DNA modifications and Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a complex neurodegenerative disease, affecting millions of people worldwide. Whilst a number of studies have focussed on identifying genetic variants that contribute to the development and progression of late-onset AD, the majority of these only have a relatively small effect size. There are also a number of other risk factors, for example age, gender and other co-morbidities, however how these influence disease risk is not known. Therefore, in recent years, research has begun to investigate epigenetic mechanisms for a potential role in disease etiology. In this chapter, we discuss the current state of play for research into DNA modifications in AD, the most well studied being 5-methylcytosine (5-mC). We describe the earlier studies of candidate genes and global measures of DNA modifications in human AD samples, in addition to studies in mouse models of AD. We focus on recent epigenome-wide association studies (EWAS) in human AD, using microarray technology, examining a number of key study design issues pertinent to such studies. Finally we discuss how new technological advances could further progress the research field.

Genetic contributions to Alzheimer's disease etiology

Alzheimer's disease (AD) is a progressive neurodegenerative disease that contributes significantly to the global disease burden, affecting in excess of 26 million people worldwide (1). Clinically, the first signs of AD manifest as a reduction in the ability to retain new information, leading to disruptions in daily routine. This is followed by difficulty in planning and solving problems, confusion related to time and/or place, speech trouble and mood and personality changes. AD is characterized by the accumulation of two proteins that contribute to the neuropathology of the disease: extracellular plaques of amyloid-β peptide (Aβ) and neurofibrillary tangles of hyperphosphorylated microtubule binding protein tau (2, 3). These neuropathological changes are thought to occur perhaps decades before clinical symptoms manifest and the disease is diagnosed (4). Moreover, given that currently prescribed medications are simply symptomatic treatments and do not modify the underlying disease process, considerable research effort is currently focussed on understanding disease etiology.

While the neuropathological manifestation of AD has been well characterized in post-mortem brain tissue, less is known about either the underlying risk factors for the disease or the exact mechanisms involved in disease progression. Given the high heritability estimates (60–80%) for AD derived from quantitative genetic analyses (5), the majority of etiological studies have focussed predominantly on genetic contributions to the disease. Indeed autosomal dominant mutations in three genes (*APP*, *PSEN1*, and *PSEN2*), which are involved in Aβ production, can explain early onset (<65 years) familial AD, however these account for only 5–10% of the total disease burden. Most cases of AD are late-onset (>65 years), non-Mendelian and highly sporadic, with susceptibility attributed to the action of common genetic variants of low penetrance. In recent years a number of genome-wide association studies (GWAS), and a subsequent meta-analysis, have nominated around 20 common variants in a number of genes including *ABCA7*, *BIN1*, *CASS4*, *CD2AP* , *CELF1*, *CLU*, *CR1*, *FERMT2*, *FRMD4A*, *HLA-DRB5*, *INPP5D*, *MEF2C*, *MS4A4E/MS4A6A*, *NME8*, *PICALM*, *PTK2B*, *SLC24A4/RIN3*, *SORL1* and *ZCWPW1 (6, 7)*. However collectively these only explain around a third of disease incidence (8), although polygenic risk scores based on these variants have been developed to predict disease risk (9). The only common variants identified to date with a modest effect size exist within *APOE*, where the *APOE* Ɛ4 variant, which arises due to single nucleotide polymorphisms (SNPs) at rs7412 and rs429358, is the largest genetic risk factor for late onset AD, with carriers of the Ɛ4Ɛ4 genotype having an odds ratio of 14.9 for developing the disease (10). Recent genome sequencing projects have identified other variants, for example rs75932628 (R47H) within *TREM2* (11, 12), however these are relatively rare within the population.

A role for epigenetics in AD

Although many of the genomic studies to date have identified robust and reproducible findings, they do not account for all of AD incidence. Furthermore, a number of disease attributes suggest a potential epigenetic contribution to etiology, for example the differential vulnerability of specific brain regions to disease, age of onset of disease, environmental influences on disease such as diet and the increased risk of developing AD in individuals with obesity and type II diabetes (13). Epigenetic processes mediate the reversible regulation of gene expression, occurring independently of DNA sequence variation, acting principally through chemical modifications to DNA and nucleosomal histone proteins and orchestrate a diverse range of important neurobiological processes. DNA methylation is the best characterized and most stable epigenetic modification modulating the transcription of mammalian genomes and has been the focus of most human epidemiological epigenetic research to date. Standard genotyping techniques are not able to distinguish between unmodified cytosine and 5-methylcytosine (5-mC), which contains a methyl-group on the 5 position of the cytosine ring. Bisulfite conversion is by far the simplest method to assess the degree of DNA methylation present in a given sample as it converts unmethylated cytosine to uracil (and to thymine through subsequent PCR), whilst 5-mC is not converted (and thus remains as cytosine in PCR). As such, bisulfite-treatment of DNA allows the differentiation between cytosine and 5-mC through downstream sequencing, amplification or array-based techniques.

