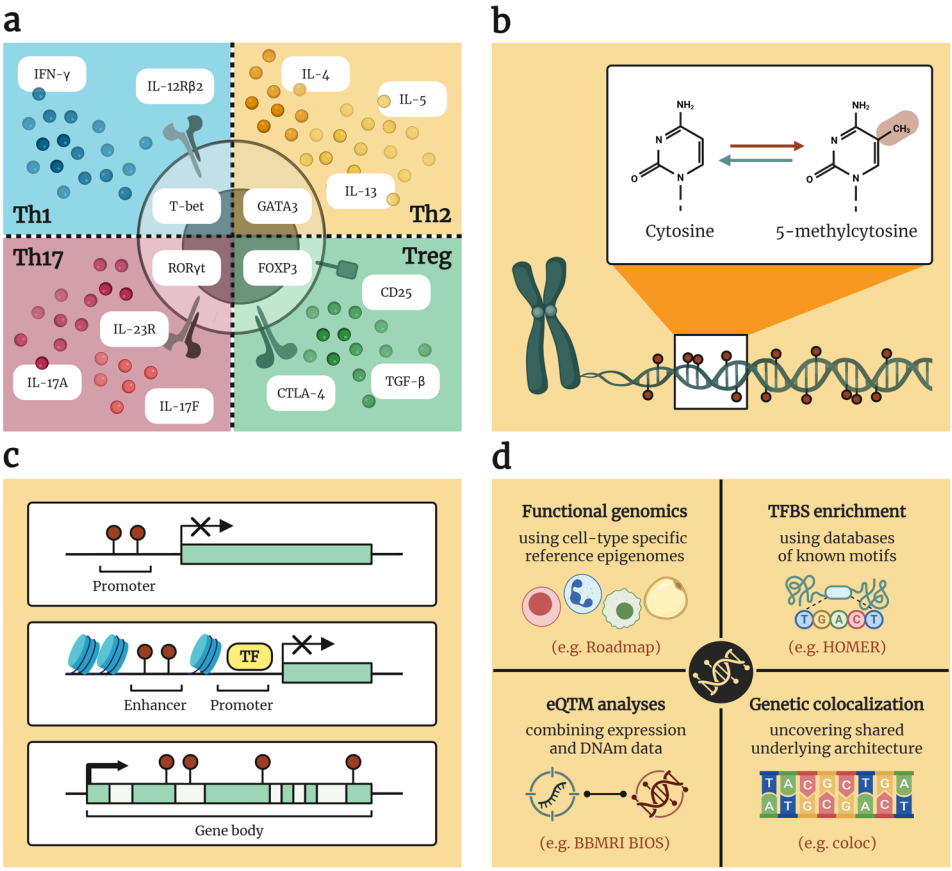

Figure 2 | Gene regulation involves co-operation between transcription factors (TFs) and the chromatin landscape. a) T-cell subtypes are defined by lineage-defining TFs, receptors, and secretions. **b)** DNA methylation (DNAm) attaches a methyl group (red) to cytosine residues. **c)** DNAm is context-dependent: it may block the binding of TFs at promoter and enhancer regions but is also linked to transcriptional activation when located in gene bodies. **d)** Large-scale integrative analyses can improve CpG-gene annotations including functional genomics, TF binding site (TFBS) enrichment, eQTM analyses, and genetic colocalization. *Figure created in BioRender.*

Methylation within gene bodies, for example, is frequently associated with active transcription, and intergenic DNAm can help preserve the boundaries of regulatory domains. Furthermore, expression quantitative trait methylation (eQTM) studies have demonstrated that DNAm correlates with expression in both directions; although associations are predominantly negative, a substantial proportion are positive, underscoring a nuanced role for DNAm in transcriptional regulation[57].

This context-dependent regulatory nature has complicated the development of reliable maps linking DNAm to target genes[58]. While CpGs are frequently annotated to the nearest gene body, this pairing is crude: it overlooks tissue-specificity, ignores local chromatin structure, and provides no insight into the regulatory factors involved. Emerging evidence supports more distal CpG-gene associations and reveals that TFs differ in their sensitivity to DNAm, highlighting the limitations of simple proximity based approaches[59,60]. Refining CpG-gene annotations will require large-scale, longitudinal, and tissue-specific analyses that combine available epigenetic, transcriptomic, and genetic data, such as those generated by the Roadmap Epigenomics and BBMRI BIOS consortia[61]. These resources offer valuable opportunities to uncover the shared regulatory architecture of molecular traits. Yet, systematic integration of evidence-based mapping is not yet routine in epigenomic studies, and functional and mechanistic interpretations continue to lag behind their scientific potential (**Fig. 2d**).

