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Review

Utility of DNA Methylation as a Biomarker in Aging and Alzheimer's Disease

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Abstract. Epigenetic mechanisms such as DNA methylation have been implicated in a number of diseases including cancer, heart disease, autoimmune disorders, and neurodegenerative diseases. While it is recognized that DNA methylation is tissue-specific, a limitation for many studies is the ability to sample the tissue of interest, which is why there is a need for a proxy tissue such as blood, that is reflective of the methylation state of the target tissue. In the last decade, DNA methylation has been utilized in the design of epigenetic clocks, which aim to predict an individual's biological age based on an algorithmically defined set of CpGs. A number of studies have found associations between disease and/or disease risk with increased biological age, adding weight to the theory of increased biological age being linked with disease processes. Hence, this review takes a closer look at the utility of DNA methylation as a biomarker in aging and disease, with a particular focus on Alzheimer's disease.

Keywords: Aging, Alzheimer's disease, dementia, DNA methylation, epigenetics

INTRODUCTION

Dementia is an umbrella term that encompasses a wide variety of neurodegenerative disorders including vascular dementia, Lewy Body dementia, frontotemporal dementia, and Alzheimer's disease (AD), which is the most common form and accounts for 60-80% of all dementia cases [1]. AD is characterized by cortical atrophy [2] and thinning [3], aggregation of hyperphosphorylated microtubule-associated tau protein (MAPT) resulting in intracellular neurofibrillary tangles, and accumulation of extracellular amyloid-β (Aβ) plaques [4]. Age is regarded as the most important risk factor for neurodegenerative diseases, including AD [5], and as the worldwide population ages, there will be an increase in the prevalence of dementia and AD. In addition to age, there are several accepted modifiable and non-modifiable risk factors for AD [6, 7]. The most common modifiable factors include physical inactivity, smoking, excessive alcohol consumption, air pollution, head injury, infrequent social contact, lower educational attainment, obesity, hypertension, diabetes, depression, and hearing impairment [8].

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Non-modifiable risk factors include sex, family history, and genetics, most notable being variation in the Apolipoprotein E (*APOE*) gene [7]. Currently, several biomarkers for dementia are available that may aid in the diagnosis of possible/probable AD [9]. Cerebrospinal fluid (CSF) obtained via lumbar puncture and brain imaging measures, such as positron emission tomography (PET), computed tomography (CT), and magnetic resonance imaging (MRI), have been the focus in the search for robust and easily accessible biomarkers for AD [10]. These methods focus on the measurement of proteins important in the disease process including tau and Aβ. Notably, while CSF and brain imaging markers have shown good predictive accuracy, particularly in preclinical studies, they are not ideal for use as routine clinical biomarkers due to their cost, level of invasiveness, and need for interpretation of results by specialists [11]. Even though the aforementioned biomarkers are useful in assisting in the diagnosis of AD, a definitive diagnosis of AD can only be made on biopsy postmortem [2]. Currently, there is an urgent need for an easily obtainable, less invasive, cost-effective routine biomarker, that is specific and sensitive to predicting disease progression.

EPIGENETICS AND DNA METHYLATION

Epigenetic modifications are heritable phenotypic changes that do not alter the DNA sequence [12]. These modifications fall into one of three categories; histone modification, non-coding RNAs, DNA hydroxymethylation, and DNA methylation [12]. Epigenetic modifications regulate gene expression patterns by altering DNA accessibility and chromatin structure [13]. Epigenetic processes are a natural occurrence and have critical roles in cellular processes such as gene regulation, chromosome stability, X chromosome inactivation, and genomic imprinting [14]. This review will focus on the most well-studied epigenetic mechanism [15], DNA methylation. Approximately 28 million cytosine-phosphate-guanine (CpG) dinucleotides are distributed unevenly throughout the mammalian genome [16]. These CpG dinucleotides are the target of an epigenetic modification known as DNA methylation, which involves the transfer of a methyl group from S-adenosyl-L-methionine (SAM) onto the 5' position of a cytosine ring, resulting in the formation of 5-methylcytosine (5mC). The covalent attachment of the methyl group is catalyzed by one of several members belonging to the enzyme family known as DNA methyltransferases (DNMT). DNA methylation is essential in several processes, including silencing retroviral elements, regulation of tissue-specific gene expression, genomic imprinting, and X chromosome inactivation. It has also been found that DNA methylation exerts different influences on gene activities in different genomic regions depending on the underlying genetic sequence. Within intergenic regions, one of the main functions of DNA methylation is to repress the expression of genetic elements that might be potentially unfavorable. These elements, when expressed, may have harmful consequences, with their replication and insertion potentially leading to gene disruption and DNA mutation [17]. Varying degrees of methylation at genes and regulatory sequences can determine the level and integrity of gene expression [16]. Importantly, unmethylated CpGs are not distributed randomly throughout the genome, but are clustered in formations called CpG islands (CGIs), usually located in the promoter regions of genes [18]. CGIs are usually unmethylated in normal cells regardless of their expression levels and gene silencing is promoted post-methylation [16]. Due to their location, they are able to regulate gene expression through silencing of transcription [19]. DNA transcription can be affected in two ways; firstly, methylation of CGIs can impair transcription factors binding and, secondly, methyl-CpG-binding proteins bind to the methylated DNA sequences [20].

Assessment of DNA methylation in neurodegenerative diseases

As stated above, DNA methylation regulates tissue-specific gene expression, complicating its exploration in neurodegenerative diseases, due to the requirement of brain tissue. Brain tissue is only available postmortem making it infeasible for large-scale or longitudinal studies, where repeated measurements are required. Additionally, the heterogeneity of brain tissue poses a barrier in terms of analysis as it is widely known that different cells possess different patterns of methylation [21]. Importantly, postmortem brain tissue only offers an insight into the end stage of processes associated with AD and may not reflect the processes involved in disease development.

To address these concerns, proxy tissues such as blood or saliva have been investigated to determine if their level of DNA methylation is reflective of

the methylation state of the tissue of interest, i.e., the brain. Specifically, are the changes observed in the tissue of interest truly reflected in peripheral tissues. Early studies sought to clarify this, with a number demonstrating conservation of DNA methylation across different tissues, using several different methodologies for the assessment of DNA methylation, including the widely used Illumina 450k array, which offered the most comprehensive coverage of the methylome available at the time (now superseded by the Illumina EPIC 850k array). A study by Horvath et al. [22] showed evidence for conservation of DNA methylation patterns across four brain regions and blood tissue, with a correlation of $r = 0.85$-0.91, using publicly available genome-wide methylation data. This finding was supported in a study assessing 80 matched blood and post-mortem brain tissues, which found that subset of probes on the Illumina 450K array were significantly correlated across tissues [21]. Walton et al. [23] have likewise published on the 450K array, reporting that 7.9% of CpGs were strongly correlated between tissues. Further, through the development of the BECon (Blood-Brain Epigenetic Concordance) tool, Edgar and colleagues [24] demonstrated that 9.7% of CpGs from the Illumina 450K array were correlated between tissues. When assessing data from methylated DNA immunoprecipitation sequencing (MeDIP-seq) a high correlation between blood and the cortex and cerebellum ($r = 0.82$ and 0.77, respectively) was observed in three individuals [25]. More recently, Braun [26] investigated correlations between brain, saliva, blood, and buccal samples obtained from patients undergoing neurosurgical resection for epilepsy, and found that DNA methylation in the brain was most highly correlated with saliva ($r = 0.90$), followed by blood ($r = 0.86$) and buccal cells ($r = 0.85$).