Epigenetic studies of mouse models of AD

There are many available murine models that have been traditionally used for studying AD. There are, however, limitations to their utility for modelling human AD, for example mice do not naturally get AD symptoms or produce amyloid plaques. Therefore, in mouse models of amyloid pathology, the amyloid is derived from human transgenes and so produces human amyloid. Currently no mouse model has all the features of AD seen in humans, but they have differing combinations of the disease characteristics, such as behavioral changes, neurodegeneration, neuropathology and

cognitive deficits at different ages (14). Although most AD cases are sporadic, transgenic animal models have relied on the utilization of genetic mutations associated with familial AD and are thus a model of the effects of the accumulation of amyloid-β (Aβ) plaques and/or neurofibrillary tangles, rather than a model of sporadic AD, where the causes are unknown. Mouse models have been made for mutations in *APP (15-17)* and knock-out and knock-in mouse models are also available for the *APP* secretases (*BACE* (18), *PSEN1*, *PSEN2* (19), *ADAM10* and *ADAM17* (20)). The 3xTg-AD mice, which contain human *APP*, *PSEN1*, and tau mutant transgenes (21), are useful as they exhibit both plaque and tangle pathology. Nonetheless, despite this issue of translation from murine models to human sporadic patients, there are many advantages to using them to study epigenetic changes beyond the advantages common of most mouse models, such as having experimental control and a short life span. For example one can assess longitudinal changes in the epigenome across specific regions of the brain in genetically identical mice. Furthermore, with specific murine models available, which have already been well characterized, one can accurately predict when pathology will start to develop and easily look for epigenetic alterations associated with behavioral, cognitive and physiological changes at different stages of pathology (14).

To date three epigenome-wide association studies (EWAS) of DNA methylation in AD transgenic mice have been published. The first by Sanchez-Mut *et al.* analyzed 12 brain regions from C57BL/6J on a genome-wide promoter DNA methylation array focussing on 762 genes associated with sensory perception, cognition, neuroplasticity, brain physiology and mental disease. They limited their investigation to two transgenic mouse models: *APP*/*PSEN1* (double-transgenic mice carrying the APPswe/PS1dE9 mutations) and 3xTg-AD, and their analysis of their non-transgenic littermates to the prefrontal cortex using only seven genes which had the largest degree of differential methylation between cerebral cortex and the rest of the brain. They observed that *Tbxa2r*, *F2rl2*, *Sorbs3* and *Spnb4* were hypermethylated in the frontal cortex of *APP*/*PSEN1* and 3xTg-AD mice and were replicated in independent samples by pyrosequencing. Further they also found *TBXA2R*, *SORBS3* and *SPTBN4* to be hypermethylated in AD Braak stage V-VI cases compared to controls (22).

Cong and colleagues performed MeDIP-chip analysis on cortex samples from *APP*/*PSEN1* transgenic mice. They identified 2346 hypermethylated CpG sites in 485 unique genes associated with AD compared to non-transgenic littermates. Subsequent pathway analyses showed differentially methylated genes were enriched in inflammatory response and disease, organismal injury and abnormalities, respiratory disease and cancer pathways (23). Another MeDIP-chip study by Agbemenyah *et al.* using hippocampal tissue from APPPS1-21 mice, which have Thy1-APP and Thy1-PS1 transgenes, found hypomethylation at the *Igfbp7* promoter was lower in transgenic mice when compared to wild-type. They also demonstrated that *Igfbp7* gene expression and IGFBP7 protein levels were also increased (24). The majority of DNA methylomic studies of AD, have however focussed on studying the human disease in post-mortem brain samples, however there are some specific issues when performing EWAS in AD brain samples, which require careful consideration.