Epigenome-wide association studies

DNAm is a stable and accessible epigenetic mark that can be profiled using minimally invasive sampling and cost-effective, microarray-based technologies[62]. These practical advantages have fuelled a wave of large-scale epidemiological investigations examining DNAm variation across populations. A core method in this field is the epigenome-wide association study (EWAS), which systematically associates DNAm with a trait of interest[63,64]. EWAS have become a cornerstone of population epigenetics, revealing DNAm signatures for a broad range of traits, including age, lifestyle factors, and immunometabolic health[14,65–67].

Methylation changes across the lifecourse have been particularly well-characterised[68]. Genome-wide analyses and twin studies uncovered a hallmark signature of global hypomethylation, particularly in intergenic and repetitive elements, accompanied by site-specific hypermethylation at CpG-rich promoters[69,70]. A subset of these predictable modifications forms the foundation of epigenetic clocks, which estimate calendar age using highly informative CpGs[14,15]. Beyond chronological prediction, these clocks also capture biological changes, including shifts in immune cell composition, mortality risk, and functional decline, with reorganization of the epigenetic landscape now established as a characteristic component of human ageing[8,71,72].

EWAS have also revealed robust associations between DNAm and immunometabolic health, linking CpG sites at key regulatory genes to body mass index (BMI), circulating lipid levels, and type 2 diabetes across multiple studies[73–75]. Notable examples include *ABCG1*, which encodes a cholesterol transporter central to lipid homeostasis and inflammation, *SREBF1*, a transcriptional regulator of lipogenesis, and *CPT1A*, a rate-limiting enzyme in mitochondrial fatty acid oxidation[65,76]. These findings reveal a central role for methylation of metabolic loci in health and disease, illustrating the power of EWAS to uncover compelling CpGs with clear biological relevance.

Yet, despite these successes, the epigenetic architecture of many clinically relevant traits and exposures remains poorly characterised. While certain risk factors, such as adiposity and smoking, have been extensively studied, many others lie unexplored[64]. As a result, gaps persist in our understanding of how DNAm contributes and responds to early shifts in immunometabolic health, and EWAS efforts will need to be expanded to capture a broader spectrum of inter- and intra-individual epigenetic variation.

Technical considerations

Although EWAS primarily yield correlational findings, their interpretation requires careful consideration of plausible underlying causal structures[77]. By directly altering the mechanical properties of DNA, CpG methylation can modulate chromatin accessibility and alter TF binding[54,60]. However, this regulatory *potential* by no means guarantees that CpGs identified in EWAS are a product of direct, functional effects within studied cells (**Fig. 3a**). Observed DNAm-trait associations may also arise from technical or biological confounding, reverse causation, or shared upstream factors (**Fig. 3b-f**)[62,78]. Distinguishing between these scenarios requires a combination of thoughtful study design, high-quality data, and analytical strategies capable of separating biological signals from technical artifacts.

Among these scenarios, technical confounding (**Fig. 3b**) is arguably the only one that is wholly undesirable. Arising from differences in sample handling, array position, or environmental conditions during processing, these effects represent variation introduced only after sample collection and therefore do not reflect underlying biology in the organism or test system sampled. As DNAm datasets expand, they become increasingly vulnerable to these confounders which, if not properly addressed, can obscure signals or even generate misleading results[79,80]. While *post hoc* adjustment strategies, such as ComBat and other normalisation techniques, are widely employed, they risk inadvertently masking genuine biological variation[81]. Consequently, proactive strategies to minimise batch effects through experimental design and randomisation are preferred. Despite this, practical frameworks and accessible tools to support researchers at the design stage remain limited, and there is a disconnect between best-practice recommendations and their routine implementation in high-throughput studies.