While evidence exists for conservation of DNA methylation between tissues, contradicting studies reporting no significant associations have been published. Specifically, a recent study reported no evidence of a correlation between DNA methylation in peripheral blood CD4 + lymphocytes and prefrontal cortex samples [27]. Currently, the evidence is unclear, and the relationship between DNA methylation in the brain and blood requires additional characterization. However, it has been suggested that a suitable peripheral biomarker does not necessarily need to mirror disease-associated changes observed in the brain and could independently reflect disease responses in the periphery.

DNA METHYLATION AND ALZHEIMER'S DISEASE

Global DNA methylation

DNA methylation can be classified as either global or gene-specific. Global methylation refers to the average percentage methylation across the entire genome, whereas gene-specific DNA methylation refers to the average percentage methylation within a specific gene. Global methylation or overall methylation of the entire genome provides an over-arching picture of methylation status in a sample [28]. However, this measurement should be used with caution as differences in global methylation can result from disease state, tissue collection site, sex, and age [29].

In a 2009 twin study, monozygotic twins discordant for AD were found to have differences in global methylation in the anterior temporal cortex and the superior front gyrus, where the twin with AD had significantly decreased levels of methylation in those two areas of the brain [30]. While both twins had similar levels of education and worked in similar fields, the twin diagnosed with AD worked extensively with pesticides, suggesting this environmental exposure may have contributed to epigenetic changes and the development of AD [30]. Several epidemiological studies have reported phenotypic discordance in monozygotic twins, with the older sibling typically reflecting more discordance for age-related diseases [31, 32]. This is hypothesized to be due to an increased rate of loss of epigenetic architecture, or epigenetic drift, which is described as an increase in DNA methylation errors across the genome during aging [33]. Similarly, several other studies have demonstrated that older monozygotic twins have greater global differences in DNA methylation patterns when compared to younger twin pairs [34–36].

Since it was recognized that DNA methylation could be cell and region-specific, researchers began utilizing immunohistochemical methods to assess brain regions typically affected by AD. In individuals with AD, significant decreases in global DNA methylation have been observed in regions of the brain typically affected by AD, such as the entorhinal cortex [37]. In contrast, within the cerebellum, a region known to be spared in AD, differences in those with AD were not observed [37]. However, in the frontal cortex, a significant site of synaptic loss in AD [38], individuals with AD have been reported to have higher levels of DNA methylation when compared to

those classified as cognitively unimpaired. Additional studies have reported increased global DNA methylation levels in the middle frontal gyrus and frontal cortex, in individuals diagnosed with AD compared to those without [39, 40].

The hippocampus is involved in memory formation and is one of the first regions of brain to display atrophy in AD [41]. Similar to the results outlined above, studies assessing DNA methylation in the hippocampus have been inconclusive with higher [42] and lower [43] levels of DNA methylation observed in those diagnosed with AD. Further, DNA methylation is reported to differ depending on the hippocampal subregion and cell type [43]. Similarly, Phipps et al. [44] demonstrated that pyramidal neurons, a particularly vulnerable cell type in AD, but not calretinin interneurons or microglia, have decreased DNA methylation in AD cases compared to controls.

As well as interrogating DNA methylation within brain regions, peripheral blood cells are becoming more common due to the ease of sample access. In 2015, Di Francesco et al. [45] evaluated global DNA methylation in peripheral blood mononuclear cells in AD patients and cognitively unimpaired controls, finding a significant increase in the global methylation levels of AD patients [45]. Additionally, higher global DNA methylation was observed in the presence of the *APOE* ε4 allele, highlighting the importance of combining epigenetic and genetic markers of AD [45]. In a study by Bjornsson et al. [46], peripheral blood was sampled from community cohorts to assess time-dependent changes in global DNA methylation over 11-16 years. Changes in DNA methylation over time varied between individuals, with both increases and decreases observed [46]. These findings are consistent with those published in studies of cancer, where hypomethylation (decreased levels of methylation) and hypermethylation (increased levels of methylation) work in tandem to activate and suppress oncogenes and tumor suppressor genes, respectively and concurrently [47]. It is possible that a similar interplay of gene-specific hypo- and hyper-methylation may be employed during AD progression.

Gene-specific DNA methylation

Initially, studies of gene-specific DNA methylation in AD focused on genes that encode the key proteins implicated in AD pathology, such as Aβ precursor protein (*APP*), presenilin 1 (*PSEN1*), microtubule associated protein (*MAPT*), and apolipoprotein E (*APOE*). The *APOE* ε4 allele is the strongest genetic risk factor for AD. The ApoE protein functions as the primary cholesterol carrier for the maintenance, growth and repair of neurons and is typically highly expressed in the central nervous system [48]. Wang et al. [49] reported hypermethylation of the *APOE* promoter region in the prefrontal cortex of AD patients, which is in line with observations of lower levels of circulating ApoE in AD patients [50]. Similarly, Karlsson [51] found an association between dementia and AD, and increased methylation levels at the *APOE* promoter. However, a number of studies have reported no significant difference in the methylation of *APOE* as a result of AD [52–55].

The analysis of DNA methylation in genes encoding other important proteins in AD pathogenesis (*APP*, *PSEN1*, *BACE1*, and *MAPT*), has likewise returned varied results. The level of DNA methylation of these genes within brain [52, 56] and blood [55, 57, 58] samples, was observed in several studies to be associated with AD diagnosis [52, 55, 58], and in other studies not associated [52, 56, 57]. Another commonly targeted gene is *BDNF*, which encodes the brain-derived neurotrophic factor (BDNF), a protein that is commonly found in decreased levels in AD and is involved in neuronal differentiation, plasticity, and survival [59]. Significantly higher levels of DNA methylation in the *BDNF* promoter in peripheral samples have been reported in patients with AD [60–62]. However, studies of peripheral blood samples have also found no significant differences in the methylation of *BDNF* between AD and controls [55, 63]. Neuroinflammation is known to occur during the development of AD [64], and as such the methylation of genes involved in this biological process have been investigated with regard to AD. Nicolia et al. [65] found evidence of hypomethylation of the promoter region of Interleukin-1β (IL-1β) in the early stages of AD, which increased in later stages of the disease, matching the DNA methylation of control participants. Conversely, the same study observed decreasing levels of Interleukin-6 (IL-6) DNA methylation with the progression of the disease [65]. Neuroplasticity and memory formation are also known to be compromised in AD, and a gene thought to be involved in these processes, Sorbin and SH3 Domain Containing 3 (*SORBS3*), has been found to be hypermethylated in the brains of AD patients [66] and mouse models of AD [67]. Finally, the assessment of genes involved in the process of DNA methylation (*DNMT1*, *DNMT3A*, *DNMT3B, MTHFR*) has revealed associations with AD diagno-

sis [49]; however, this finding was not replicated in a subsequent study [57].

The development of large-scale DNA methylation arrays has allowed for the unbiased assessment of site-specific DNA methylation across the epigenome. An epigenome-wide association analysis of longitudinal peripheral blood samples identified differentially methylated genetic loci, near known AD risk genes including *BDNF, BIN1,* and *APOC1*, to be associated with the diagnosis of mild cognitive impairment and AD in the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort [62]. More recently, an additional study in the ADNI cohort identified a CpG site cg00386386, mapped to *MED22*, as associated with cognitive decline as measured by the Pre-Alzheimer's Cognitive Composite (PACC) [68].