The importance of study design for EWAS in human tissues

Although assessing epigenomic variation is relatively straightforward, there are a number of caveats when compared to genomic studies. First, and foremost, epigenomic variation is tissue specific, and as such it is important to specifically examine the tissue of interest (25). Given that AD is a progressive neurodegenerative disorder, with the spread of neurofibrillary tangles throughout the brain being well documented, from the transentorhinal region through the cortex, one would expect different regions of the brain to show disease-specific alterations at different stages of the disease process. This thus poses the question as to where would be the ideal brain region to profile; areas of the brain that are affected early in AD would have large amounts of neuronal loss, whilst other

regions may not exhibit disease pathology. As such, the profiling of multiple brain regions, representing the spectrum of pathology, is optimal as it would allow spatio-temporal mapping of disease-related changes. Further by profiling multiple brain regions, one could look for patterns of epigenetic changes prior to neuropathology, to attempt to assess causality.

Second is the issue of differences in cell abundance when assessing epigenetic variation in heterogeneous tissue such as the brain (26). This is particularly pertinent for diseases such as AD, which are characterized by neuronal cell loss and gliosis. Given that distinct cell types have potentially different epigenomes, it is important to acknowledge this in analyses. By comparing epigenetic changes at a population level in DNA extracted from whole tissue, which is a collection of cell types with potentially different methylomes, one will be assessing the percentage of cells which do, or do not, have a methylated cytosine at a specific position. This means that cell specific changes in heterogeneous cell populations could be diluted by unaffected cell types or could be a combination of small changes in many cell types. Some studies have used bioinformatic approaches to provide a proxy measure of neuron/glia proportions (27) and include this as a covariate in analyses, however the optimal study would use a method such as fluorescence-activated cell sorting (FACS) or laser capture microdissection (LCM) to yield pure populations of different cell types prior to epigenomic profiling. However, such methods are labor intensive, slow and expensive, and are thus not generally feasible for large cohort studies. Third, sample size is another important consideration for EWAS. Although it is generally appreciated that numbers required for EWAS are considerably smaller than for a standard GWAS, with ~75 samples per group giving sufficient power to detect modest changes (~5%) in DNA methylation. It is however imperative that EWAS are tissue-specific, and as such it can be challenging to access sufficient numbers of highly characterized brain samples from specific brain regions to ensure adequate power. Related to this issue are co-diagnoses of AD with other dementias. Many post-mortem diagnoses of AD are made in combination with other dementias such as Lewy body dementia (LBD) or Vascular dementia (VD). As such getting sufficient numbers of donor AD samples for analysis, in the absence of other dementias, can be difficult. Although it is of interest to identify molecular mechanisms associated with dementia, it is also important to identify disease-specific signatures when looking for new pharmacological targets. Finally, many dementia sufferers die with a systemic infection, in fact one study of post-mortem records showed that 80% of AD patients had an infection at the time of death (28). This could also be a confounder in the analysis of data as infections could elicit the activation of pro-inflammatory pathways within the brain. There are thus many issues to consider when planning, designing and performing an epigenomic study on any disease, but particularly in age-related neurodegenerative diseases such as AD.

Aside from identifying novel mechanistic pathways involved in the etiology of AD in the brain, epigenomic analyses could allow the development of novel translational clinical tools for AD. Although there is growing interest in the identification of novel epigenetic biomarkers for the disease in blood, there are other important considerations for these types of studies. A number of environmental exposures have been associated with DNA methylation changes in blood cells, for example, smoking (29), exposure to environmental chemicals (30) and diet (31), which may not have the same effect in disease-relevant tissues and blood may be more susceptible to changes due to the environment. Furthermore, the timing of sampling could be important, for example normal aging can alter the epigenome (32) and may have differing effects in different tissues. This can be somewhat adjusted for with the use of “epigenetic age” tools, which allow one to estimate biological age as a result of age sensitive DNA methylation marks, and that can then be used for analysis adjustment (32). Using peripheral tissues to find detectable disease-associated differences is a goal of most studies due to the fact that neurodegeneration starts decades before clinical diagnosis. To date however, no robust epigenetic biomarkers have been identified in blood even though accessing

larger samples numbers is easier than for brain and relatively non-invasive. The potential of using blood or peripheral tissues to develop epigenetic biomarkers is still feasible given the correlation of DNA methylation between blood and brain for some, but not all, genetic loci (33). Finally, longitudinal studies would be useful in being able to identify epigenetic biomarkers of disease progression and neuropathology.