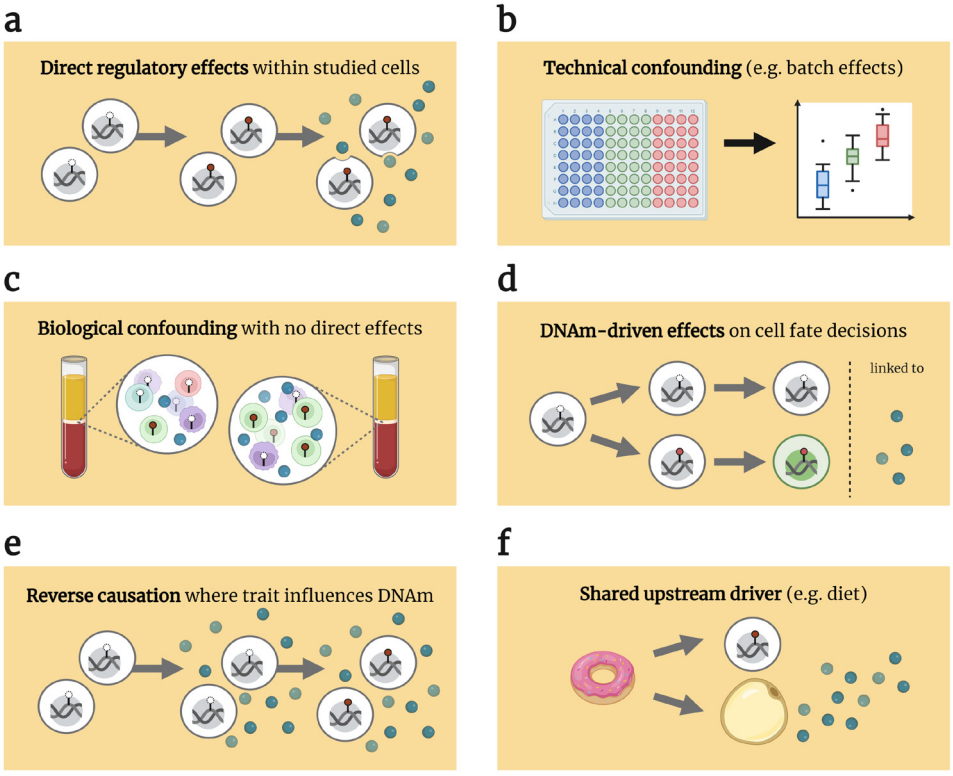

Figure 3 | Six potential causal structures. a) Direct regulatory DNAm effects within studied cells alters expression and thereby influences the trait. **b)** Associations between the trait and technical factors induce systematic DNAm differences. **c)** Associations between cell composition and the trait lead to apparent DNAm differences at CpGs that mark cell types but are not linked to their differentiation. **d)** DNAm directed cell fate decisions lead to changes in cell-type composition associated with the trait. **e)** The trait influences DNAm in studied cells. **f)** Shared upstream factors like diet cause independent effects on DNAm in studied cells and the trait, including through DNAm effects in a non-studied cell type. *Figure created in BioRender.*

Since DNAm patterns are tightly linked to cellular identity, biological confounding poses another major consideration for EWAS[82,83]. Blood, the most accessible and widely studied tissue in epigenetic epidemiology, offers rich opportunities for large-scale analysis, but its cellular heterogeneity complicates interpretation. DNAm-trait associations may arise from shifts in immune cell proportions (**Fig. 3c**) rather than within-cell differential methylation, and this is further complicated by methylation's ability to direct cell fate decisions (**Fig. 3d**)[84,85]. Although routine clinical tests measure only a small number of immune cell categories, recent developments in algorithmic deconvolution now allow estimation of a much wider spectrum of cellular subtypes across multiple tissues[86–88]. This has greatly improved the available resolution for disentangling drivers of observed results. Crucially, however, changes in sample composition are also biologically meaningful readouts, such as the shifts in immune cell populations that occur

with age[24]. As such, EWAS interpretation must carefully distinguish between cellular variation that introduces bias and that which reveals key aspects of immune or physiological function.

Processing and analysing DNAm data remains a rapidly evolving science, driven by novel platforms and technological innovations. Bioconductor packages, such as *methylAid*[89], *omicsPrint*[90], and *bacon*[91] provide powerful, interactive frameworks for monitoring data quality, verifying sample identify, and reducing bias in analysis. Yet, computational pipelines and packages require regular updates, and optimal analytical choices vary with study aims and designs[92,93]. Flexible, scalable, and reproducible workflows, supported by clear and accessible documentation, are essential to empower researchers to keep pace with advances while ensuring high-quality analysis. Even so, modular and transparent resources remain scarce and the steep learning curve of DNAm data processing and analysis continues to limit broad participation.