DNA METHYLATION DYNAMICS DURING AGING

Epigenetic factors, when compared to genetic factors, could be more appropriate in explaining the observed anomalies in LOAD, as DNA methylation patterns are altered during development. In particular, the epigenome is susceptible to deregulation during early embryonal and neonatal development, puberty and most notably, old age [69], the greatest risk factor for AD. Numerous studies have aimed to categorize the cellular and molecular characteristics of aging [70]. Epigenetic alterations represent one of the most crucial mechanisms driving the deterioration of cellular functions, which can be observed during aging and age-related diseases [71]. Specifically, changes in the methylome have been observed to occur over time and are thought to be one of the driving factors for the development of age-related diseases [72]. Epigenetic changes either occur randomly or are driven by internal and external influences [71]. This may explain why aging patterns can be vastly different between genetically identical individuals, such as identical twins, or between animals with similar genetic makeups [34, 73]. Studies in mammals and single cellular models (e.g., *Saccharomyces cerevisiae*) reveal that the epigenome suffers a progressive loss of architecture during aging, resulting in changes in chromosomal architecture, genomic integrity, and gene expression patterns [71].

Epigenetic drift

DNA methylation changes with age, regardless of the input of maintenance DNA methyltransferases. It has been hypothesized that a complex interplay of genetic, environmental (diet and lifestyle), and stochastic factors (defects in the transmission of epigenetic information through cell division) result in epigenetic drift over time, with the contribution of these factors increasing with age [74]. Epigenetic drift is non-directional, with both hyper- and hypomethylation being observed. Further, it is non-uniform across the genome and variable within individuals of the same age [75]. While epigenetic drift is known to result in unpredictable differences between the methylomes of aging individuals, some changes involve specific genetic regions and are age-associated. This indicates that DNA methylation changes are not purely random but have a role in the aging process.

Age-associated differentially methylated positions/regions

The development of high throughput arrays, such as the Illumina 27k, 450k, and EPIC arrays, has provided an impetus to studying age-specific DNA methylation patterns. These investigations reveal that epigenetic drift appears to be restricted to specific sites in the genome (referred to as age-associated differentially methylated positions/regions; a-DMPs/a-DMRs) and is not purely random [74]. Specific age-related regulatory mechanisms are thought to be involved in age-associated hyper- and hypomethylation of sites detected in a number of epigenome-wide association studies (EWAS) [74]. A study by Bormann et al., in human skin, found that methylation deteriorated with age, with hypermethylated and hypomethylated sites trending towards an intermediate (50%) level of methylation, with a smaller dynamic range [76]. While age-associated, tissue-specific changes in DNA methylation have been identified [77, 78], consistent tissue-type independent a-DMPs have been reported in multiple tissues and cell types [22, 79], and in stem cells [77]. Notably, the causes and functional consequences of age-related changes in DNA methylation, and their relationship to aging, longevity, and disease, are yet undermined [79]. It has been speculated that the identification of age-associated DNA methylation differences between diverse groups of individuals could aid in determining the epigenetic basis of aging and age-related health disparities [80]. Recently, these age-associated changes have been leveraged to aid in the development of DNA methylation clocks.

EPIGENETIC AGE AND DNA METHYLATION CLOCKS

There is great potential for the use of DNA methylation clocks as biomarkers for disease risk and predictors of life expectancy, morbidity, and mortality. Biological aging, like chronological aging, is associated with disease, morbidity and mortality, health, and cognitive and physical decline. Indeed, the varied progression in biological age may allow for the prediction of the aspects of aging outlined above. Measures of biological aging are in development for use in the prediction of disease onset, risk, and progression [81]. Several potential biomarkers of aging, including telomere length, metabolomic, transcriptomic and proteomic markers, and of interest here, DNA methylation, have been proposed [70, 82]. While many biomarkers only capture certain aspects of aging, an increasing body of literature suggests that manifestations of aging are due to epigenetic processes [83]. The "epigenetic clock" is a DNA methylation-based estimate of biological age and has been strongly correlated with chronological age. A measure of age acceleration can be calculated based on the difference between an individual's biological age (estimated) and chronological age (actual) [84]. Accelerated aging has been linked to an increase in the pathological hallmarks of AD, reduced cognitive and physical fitness and an increase in all-cause mortality.

Approximately 28 million CpG sites exist within the human genome, usually located in clusters of hundreds or thousands, known as CpG islands. Epigenetic clocks have been developed using a select number of these CpG sites, widely distributed throughout the genome. An epigenetic clock, or a DNA methylation clock, is built from DNA methylation markers strongly correlated with chronological age ($r \geq 0.8$) [82]. The process of building a DNA methylation clock utilizes supervised machine learning methods such as lasso (least absolute shrinkage and selection operator), elastic net or ridge and training against chronological age. During this process, an informative and predictive set of CpGs is identified [81]. Newer generation epigenetic clocks are specifically trained on age-related biological and health-related measures.

Broadly, epigenetic clocks have been classified as first-generation chronological DNA methylation (DNAm) clocks or second-generation biological DNAm clocks. Chronological DNAm clocks reflect age-related DNAm changes that are common among individuals and reflect the intrinsic aging process. In a pioneering study, Bocklandt et al. [85] demonstrated that DNA methylation levels from saliva could generate an accurate age prediction. Since then, numerous DNAm clocks have been developed that accurately estimate the chronological age of a sample based on DNA methylation levels within the blood and other tissue and cell types (Table 1) [83]. The DNAm clocks developed by Horvath [83] and Hannum [100] were built using similar regression models, are correlated with age and have the ability to predict all-cause mortality. The most well validated of the DNAm clocks is the Horvath clock, which is predictive in all cell types, and encompasses 353 CpGs all weakly correlated with age but when combined are highly predictive of chronological age [83]. The Hannum clock is also well correlated with age, comprising of 71 CpGs sites; however, while works in blood, it requires further calibration for use in other tissues. More recently, the Zhang DNAm clock was developed correcting for cellular composition so it can be accurately used in blood and non-blood samples [86]. Currently there is a movement towards the development of clocks encompassing a smaller number of CpG sites to minimize cost/increase time efficiency [87]. However, these estimations have been demonstrated to be unreliable when used for multiple tissues [82], and have high average errors in their age predictions when compared to clocks with a greater number of CpG sites [74, 88–90].

Biological DNAm clocks, considered "second generation" clocks, are reflective of DNAm changes that vary between individuals and may highlight age-related phenotypes and external factors that may influence age-related DNAm [91]. Specifically, these clocks have been developed against age-related biological processes such as mitotic activity (e.g., epiTOC [92]; MiAge [93]), clinical measures of phenotypic aging (PhenoAge [94]), telomere length (DNAmTL [95]), plasma biomarkers and physiological and stress factors (GrimAge [96]), and all-cause mortality (Zhang [97]). Finally, the Dunedin Pace of Ageing clock, has incorporated the rate of change of 18 blood chemistry and organ system function measurements into the calculation of its score to improve predictive utility [98].