DNA Methylomic studies of human AD

Until relatively recently, published literature examining a role for epigenetic modifications in AD development had been largely limited to either speculative reviews, or a limited amount of empirical research focussed on candidate genes or global changes. Whilst some global methylomic studies using antibodies to detect DNA methylation, have shown reductions in DNA methylation in the entorhinal cortex (34), temporal neocortex (35) and hippocampus (36) of AD sufferers post-mortem, other studies have reported conflicting results (37-39). A number of candidate gene studies have also been carried out in human tissue to try and identify AD associated methylation changes. There have been many candidate gene studies on blood on *5-LOX* (40), repetitive elements Alu, LINE-1, and SAT-α (41, 42), *FAAH* (43), *PIN1* (44), *SNAP25* (45), *SORL1* and *SIRT1* (46) in small numbers of samples but some of these were variable in their results. Other candidate gene studies have used brain tissue samples from AD cases and controls to assess DNA methylation. These have used assays for *HSPA8* and *HSPA9* (47), *ACE*, *APOE*, *APP*, *BACE1*, *GSK3B*, *MAPT*, and *PSEN1* (48-51), *CNP* and *DPYSL2* (52), *PP2AC* (53), *RAGE*, *ADORA2A* and *UCHL1* (51) with associations with AD being found in *APP*, *GSK3B*, *MAPT*, *PP2AC* , *APOE*, *DNMT1*, *MTHFR*, *5-LOX*, *FAAH* and *PIN1*. Meanwhile two MethylLight PCR studies assessed DNA methylation in AD, the first in 50 candidate genes, and the second accessing promoter methylation for a small selection of genes (*COX-2*, *BDNF*, *NF-κβ*, *CREB*, *DBNL*, *SYP*, *ALOX12* and genes associated with p450 epoxygenase), in a limited number of AD samples (54, 55).

However, in recent years, advances in epigenomic technology have allowed the quantification of DNA methylomic variation in a number of complex disease phenotypes, including AD (**Table 1**). The workhorses for epigenome-wide association studies (EWAS) have been the Illumina Infinium 27K array, the 450K beadarray, and their recent successor, the EPIC 850K array, which are cost-effective approaches to screen methylomic variation at ~27,000, ~450,000 and ~850,000 methylation sites in the human genome respectively. Other methods, such as methylated DNA immunoprecipitation sequencing (MeDIP-seq), whole genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS) are also available, however due to the prohibitive cost of sequencing the human genome at sufficient depth, have not been widely utilized in epigenetic epidemiological studies of AD. The first empirical EWAS, by Bakulski and colleagues, used the Illumina Infinium 27K Beadarray to quantify DNA methylation at ~27,000 CpG sites in the frontal cortex of 12 late-onset AD donors and 12 cognitively normal matched control subjects. They identified 948 nominally significant DNA methylation differences mapped to 918 unique genes with an average methylation difference between AD cases and controls of 2.9%. Interestingly their most significant loci, with a 7.3% reduction in AD, resided in the *TMEM59* gene, which is believed to be involved in APP post-translational glycolytic processing (56). Using the same technology Sanchez-Mut and colleagues examined hippocampal samples from five control donors to those with early-stage AD (Braak I-II), mid-stage AD (Braak III-IV) and late-stage AD (Braak V-VI) (57), demonstrating a >25% methylation difference between controls and Braak stage V–VI at single loci in *CLDN15* and *QSCN6* and two loci in *DUSP22*.

The advent of the Illumina Infinium 450K Beadarray has since allowed more in depth studies of DNA methylomic differences in AD. Two independent back-to-back publications both demonstrated highly robust and reproducible alterations in four genes not previously associated with AD, namely

ANK1, *RHBDF2*, *RPL13* and *CDH23* (58-60). De Jager *et al.* used a large cohort of 708 prefrontal cortex samples to examine DNA methylomic differences associated with neuritic plaque burden. They identified 71 differentially methylated probes, 11 of which were replicated by Lunnon *et al.* Differentially methylated loci associated with neuropathology included genes such as *RNF34*, *CDH23*, *SLC2A1*, *COQ7* and the *HOXA* gene cluster and each of the 71 CpGs explained on average 5% of the variance in neuritic amyloid plaque burden, with a range of 3.7% to 9.7% (58). In this study, they also attempted to look for altered gene expression with AD pathology in the replicated differentially methylated genes in independent temporal cortical samples. They found that *ANK1*, *CDH23*, *DIP2A*, *RHBDF2*, *RPL13*, *SERPINF1* and *SERPINF2* all showed differential gene expression with amyloid burden providing some evidence of further reaching consequences as a result of these DNA methylation differences.