Yet, despite these ongoing methodological challenges, the expanding availability of large-scale DNAm data offers opportunities to aggregate EWAS findings across populations. In this context, meta-analysis has become an indispensable tool, enhancing the statistical power available to detect modest but consistent DNAm-trait associations. Scalable software such as *METAL* facilitates the integration of summary statistics while quantifying cohort-specific heterogeneity, allowing researchers to detect robust signals that may not reach significance in individual studies[94]. To fully capitalise on EWAS meta-analysis, careful consideration of plausible causal structures will be required alongside collaborative efforts and consortia aimed at elucidating how the DNAm landscape varies with complex traits.

Functional interpretation

Most EWAS to date remain largely descriptive and there is a pressing need to move beyond site discovery towards in-depth functional interpretation[77]. While genic annotation of CpGs, particularly when strengthened by tissue-specific reference epigenomes and TF binding site information, can offer preliminary clues about regulatory potential, this alone is insufficient to contextualize high-dimensional results. Achieving meaningful biological interpretation requires a more systematic and integrative approach. Yet, despite growing enthusiasm and the increasing availability of relevant resources, such follow-up remains inconsistently applied. This limits the field's capacity to generate mechanistic insights and leaves the full potential of EWAS unrealized.

One promising avenue to address this gap is to use curated databases containing gene sets and prior EWAS findings (**Fig. 4a,b**)[95,96]. These resources can be systematically interrogated through enrichment analyses to determine whether differentially methylated CpGs and their putative target genes converge on shared biological

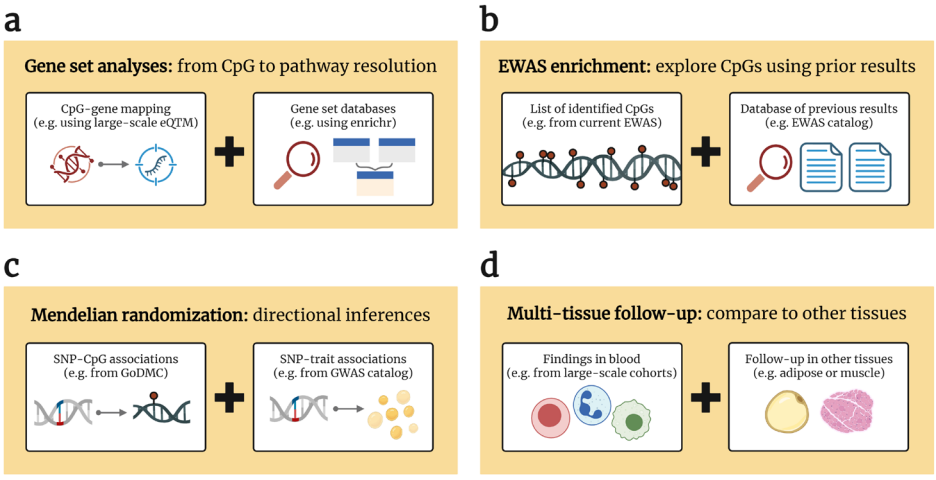

Figure 4 | Follow-up analyses available for epigenome-wide studies including a) gene-set analyses, **b)** EWAS enrichment analysis, **c)** bidirectional two-sample Mendelian randomization, and **d)** multi-tissue investigations and interventions. *Figure created in BioRender.*

processes or pathways. This can illuminate underlying mechanisms, reveal points of regulatory control, and help prioritize loci for functional follow-up. By embedding biological and clinical context into epigenomic workflows, researchers can shift from the isolated nucleotide resolution of CpGs back up to interpretable, systems-level biology.