Age acceleration and health and disease

As outlined above, and summarized in Table 1, DNAm clocks have been developed using several different training models and as such reflect different

Table 1
Summary of DNA methylation clocks

Clock	Gen.	No. of CpGs	Age Range	Platform/Array	Method	Tissue	*n*	Model	Outcome	Ref.
DNAm-age	First	3	18-70	Illumina Infinium HumanMethylation 27K	Bisulphite conversion (EZ DNA Methylation Kit; Zymo)	Saliva	68	Lasso penalized regression	Association with chronological age	[85]
Epigenetic aging signature	First	19	16-72	Illumina Infinium HumanMethylation 27K	Used publicly available data - mixed	Mixed	130	Linear regression model	Association with chronological age	[88]
Passage Number	First	6	-	Illumina Infinium HumanMethylation 27K	Bisulphite conversion (EZ DNA Methylation Kit; Zymo)	Dermal fibroblasts & mesenchymal stem cells	51	Pavlidis Template Matching	Track passage number in fibroblast cell culture	[88]
EVOLV2 epigenetic marker of age	First	1	9-99	Illumina Infinium HumanMethylation 450K	Bisulphite conversion (EZ DNA Methylation Gold Kit; Zymo)	Blood	501	Spearman correlation	Association with chronological age	[147]
Florath age predictor	First	17	50-75	Illumina Infinium HumanMethylation 450K	Bisulphite conversion (EZ DNA Methylation Kit; Zymo)	Blood	400	Linear regression model	Association with chronological age	[148]
DNAm-age	First	353	0-101	Illumina Infinium HumanMethylation 27K & 450K	Used publicly available data - mixed	Mixed	8,000	Elastic Net regression	Association with chronological age, age-related disease, all-cause mortality, cancer, neurodegenerative phenotypes	[83]
DNAm-age	First	71	19-101	Illumina Infinium HumanMethylation 450K	Bisulphite conversion	Blood	656	Elastic Net (combination of Lasso and ridge regression)	Association with chronological age and all-cause mortality	[101]
DNAm-age	First	3	0-78	Illumina Infinium HumanMethylation 27K & 450K	EpiTect Bisulphite Kit; QIAGEN	Blood	575	Multivariate linear model	Association with chronological age, clinical/lifestyle factors, telomere length, related disease phenotypes	[149]

(*Continued*)

Table 1
(*Continued*)

Clock	Gen.	No. of CpGs	Age Range	Platform/Array	Method	Tissue	*n*	Model	Outcome	Ref.
DNAm-age	First	99	19-101	Illumina Infinium HumanMethylation 27K & 450K	EpiTect Bisulphite Kit; QIAGEN	Blood	656	Multivariate linear model	Association with chronological age, clinical/lifestyle factors, telomere length, related disease phenotypes	[149]
DNAm-age	First	102	0-78	Illumina Infinium HumanMethylation 27K & 450K	EpiTect Bisulphite Kit; QIAGEN	Blood	575	Multivariate linear model	Association with chronological age, clinical/lifestyle factors, telomere length, related disease phenotypes	[149]
Huang age predictor	First	5	9-75	Pyrosequencing	EpiTect Bisulphite Kit; QIAGEN	Blood	89	Linear regression model	Association with chronological age – for forensic use	[90]
Zbiec-Piekarska age predictor	First	5	2-75	Pyrosequencing	EpiTect Bisulphite Kit; QIAGEN	Blood	420	Multivariate linear model	Association with chronological age – for forensic use	[89]
Buccal Cell Signature	First	5	1-85	Pyrosequencing	Bisulphite conversion (EZ DNA Methylation Kit; Zymo)	Buccal Cells	55	Multivariate linear model	Epigenetic age predictor	[150]
Cho Model 2	First	5	20-74	Pyrosequencing	Bisulphite Conversion (EZ DNA Methylation - Lightning ™ Kit; Zymo)	Blood	100	Multivariate linear model	Association with chronological age	[151]
DNAm-age from Saliva	First	7	18-65	Illumina Infinium HumanMethylation 450K & SNaPshot	Bisulfite Conversion	Saliva	54	Linear regression model	Enabled age prediction in saliva with high accuracy	[152]
Skin and Blood	First	391	0-94	Illumina Infinium HumanMethylation 450K & EPIC	Used publicly available data - mixed	Mixed	896	Elastic Net regression	Prediction of age	[153]
Zhang age predictor	First	514	2-104	Illumina Infinium HumanMethylation 450K & EPIC	Used publicly available data - mixed	Blood and Saliva	13,661	Elastic Net	Association with chronological age	[86]

Zhang age predictor	First	319,607	2-104	Illumina Infinium HumanMethylation 450K & EPIC	Used publicly available data - mixed	Blood and Saliva	13,661	Best Linear Unbiased Prediction	Association with chronological age	[86]
PedBE Clock	First	94	0-20	Illumina Infinium HumanMethylation 450K & EPIC	Used publicly available data	Buccal epithelial cells	1,721	Elastic Net	Tool for measuring biological age in children	[154]
DNAm-age	First	5	19-70	SNaPShot	Bisulphite conversion (Imprint™ DNA Modification Kitl Sigma-Aldrich)	Blood, saliva, and buccal cells	448	Linear Regression	Age prediction	[155]
DNAm-age	First	5	24-86	Bisulphite PCR	Bisulphite conversion (EZ DNA Methylation Gold Kit; Zymo)	Blood	51	Linear Regression	Age prediction for forensic use in deceased individuals	[156]
Blood-Bone-Tooth Age Prediction Model	First	43	1-94	Sanger Sequencing	Bisulphite conversion (EZ DNA Methylation Gold Kit; Zymo)	Blood, bone, and tooth	185	Multivariate linear model	Age prediction for forensic use in alive and deceased individuals	[157]
PC Horvath 1 PC Horvath 2 PC Hannum PC PhenoAge PC GrimAge PC DNAmTL	Second	PCs trained on 78,464 CpGs	0-105 -0.3-101 19-101 21-100 24-92 24-92	Illumina Infinium HumanMethylation 450K & EPIC & Elysium custom array	Used publicly available data - mixed	Mixed	4,297 895 656 4,505 2,754 2,754	Principle components and elastic net regression	PC clocks show reduced technical noise allowing for improved detection of clock associations, intervention effects and reliable longitudinal tracking	[158]
Cortical DNAm Clock	Second	347	1–104	Illumina Infinium HumanMethylation 450K array	Used publicly available data - mixed	Human cortex	1,397	Elastic Net regression	Clock outperforms previously reported clocks	[138]
Boroni Skin	Second	2,266	18–95	Illumina Infinium HumanMethylation EPIC array	Used publicly available data	Human skin	508	Elastic Net regression	Skin specific DNAm age predictor	[159]
epiTOC	Second	385	19–101	Illumina Infinium HumanMethylation 450K array	Used publicly available data	Blood	656	Linear regression model	Association with mitotic age, cancer	[92]

(*Continued*)

Table 1
(*Continued*)

Clock	Gen.	No. of CpGs	Age Range	Platform/Array	Method	Tissue	*n*	Model	Outcome	Ref.
Epigenetic age predictor	First	99	0–78	Illumin Infinium HumanMethylation 27k array	Lothian Birth Cohorts 1921 and 1936	Blood	575	Cox linear regression	Association with life expectancy	[160]
Biological age predictor	First	8	–	Methylation-sensitive single-nucleotide primer extension (MS-SNuPE)	Used publicly available data - mixed	Blood	390	Multiple linear regression	Simplified assay for epigenetic age estimation	[161]
All-cause mortality	Second	10	31–82	Illumina Infinium HumanMethylation 450K array	Bisulphite conversion	Blood	1,000	Lasso penalized regression	Association with all-cause CVD, cancer, smoking behavior	[97]
MiAge	Second	286	–	Illumina Infinium HumanMethylation 450K array	Used publicly available data - mixed	8 Cancer cell types	4,020	Linear regression model	Association with mitotic age, cancer outcome and survival prediction	[93]
DNAm PhenoAge	Second	513	>20	Illumina Infinium HumanMethylation 27K, 450K & EPIC array	Used publicly available data - mixed	Blood	9,926	Cox penalized regression	Association with all cause and cause specific mortality, telomere length, survival, smoking status	[94]
DNAm GrimAge	Second	1,030	-	Illumina Infinium HumanMethylation 450K & EPIC array	Used publicly available data - mixed	Blood	1,731	Elastic Net, Cox regression	Association with morbidity and mortality, survival, cognitive decline, telomere length	[96]
DNAmTL	Second	140	22-93	Illumina Infinium HumanMethylation 450K & EPIC array	Used publicly available data - mixed	Blood	2,256	Multiple regression models	Association with chronological age, telomere length, morbidity and mortality, physical function	[95]