Meanwhile Lunnon and colleagues used a cross-tissue approach to assess DNA methylomic changes in AD in a range of brain regions representing the spectrum of AD pathology in a discovery cohort of 117 individuals (59). They initially focused on the entorhinal cortex, as it shows neuropathology in the early stages of disease, to identify a number of differentially methylated loci associated with Braak stage, a standardized measure of neurofibrillary tangle deposition. They then examined other matched brain regions from the same donors, namely the prefrontal cortex and superior temporal gyrus to identify cross-cortex differences in the identified top loci from the entorhinal cortex. Probes in *ANK1*, *SLC15A4*, *MEST* and *TMX4* were only seen to replicate in the prefrontal cortex, superior temporal gyrus or both (59), with probes in *PCBD1, MLST8 and ZNF512* not being significant in other cortical tissues and probes in *SIRT6* and near *CLYBL* being significant in the cerebellum, a region of the brain largely protected from neurodegeneration. They utilized two independent replication cohorts to validate their findings, one again utilizing the Illumina Infinium 450K Beadarray to profile genome-wide methylomic differences in the prefrontal cortex and superior temporal gyrus in a cohort of 147 individuals, and the other utilizing pyrosequencing in the entorhinal cortex, prefrontal cortex and superior temporal gyrus of 62 individuals. The authors showed an extended region of hypermethylation in the *ANK1* gene in AD cortex that spanned at least six CpG sites.

Interestingly *ANK1* encodes a brain-expressed protein involved in compartmentalization of the neuronal plasma membrane but has not previously implicated in AD. *ANK1* is primarily expressed in red blood cells but is also expressed in brain and muscle and is thought to play a role in cell-surface protein binding to the underlying spectrin-actin cytoskeleton and is used in cell motility, activation, proliferation and contact. To date, *ANK1* is the most robust AD-associated DNA methylation difference observed in the brain (61) (**Figure 1**). Further studies from the same groups have since built on these now publically available EWAS datasets. Chibnik *et al.* have examined DNA methylomic variation in AD loci nominated from GWAS, demonstrating that DNA methylation at 17 CpG sites spanning six AD-risk genes (*BIN1, CLU, ABCA7, MS4A6A, CD2AP* and *APOE*) show an association with amyloid burden, independent of genotype, and collectively explain 16.8% of variability in neuritic plaques (62). Smith and colleagues examined DNA methylation at a locus within the *TREM2* gene, showing consistent hypermethylation in three different cohorts in the superior temporal gyrus, which appeared to be independent of the SNP previously implicated in the disease (63). Finally, Watson *et al.* used the Illumina Infinium 450K Beadarray to identify AD related DNA methylation changes in the superior temporal gyrus in 34 AD cases and 34 matched controls (64). They identified 479 differentially methylated regions (DMRs, clusters of significantly differentially methylated positions) encompassing 4,565 CpG sites, with the majority of differentially methylated positions being hypermethylated. They also showed overlap between their most significant DMRs and the Lunnon *et al.* and De Jager *et al.* studies, with eight of their top 25 DMRs containing genes having differentially methylated positions in the previously published studies (*LOC100507547*, *PRDM16*, *PRRT1*, *C10orf105*, *CDH23, PPT2*, *PPT2-EGFL8* and *RNF39*) (58, 59).

A role for other DNA modifications in AD?