Yet even biologically plausible associations do not imply causality. A central challenge in epigenetic epidemiology is determining whether differential methylation plays a direct role in shaping cellular phenotypes, or if it instead reflects a different underlying causal structure such as reverse causation (**Fig. 3e**) or shared upstream drivers (**Fig. 3f**). Robust study designs and causal inference approaches can assist in disentangling these possibilities: bidirectional two-sample Mendelian randomization provides a powerful statistical framework to assess directionality of associations by leveraging genetic instruments as proxies for exposure and outcome (**Fig. 4c**)[82], tissue- or cell-type specific follow-up can assess whether blood-based signals mirror changes in other relevant compartments (**Fig. 4d**)[78], and intervention studies, in which exposure precedes DNAm by design, offer a valuable window into causality by revealing if epigenetic control is responsive to established modulators of immunometabolic health.

When applied in concert and thoughtfully interpreted, these integrative approaches will allow researchers to move beyond CpG associations, highlighting focal points for mechanistic investigations and generating testable hypotheses about how epigenetics and immunometabolic health interact. In doing so, such frameworks have potential to transform the field of epigenetic epidemiology from a cataloguing exercise into a mature, mechanistically grounded discipline that advances translational research.

Outline of this thesis

A central feature of ageing is a progressive decline in immunometabolic health, amplifying the risk of certain conditions in older adults. This thesis aims to examine the epigenetic component of such immunometabolic health variation through cohort and intervention studies, using in-depth functional epigenomics to gain fresh insights into the mechanisms that limit or lead to the pathogenesis of age-related disease.

In *Chapters 2 and 3*, we explore DNAm signatures of circulating adipocytokines, addressing key knowledge gaps in the epigenetics of immunometabolic risk. **Chapter 2** focuses on IL-6, a central inflammatory mediator that increases with age and is predictive of cardiovascular events and all-cause mortality[9,10]. By combining blood-based data from over 4,000 individuals, we identify CpG sites associated with circulating IL-6 levels and assess their regulatory potential. Furthermore, we uncover the shared molecular architecture of DNAm, expression, and IL-6 through integration with transcriptomic and genetic data, including large-scale quantitative trait loci databases and genome-wide association studies. In this manner, the resulting epigenetic signature is connected to immunometabolic risk and disease, with three complementary causal inference approaches placing CpGs along the aetiological pathway from elevated inflammation to IL-6 related conditions, such as increased BMI and inflammatory bowel disease.

In **Chapter 3** we conduct a meta-analysis of five European cohorts to explore links between DNAm and serum adiponectin and leptin, two circulating adipokines with established regulatory roles in ageing and immunometabolic disease. Using comprehensive functional annotation, integration with BIOS consortium data, and follow-up in adipocytes, we carefully consider the directionality, tissue-specificity, and broader relevance of our findings for immunometabolic health, including by exploring causal effects on lipid levels and type 2 diabetes.

Recognising the common challenges in epigenome-wide studies, *Chapter 4 and 5* focus on improving analytical workflows and data quality for use in a novel experimental setting. In **Chapter 4** we present statistical software designed to proactively minimise batch effects in high-throughput genomic studies with special attention for accessible implementation and paired designs. **Chapter 5** introduces *DNAmArray*, a reproducible and modular workflow with extensive documentation that streamlines quality control, preprocessing, and analysis in EWAS using existing packages, including *methylAid*[89], *omicsPrint*[90], and *bacon*[91], ensuring high-quality DNAm data for downstream analyses.

In contrast to observational cohort study designs, interventions can examine alteration to immunometabolic health and molecular traits in parallel. Therefore, in **Chapter 6** we evaluate multi-tissue epigenomic responses to a combined lifestyle intervention, where older adults improved their health through a combination of calorie restriction and increased physical activity. We comprehensively characterise epigenetic effects in

fasted blood, subcutaneous adipose tissue, and skeletal muscle, linking them to changes in immunometabolic health markers, including BMI, body fat percentage, grip strength, and serum adipocytokine levels. Furthermore, we incorporate epigenetic clocks to assess the efficacy of DNAm-based algorithms for capturing intervention-driven effects, demonstrating value for combining experimental and computational methods in epigenomic studies.

The general discussion in **Chapter 7** synthesizes our main findings within the broader context of immunosenescence and inflammaging. We discuss the importance of integrative approaches in large-scale EWAS, emphasizing the role of functional annotation and causal inference in moving beyond CpG associations. By comparing findings across *Chapters 2, 3, and 6*, we identify shared epigenetic indicators of health and focal points for follow-up investigations. In closing, we offer fresh perspectives for future directions and outline next steps on the path towards a more mechanistic and translational epigenetic science.

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