DunedinPoAm	Second	140	-	Illumina Infinium HumanMethylation 450K & EPIC array	Used publicly available data - mixed	Blood	1,037	Elastic Net	Blood DNAm measure sensitive to variation in pace of biological aging in individuals of same age	[98]
epiTOC2	Second	163	19-101	Illumina Inifinium HumanMethylation 450K, Whole genome bisulfite sequencing, RNA-SeqV2	Used publicly available data - mixed	Mixed	>2,000	Formal dynamic model	Prediction of increased mitotic rate in cancer	[162]
Hypoclock	Second	-	-	Whole genome bisulfite sequencing, Illumina Inifinium HumanMethylation 450K	Used publicly available data - mixed	Mixed	-	Gaussian mixture model	DNA methylation loss in late-replicating domains linked to mitotic cell division	[163]
DunedinPACE	Second	173		Illumina Infinium HumanMethylation 450K & EPIC array	The Dunedin Study, the Understanding Society Study, the Normative Ageing Study, the Framingham Heart Study & the Environmental Risk Longitudinal Twin Study	Mixed	1,037	Elastic Net regression	Associated with morbidity, mortality, and disability. Novel blood biomarker of the age of pacing for gerontology and geroscience.	[164]
GrimAge 2	Second	1,030		Illumina Infinium HumanMethylation 450K & EPIC array	Used publicly available data - mixed	Mixed	13,399	Elastic Net, Cox regression	GrimAge 2 outperforms GrimAge and is an epigenetic biomarker of mortality and morbidity risk	[165]

aspects of disease and the aging process. It has been hypothesized that DNA methylation age (DNAm age) measures the cumulative effect of an epigenetic maintenance system [83]. Studies have demonstrated that DNAm age of blood predicts all-cause mortality in later life, which suggests that methylation age is a process that reflects aging [99]. However, it is more probable that the epigenetic clock is a result of the emergent properties of the epigenome [83]. Several studies have proven the usefulness of using age acceleration as a measure for healthy biological aging. For example, semi-supercentenarians and their offspring have a lower biological age compared to chronological age, or age deceleration, when compared to age-matched controls [100]. Similarly, women have shown to have less significant age acceleration compared to men, which is in line with their representative longer lifespans [101, 102].

Moreover, a number of studies have demonstrated the utility of age acceleration as a predictor of disease risk and mortality. A study assessing four longitudinal cohorts identified an association between all-cause mortality and increased epigenetic age in blood [103]. Further, a number of genetic syndromes have been reported to accelerate biological aging, such as Down syndrome [104] and Werner's syndrome [105]. There is ample evidence of premature biological aging in those with viral infections such as HIV [106, 107], individuals with decreased mental and physical fitness [108], body mass index and metabolic disease [109], non-alcoholic steatohepatitis [110], obesity [111], insomnia [112], and extreme stress [113]. Additionally, individuals with neurodegenerative diseases such as Parkinson's disease [114], Huntington's disease [115], and AD [116] show accelerated biological aging.

METHYLATION AGE AND AD PHENOTYPES

The limited research which has been undertaken to explore the relationship between DNAm age and AD related phenotypes has yielded inconsistent findings. The association between DNAm age and AD phenotypes has primarily been characterized by measures of DNA methylation within the blood [108, 117–125], while only one study investigated brain samples [116].

Further, research has focused largely on "first generation" estimates of DNAm age, specifically the Horvath and Hannum clocks, which have been trained to approximate chronological age. Age acceleration, as estimated using the Horvath DNAm age clock in blood, has been associated with lower cognition cross-sectionally [108, 116], longitudinal cognitive decline [122, 123], and reduced white matter integrity. Additionally, Horvath age acceleration in prefrontal cortex samples has been associated with diffuse and neuritic Aβ plaques and higher global Aβ burden [116]. Despite these findings, a number of studies have also reported no significant association between Horvath DNAm age acceleration and cognitive functioning or brain volume, both cross-sectionally [117, 121] and longitudinally [108, 121]. Likewise, inconsistent findings have been reported when assessing DNAm age using the Hannum estimation. Hannum age acceleration has been associated with smaller hippocampal volume [125], but conversely, increased brain connectivity and decreased mean diffusivity (improved axonal integrity) in MRI quantified studies [117]. Additionally, several studies have failed to identify any significant associations between Hannum age acceleration and cognitive functioning [117, 121] or longitudinal cognitive decline [121, 124].

The association between "second generation" biological DNAm age estimates and AD phenotypes has also been explored, although less broadly. GrimAge acceleration has been associated with lower cognitive ability, accelerated cognitive decline, lower intelligence test scores, lower white matter and grey matter volumes, total brain volume, and higher volume of white matter hyperintensities [119]. However, both DNAm telomere length and the Pace of Ageing score were not associated with cognitive decline [120]. Inconsistency in the current literature underscores the need for further investigation of DNA methylation before it can be considered as a robust biomarker for aging-related disease such as AD.

CONSIDERATIONS WHEN UTILIZING DNA METHYLATION

The "tissue issue"

The studies outlined above, and summarized in Table 2, have presented DNA methylation findings from a range of tissue and cell types. As is evident in Table 2, there has been a trend towards large-scale discovery studies (EWAS) using peripheral blood and a shift away from studies exploring global methylation. When exploring these findings, it is important to consider that DNA methylation levels at specific CpG