Although DNA methylation has been the focus of published research to date, a number of additional DNA modifications are now starting to receive considerable attention. 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) were originally thought to represent intermediates in the demethylation of 5-mC to un-modified cytosine (65) (**Figure 2**). However, recent evidence suggests that they may represent independent epigenetic marks. There has been particular interest in 5-hmC in the context of brain disorders as it appears to be found at relatively high levels in the brain compared to other tissues (66, 67) and is particularly enriched in the vicinity of genes with synapse-related functions (68). Until recently, studies of 5-hmC in AD brain have been limited to global profiling methods, two of which have shown global decreases in the hippocampus (36), entorhinal cortex and cerebellum (69), although another study showed increased 5-hmC in the middle frontal gyrus and middle temporal gyrus (38), whilst another revealed no difference in the entorhinal cortex (37). Given that we know that DNA methylomic differences in AD are loci and tissue-specific, and that global studies of alterations in DNA methylation levels were inconclusive, this demonstrates the importance of assessing DNA hydroxymethylation levels at single base resolution. Interestingly, all of the published EWAS of DNA methylation in AD to date have utilized bisulfite converted DNA, however this treatment is unable to convert either 5-mC or 5-hmC to uracil (70). As such, all of the published EWAS of AD actually represent a summative measure of these two modifications. A recently published adaptation to bisulfite treatment has allowed the simultaneous measurement of 5-mC and 5-hmC in a sample. Oxidative bisulfite technology uses a selective chemical oxidation to accurately distinguish between 5-mC and 5-hmC by first converting 5-hmC into 5-fC and through bisulfite conversion this is converted to uracil. As such, this allows the quantification of 5-mC in the absence of confounding by 5-hmC. Further, by profiling bisulfite and oxidative bisulfite treated DNA in parallel, one can generate a quantitative measurement of 5-hmC by subtracting the oxidative bisulfite data from the bisulfite data. This method has been utilized together with the Illumina Infinium 450K Beadarray to assess 5-mC and 5-hmC across different regions of the human brain (71-73), however there are nuances to the method, for example technical artefacts can sometimes result in negative 5-hmC values. Two current studies in AD that are expected to be published soon are using this approach to quantify true 5-mC and 5-hmC measures in AD brain. Smith *et al.* have profiled both modifications in the entorhinal cortex and cerebellum of 96 individuals ranging from Braak 0 to Braak VI, whilst Roubroeks *et al.* have assessed the middle temporal gyrus in a similar number of individuals.

Looking to the future

Although the published EWAS in AD have shown a number of robust and reproducible DNA modification changes in disease, it is to be expected that technological advances will allow more in depth assessments. The recent release of the of Illumina Infinium Methylation EPIC 850K Beadarray now allows the simultaneous assessment of ~850,000 methylation sites in the human genome. In addition, the falling cost of next generation sequencing means that methods such as RRBS and WGBS are becoming more affordable for cohort studies. Similarly the advent of third generation sequencing technologies such as the PacBio RS II from Pacific Biosciences allows for whole genome sequencing and targeted sequencing. These methods have the potential to detect different DNA modifications during standard sequencing as well as allowing for single-base and DNA-strand resolution. Targeted and whole genome sequencing approaches could also be utilized to assess epigenetic variation in mitochondrial DNA (mtDNA). Mitochondrial dysfunction has been proposed to be a potential mechanism in the development of AD, which has been reported in various studies (74, 75). Interestingly, mitochondria possess their own circular genome of 16.6Kb, which is separate from the nuclear genome, and contains 37 genes (76). However, with no coverage of the

mitochondrial genome on the Illumina Infinium Beadarrays, a potential role for mtDNA modifications has not been examined in the AD EWAS published to date (77, 78).

By far the major criticism of epigenetic studies in various diseases relates to the issue of causality. Unlike genetic variation, it is not known whether disease-associated epigenetic changes represent a cause or a consequence of disease. Methods such as Mendelian randomisation (MR) with existing "omics" datasets could provide some evidence for the direction of effect of the epigenetic changes observed but, more recently, there have been suggestions of using genetic editing techniques to determine causality. New technologies such as Clustered regularly interspaced short palindromic repeats (Crispr)/Cas9 allow researchers to impose genetic modifications to DNA and observe the results in cell lines and model organisms (79). This technology could be lent to loci-specific epigenetic editing via the use of targeting proteins to methylate of demethylate target sites, and then testing whether this accelerates or reverses disease pathology. Previously, CRISPR technology has been used to target nuclease activity to introduce single or double strand breaks in DNA, target transcriptional transactivation and regulate gene expression. Genome editing with CRISPR targets enzyme activity to specific target DNA sequences depending on the specificity of guide RNAs in the CRISPR complex. These methods could be used to alter DNA methylation levels in model organisms or cell lines to replicate the differences seen in human EWAS studies to attempt to establish causality and the effects of disease associated changes. Ultimately, even if such studies prove that a nominated locus is not causal in disease, it does not make EWAS any less worthwhile, as even identifying consequences of disease will teach us more about the disease process.