Table 2
Summary of DNA methylation studies in AD

Phenotype	Tissue	Sample size	Age range / mean age	Platform	Gene/s	Outcome	Ref.
AD	Frontal cortex	1 case	-	Southern blot	Targeted	Hypomethylation of *APP* gene in AD	[166]
AD	Brain tissue	72 (48 case, 24 control)	52-93	Immunohistochemistry	Targeted genes	Methylation levels of *PP2AC* decreased in AD-affected brain regions	[167]
AD	Brain tissue	125	17 weeks-104	MethyLight PCR	Targeted genes	AD-associated differences in *SORBS3* and *S100A2,* DNA methylation dynamically regulated throughout life span	[66]
AD	Prefrontal cortex and lymphocytes	34 brain (24 AD, 10 control); 10 lymphocytes (6 AD, 4 control)	AD 81, control 80	MALDI-TOF mass spectrometry	Targeted genes	Age-specific epigenetic drift in AD, significant interindividual epigenetic variability in *PSEN1, APOE, MTHFR, DNMT1*	[49]
AD	Temporal neocortex	2 (1 case, 1 control)	Case 76, control 79	Immunohistochemistry	Global	Reduced levels of DNA methylation in the temporal neocortex of the AD twin	[30]
AD	Frontal cortex & hippocampus	66 (43 case, 26 control)	46-93	MALDI-TOF	Targeted genes	No differences in percentage methylation between cases and controls	[56]
AD	Entorhinal cortex layer II	40 (20 case, 20 control)	60-97	Immunohistochemistry	Global	DNA methylation status in entorhinal cortex layer II neurons is highly diminished in AD	[37]
AD	Brain tissue	6	-	Bisulfite sequencing	Targeted genes	No associations of *APP* with AD	[168]
AD	Peripheral blood	83 (43 case, 38 control)	55-87	Quantitative bisulfite-PCR pyrosequencing	Targeted repetitive elements	LINE-1 methylation increased in AD compared to controls	[169]
AD	Frontal cortex	20 (10 case, 10 control)	Case 70.60, control 70.20	RT-PCR	Targeted genes & global methylation	Hypomethylation of *COX-2* and *NF-κB* promoter in AD, hypermethylation of *BDNF* and *CREB*, increased global methylation in AD	[40]
AD	Frontal cortex	50 discovery (12 case, 12 control); 26 validation (13 case, 13 control)	Case 78.2, control 78.3	Illumina Infinium HumanMethylation 27K array	EWAS	Hypomethylation of *TMEM59* in AD	[170]
AD	Peripheral blood mononuclear cells	66 (33 case, 33 control)	Case 79.47, control 79.98	Methylation-specific primer RT-PCR	Targeted genes	Decreased DNA methylation at *FAAH* gene promoter	[171]
AD	Peripheral blood mononuclear cells	60 (32 case, 28 control)	-	Methylation-specific primer RT-PCR	Targeted genes	Promoter methylation of *PIN1* in AD increased compared to controls	[172]

(*Continued*)

Table 2
(*Continued*)

Phenotype	Tissue	Sample size	Age range / mean age	Platform	Gene/s	Outcome	Ref.
AD	Entorhinal, auditory cortices, hippocampus, and blood	99 (44 case, 55 control)	Case blood 75.47, case brain 80.1, control blood 72.09, control brain 78.5	Mass spectrometry	Targeted genes	No AD-associated differences in *SNAP25* promotor DNA methylation	[173]
AD	Entorhinal, auditory cortices, hippocampus, and blood	22 brain (12 case, 10 control); 84 blood (36 case, 48 control)	Case blood 75.4, case brain 81, control blood 72.1, control brain 78.5	MALDI-TOF mass spectrometry	Targeted genes	Brain *SORL1* DNA methylation was significantly higher in brain than in blood in both cases and controls	[174]
AD	Brain and blood	57 blood (34 case, 23 control); 22 brain (12 case, 10 control)	Blood – case 75.47, control 72.09; Brain – case 81, control 78.50	Mass spectrometry	Targeted genes	No differences in DNA methylation between cases and controls	[175]
AD	Brain tissue	391 (170 cases, 221 control)	Case 78.68, control 76.59	Pyrosequencing	Targeted genes	Hypermethylation in APP, MAPT and GSK3B in sporadic AD, which is more prominent in *APOE* ε4	[52]
AD	Hippocampus	20 (10 case, 10 control)	Case 75.36, control 77.91	Immunohistochemistry	Global	Decreased global DNA methylation in the hippocampus of AD	[43]
Preclinical AD/AD	Hippocampus/ parahippocampus and cerebellum	17 (12 case, 5 control)	Preclinical 89.4, AD 77.4, control 84.6	Immunohistochemistry	Global	Altered DNA methylation patterns in vulnerable brain regions prior to onset of clinical symptoms	[42]
AD	Entorhinal, auditory cortices, hippocampus	22 (12 case, 10 control)	Case 81, control 78.5-	Mass spectrometry	Targeted genes	No AD-associated differences in promoter methylation	[176]
AD	Peripheral blood	55 (27 case, 28 control)	Case 79.47, control 79.98	Fluorescence-based RT-PCR	Targeted genes	Decreased DNA methylation at the *5-LOX* gene promoter in AD compared to controls	[177]
AD	Gray matter	58 (26 case, 26 control)	51-98	High content analysis method	Global methylation	Increase in global 5mC and 5hmC levels in AD	[39]
AD	Brain tissue and peripheral blood	166 (122 brain discovery, 57 blood; 144 brain replication)	67-105	Illumina Infinium HumanMethylation 450K array	Targeted genes	*ANK1* differentially methylated and associated with neuropathology in the entorhinal cortex	[54]

AD	Brain tissue	825 (708 discovery, 117 test)	>53	Illumina Infinium HumanMethylation 450K array	EWAS	71 CpGs associated with AD pathology, 11 validated in independent cohort	[53]
AD	Middle frontal gyrus and middle temporal gyrus	58 (29 case, 29 control)	54-98	Immunohistochemistry	Global	Global hypermethylation in AD compared to controls	[39]
AD	Peripheral blood	58 (28 case, 30 control)	Case 77.4, control 74.9	Methylation-sensitive high-resolution melting quantitative assay	Global	No differences in LINE-1 methylation between AD and controls	[178]
AD	Hippocampus	20 (discovery 15 case, 5 control; validation – 25 case, 25 control)	Discovery – case 82.4, control 82.4; Validation case 79.1, control 64.2	Illumina Infinium HumanMethylation 27K array	EWAS	AD-associated hypermethylation of promoter region of *DUSP22*	[179]
AD	Dorsolateral pre-frontal cortex	740	Mean age at death 88	Illumina Infinium HumanMethylation 450K array	Targeted genes	16 CpGs associated with neuritic amyloid plaques from 11 AD susceptibility gene regions	[180]
AD	Dorsolateral prefrontal cortex	740	66-108.3	Illumina Infinium HumanMethylation 450K array	Targeted genes	Brain DNA methylation in 5 AD-risk loci is associated with pathological AD	[181]
AD	Entorhinal cortex and cerebellum	26 (14 case, 12 control)	38-99	Immunohistochemistry & ELISA	Global	No differences in global methylation between AD and controls	[182]
AD	Hippocampus	42 (30 case, 12 control)	19-98	Bisulfite cloning sequencing further measured by 5hmC	Targeted genes	*TREM2* methylation was significantly higher in AD compared to controls	[183]
AD	Peripheral blood	260 (80 case, 160 control)	65-96	Melting Curve Analysis-Methylation Assay (MCA-Meth)	Targeted genes	*UQCRC1* was highly methylated in AD, *UQCRC1* was significantly associated with gene expression of *NRD1, DDT, CTSB* and *CTSD*	[184]
AD	Superior temporal gyrus	390	Cohort 1 – case 85.4, control 77.6; Cohort 2 – case 88.0, control 82.1; Cohort 3 – case 78.4, control 84.1	Illumina Infinium HumanMethylation 450K array & pyrosequencing	Targeted genes	AD-associated hypermethylation in CpG site 289bp upstream of *TREM2* in 3 independent cohorts	[185]

(*Continued*)

Table 2
(*Continued*)

Phenotype	Tissue	Sample size	Age range / mean age	Platform	Gene/s	Outcome	Ref.
AD	Superior temporal gyrus	68 (34 case, 34 control)	66–92	Illumina Infinium HumanMethylation 450K array	EWAS	Identified 479 DMRs, with the majority being hypermethylated	[186]
AD	Inferior temporal gyrus	20 (15 case, 5 control)	58–91	Immunohistochemistry	Targeted	Epigenetic dysregulation occurs in astrocytes and neurofilament-positive pyramidal neurons in AD	[44]
MCI	Peripheral blood	96 (48 MCI, 48 control)	–	Bisulfite Pyrosequencing, Dual Luciferase Assays	Targeted genes	Hypermethylation of *OPRK1* in females, *OPRM1* differential methylation associated with MCI	[187]
AD	Discovery - Prefrontal cortex & superior temporal gyrus; test - mixed	1004 (147 discovery, 857 test)	64–95	Illumina Infinium HumanMethylation 450K array	Targeted genes	Increased DNA methylation associated with AD neuropathology	[188]
AD	Frontal cortex, temporal cortex, & occipital cortex	159 (91 case, 68 control)	18–97	Illumina Infinium HumanMethylation 450k array	EWAS	Identified numerous genes with cell-type-specific methylation signatures	[189]
AD	Peripheral blood	84 (45 case, 39 control)	AD 73.56, control 75.33	Illumina Infinium HumanMethylation EPIC array	EWAS	Hypomethylation of *B3GALT4* and *ZADH2* associated with the level of Aβ and tau in CSF	[190]
AD	Cortical pyramidal layer	32 (18 case, 14 control)	AD 77.22, control 70.29	Illumina Infinium HumanMethylation 450k array	EWAS	Differential hypermethylation in several genomic regions, including *HOXA3, GSTP1, CXXC1-3 & BIN1*	[191]
AD	Brain tissue	51 (40 case, 11 control)	41–99	Illumina Infinium HumanMethylation 450k array & whole genome bisulfite sequencing	EWAS	Hypomethylation of *KIAA0566* associated with age and presence of NFT pathology	[192]
AD	Prefrontal cortex	101	54–105	Bisulfite padlock probe technique	EWAS	1224 differentially methylated enhancer regions identified, enhancement in the *DCSAML1* gene which targets *BACE1*	[193]
AD	Hippocampus, entorhinal cortex, dorsolateral pre-frontal cortex and cerebellum	73 (24 case, 48 control)	53–80	Illumina Infinium HumanMethylation 450k array	EWAS	858 differentially methylated sites, annotated to 772 genes, some novel. Sites were overrepresented in AD genetic risk loci	[194]

AD	Brain tissue	60	Mean age entorhinal cortex 77.7, superior temporal gyrus 77.6, cerebellum 78.4	Bisulfite pyrosequencing	Targeted genes	DNA hypermethylation in the entorhinal cortex	[195]
AD	Middle temporal gyrus and peripheral blood	80 brain tissue (45 case, 35 control); 96 peripheral blood (54 AD converters and 42 non-converters)	–	Illumina Infinium HumanMethylation 450K array	Targeted genes	Differential methylation in *OXT* promoter in brain of AD, same region in blood associated with converters	[196]
AD	Brain tissue	296 (96 discovery, 104 validation cohort 1, 96 validation 2)	Discovery 81.2; Validation 1 84.9; Validation 2 85	Illumina Infinium HumanMethylation 450K array	EWAS	*WNT5B* differentially methylated; two differentially methylated regions in *ANK1* & *ARID5B*;	[197]
AD	Brain Tissue	706	88 (mean age)	Illumina Infinium HumanMethylation 450K array	Targeted genes	249 & 115 variably methylated probes associated with Amyloid-β & Neurofibrillary tangles – most not overlapping with DMPs	[198]
AD	Hippocampus	38 (26 case, 12 control)	19-98	Illumina Infinium HumanMethylation 450K array	Targeted genes	118 differentially methylated positions identified in AD hippocampus	[199]
AD	Dorsolateral pre-frontal cortex &	1221	41-104	Illumina Infinium HumanMethylation EPIC array	EWAS	334 DMPs associated with AD pathology	[200]
MCI/AD	Peripheral blood	284 (AD 86, MCI 109, control 89)	AD 76.8, MCI 75.1, Control 73.8	Illumina Infinium HumanMethylation EPIC array and pyrosequencing	EWAS	12 AD-associated hypermethylated probes identified in *HOXB6* and validated by pyrosequencing	[201]
AD	Peripheral blood	653	AD 77.19, MCI 72.58, 76.23	Illumina Infinium HumanMethylation EPIC array	EWAS	Identified differentially methylated loci were near brain/neurodegeneration related genes	[62]
AD	Brain tissue	134 (72 case, 62 control)	AD 81.00, Control 80.50	Illumina Infinium HumanMethylation EPIC array	EWAS	22 DMPs and 30 DMRs associated with pathology, novel DMPs and DMRs discovered, replicated in independent cohort	[202]

(*Continued*)

Table 2
(*Continued*)

Phenotype	Tissue	Sample size	Age range / mean age	Platform	Gene/s	Outcome	Ref.
AD	Peripheral blood	96 test & 96 replication (48 case, 48 control), 95 non-AD dementia	Test - AD 72.7, control 71.9; Replication – AD 70.5, control 70.2	Sanger Sequencing	Targeted genes	*CR1*, *CLU* & *PICALM* methylation significantly lower in AD	[203]
AD	Prefrontal cortex, entorhinal cortex, and superior temporal gyrus	37 (29 case, 18 control)	AD 84.2, control 88.4	Illumina Infinium HumanMethylation 450k array	EWAS	Psychosis-associated epigenetic changes in a number of loci, genes enriched in schizophrenia-associated genetic and epigenetic variants	[204]
AD	Peripheral blood and brain tissue	50636 (7540 case, 43096 control)	–	Illumina Infinium HumanMethylation 450k array	Summary-based EWAS	152 CpGs AD-associated genes corresponding to 113 genes. 10 genes had significant probes in both blood and AD-specific analyses	[205]
AD	Peripheral blood	452	56–80	Illumina Infinium HumanMethylation 450K array	EWAS	238 gene network identified, including *APP* and several novel candidate genes	[206]
AD	Brain tissue	2116 (1455 discovery, 661 replication)	>65	Illumina Infinium HumanMethylation 450K array	Meta-analysis of six EWAS	220 CpGs associated with neuropathology, annotated to 121 genes, 84 novel	[207]
AD	Middle temporal gyrus	296 (198 case, 98 control)	AD 83.8, control 82.2	Illumina Infinium HumanMethylation 450K array	EWAS	5246 CpGs and 832 DMRs, some overlap with previous EWAS, some novel	[208]

Table 3
Advantages and Disadvantages of commonly used cell types

	Advantages	Disadvantages
Organ of interest (e.g., brain)	Desired organ/tissue/cell directly targeted	Need for highly trained professionals, Relatively expensive compared to other methods, Difficult/not feasible to obtain samples in some instances, e.g., brain tissue, Heterogenous in cellular makeup, Cell numbers change in disease
Whole blood	Minimally invasive, cost-effective, Minimally trained professionals required, Repeated sampling possible (longitudinal disease/intervention/treatment tracking possible)	Heterogenous in cellular makeup, Some special training required i.e., phlebotomy, Potentially peripheral to target of interest
Buccal cells	Non-invasive, Cost-effective, Easily obtained – no specially trained professionals required	Potential to be low in cellular material, Contamination from food, beverages, kissing, etc., Heterogenous in cellular makeup, Potentially peripheral to target of interest
Mixed	Ability to pool multiple publicly available sources of data for increased sample size and analytical power	Heterogenous cell types makes interpretation of results difficult – can only draw general conclusions. Methods of sample collection/sample handling/bisulfite conversion/data collection may differ between datasets – increased variance due to large number of uncontrollable variables
Saliva	Non-invasive and convenient sampling, repeated sampling possible, easy collection without the need for special training	Potential to be low in cellular material, contamination from food, beverages, etc., Heterogenous in cellular makeup, Potentially peripheral to target of interest
Skin	Relatively non-invasive in some instances, i.e., skin scrapings, Experiments can be replicated *in vitro* with high fidelity	Potentially invasive, i.e., in the instance of skin biopsies, Heterogenous in cellular makeup, Skin is very exposed to environmental stimuli such as the sun (UV exposure) and pollution
Stem Cells	Embryonic stem cells (ESCs) can be cultured indefinitely without compromising pluripotency, Can be differentiated into different cell types *in vitro*, Homogenous	Induced pluripotent stem cell (iPSCs) reprogramming is long, complex and inefficient, Non-CpG methylation needs to be considered when using ESCs, Low passage human iPSCs retain 'epigenetic memory', which biases characteristics of tissue of origin, Non-physiological methylation patterns introduced *in vitro*

sites typically cannot be compared across different tissue types and DNA methylation at many CpG sites is only weakly correlated across tissue types such as the brain (cortex and cerebellum) and blood [21]. Tissue specificity is one of the most common uncertainties that arise when using peripheral tissues in association studies of DNA methylation. When considering the utility of DNA methylation markers in either the target organ, the brain, or the periphery, the blood, it is important to be aware of the challenges posed by sample types (Table 3).

When assessing brain tissues in epigenetic clock analysis of neurodegenerative or neuropsychiatric disorders, limited information is available regarding the specific brain regions and cell types present, which has been reflected in differences in epigenetic patterns and gene function [25]. However, with regard to cell-type specific methylation, computational methods, such as the Houseman [126] (cellular proportion estimates in blood) and Guintivano [127] (neuronal and non-neuronal proportion estimates) methods have been developed to address the issues resulting from varying cell-type proportions between individuals [127, 128]. The gold standard for controlling for cell proportions is using sorted cell populations such as laser capture microdissection or fluorescence-activated cell sorting; however, these are relatively expensive options and hard to achieve logistically in large scale studies. Importantly, brain tissue is typically obtained postmortem, which needs to be considered when interpreting data [129]. Specifically, are epigenetic patterns observed *post-mortem*

consequence of the disease process itself, rather than causal? Additionally, detected epigenetic signals could be due to the cause of death, tissue pH and the premortem agonal state [130]. Careful study design via targeted validation employing cell sorting within the tissue of interest can help isolate cell type-specific changes, resulting in a clearer explanation of observed biological effects, such as which epigenetic markers mediate risk for disease or associate with a phenotype. Characterizing and exploring the effects of cellular heterogeneity is a crucial step in the analytical pathway of methylome-wide DNAm data in heterogenous tissues, especially peripheral blood, thus we recommend that it not be overlooked. Moving forward, more research focus needs to be placed on cross-tissue study designs to identify DMPs/DMRs common across two or more cell types such as brain tissue and peripheral blood to uncover common correlations. If the aforementioned limitations can be overcome, the next major issue is sample availability and the capacity to undertake appropriately powered studies to detect associations, which may be subtle.

Conversely, sampling of peripheral tissue offers many advantages over target tissues, such as ease of access, large sample sizes, replication opportunities and longitudinal sampling, both prior to disease onset and during treatments and/or interventions. However, as with brain samples, several limitations need to be considered when performing and interpreting analyses utilizing DNA methylation results from peripheral blood. In addition to tissue specificity, cellular heterogeneity is a significant potential confounder of quantifying DNA methylation levels in the blood and should be considered when interpreting results. Whole blood is composed of many different cell types, including neutrophils, lymphocytes, monocytes, eosinophils and others, and the proportions of these cell types can be influenced by numerous factors such as bacterial or viral infections, inflammation, diet, stress, medication, and environmental exposures [131]. There is an increasing number of studies utilizing peripheral DNA methylation; however, very few take into consideration the proportions of different cell types, and thus do not control for them in analyses, which likely drives some observed associations or masks potential differences between groups [132].

There is currently little evidence to suggest that AD-related epigenetic modifications in the brain are reflected in the periphery [133]. Several studies have measured DNA methylation in specific brain regions and in blood; however, only a few studies reported changes in both tissues, which are speculated to be driven by underlying genetic repeats within the *C9orf72* gene [134–136]. While a peripheral biomarker, such as one identifiable in blood, has been proposed as a useful surrogate marker to monitor disease status in major organs or systems, it could independently reflect disease processes in the periphery and be important in disease tracking and prognosis.

Underestimation of age in epigenetic clocks

Several studies have now reported an underestimation of age [137–140], with deviations in biological age from chronological age particularly noticeable in the higher age brackets. This observation is likely due to several contributing factors. One hypothesized factor contributing to an underestimation of age is the saturation of methylation sites, where large proportions of sites are approaching the lower (0%) and upper (100%) limits of methylation, caused by epigenetic drift in aging individuals [141]. Dhingra et al. [139] speculate that differences in results between epigenetic clocks within the same cohort are likely caused by missing probes between arrays, irrespective of probe imputation procedures leading to an underestimation of age. Additionally, survivorship bias, a type of selection bias, may be driving the underestimation of age, particularly in cohorts with relatively high mean age. Individuals with a negative age acceleration are maintained within the sample, and those with a positive age acceleration, are not present within the sample, leading to a skewed cohort. The underlying populations on which the clocks are trained are also an important factor to consider when interpreting results. For example, the Hannum and Horvath clocks were trained on chronological age and given their overall high accuracy in estimating age, they are likely not ideal estimators of biological age. A clock that is capable of predicting chronological age perfectly would contain no information on variation in biological age at an individual level [16]. For this reason, clocks such as the PhenoAge clock, which were trained on age-related and disease phenotypes in combination with chronological age, are preferred when investigating biological age more directly. Further, DNAm age estimates may be susceptible to a ceiling effect, where a plateau is observable. The Horvath clock requires an age transformation past a certain age threshold, indicating that the relationship between chronological age and estimated age is not linear and, past a certain age, estimates need to be

treated differently [83]. Recently machine learning and deep learning approaches have been employed in an attempt to mitigate shortfalls such as the ones mentioned here, and have observed more accurate age predictions for older individuals than the original clocks [142].

CONCLUSION

DNA methylation has been implicated in a number of diseases in the last decade including, cancer [143], heart disease [144], autoimmune disorders [145], and neurodegenerative diseases [146]. Many studies, leveraging both global and site specific CpG DNA methylation, have provided evidence for age-associated changes in DNA methylation. However, more recently, clusters of CpG sites whose DNA methylation status provide an accurate measure of chronological age have been identified. Together these sites provide the foundation for epigenetic clocks. Although initially, the clock's main purpose was to serve as a biomarker of chronological age, there is growing evidence suggesting that epigenetic clocks may have value as biomarkers of biological age. Emerging research supports the idea that age and age-associated diseases or phenotypes are characterized by increased age acceleration in comparison to healthy individuals. Consequently, research focus has been placed on epigenetic clocks as biomarkers to detect aging and aging-associated diseases. The utility of peripheral blood as a biomarker of diseases in specific organs is often questioned, given cells in the periphery may not accurately capture and reflect more central disease processes. However, a good peripheral biomarker does not have to mirror disease-associated changes in the brain. Rather, it could represent a co-occurring peripheral response to central pathology. Despite its limitations, peripheral blood remains the most obvious surrogate tissue for epigenetic studies. DNA methylation is a highly promising biomarker for AD disease-associated pathology. Further investigation of its association with disease trajectories will be essential in identifying individuals at risk of age-related diseases, thus optimizing treatment and intervention strategies.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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