Conclusion

The role of epigenetic mechanisms in AD is still a research field in its infancy, and particularly how epigenetic DNA modifications could contribute to the cause and progression of disease is still yet to be explored. Currently epigenetics has not been well studied in regards to AD and there are only a handful of studies which provide any empirical data. Due to the relative ease and affordability of EWAS however, the amount of data being generated is increasing for both 5-mC and 5-hmC. Replication is integral to finding robust epigenetic changes and so far, a few replicable differences have been observed in relevant brain tissues, such as in *ANK1* and genes in the *HOXA* gene cluster. More work is needed and combining multiple EWAS datasets into meta-analyses to provide strong evidence for the contribution of DNA methylation to disease progression is warranted. Although EWAS using bisulfite treated DNA are a combination of measures of both 5-mC and 5-hmC, using oxidative bisulfite methods give a truer measure of 5-mC as well as allowing the quantification of 5-hmC. As of now, there are no AD epigenetic peripheral tissue biomarkers for AD, which is a major goal for dementia research. As epigenetic mechanisms are malleable and changeable over the course of development, life, exposure to environmental influences and normal aging, it provides an attractive target for a proxy of disease progression and a target for drugs. By combining epigenetic measurements in peripheral tissues, such as blood, with neuroimaging and clinical assessments, we can associate what is happening in the brain to blood. As with many diseases that are believed to have both genetic and environmental components it is important to integrate different data modalities to generate a full picture of AD risk. Integrating genomic, epigenomic and transcriptomic data will allow the identification of methylation and expression quantitative trait loci (mQTLs/eQTLs), showing how genetic variation may influence methylation and expression in a tissue and disease-specific manner.

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Figure 1 – *ANK1* shows hypermethylation associated with Braak stage across multiple studies. DNA methylation (beta) difference associated with Braak stages at two probes in the *ANK1* gene (cg11823178 and cg05066959) was observed in the prefrontal cortex (PFC), entorhinal cortex (EC) and superior temporal gyrus (STG), but not in the cerebellum in four cohorts. London 1 (N=117) and Oxford (N=62) cohort data were taken from Lunnon *et al* (59), MAP/ROS cohort data (N=708) was taken from De Jager *et al*(58), Mount Sinai cohort data was taken from Lunnon *et al* (59) *(*N=147*)*. London 2 cohort data (N=94) is currently unpublished data from our group. Consistent AD associated hypermethylation was observed across all cortical tissues but not in the cerebellum.

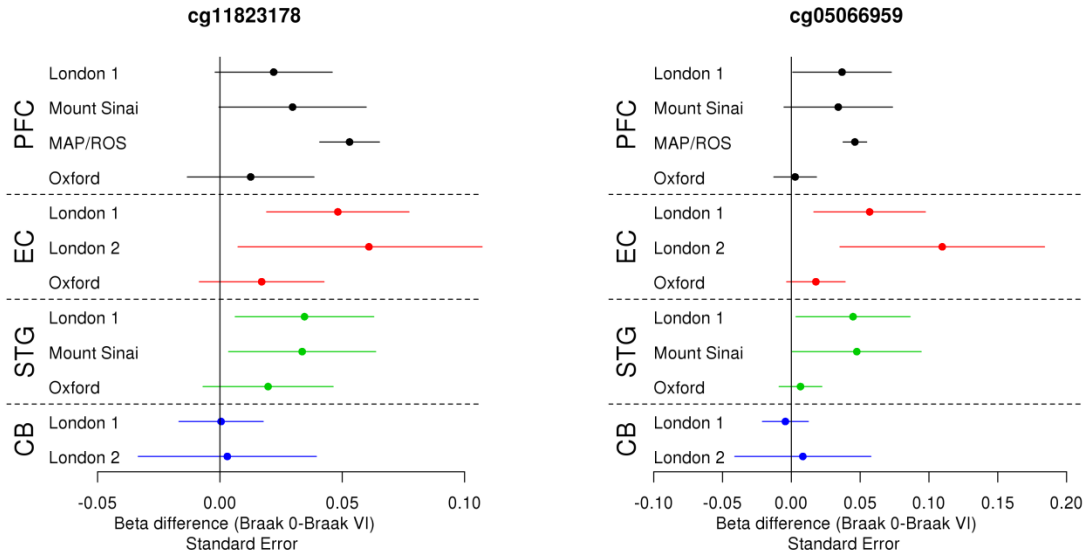

Figure 2 - Epigenetic DNA modifications. DNA methyltransferases (DNMTs) can add and maintain methyl groups on cytosine to create 5-methylcytosine (5-mC). Through the action of the Ten-Eleven Translocation (TET) family of DNA hydroxylases, the process of active DNA demethylation occurs. This occurs via 5-hydroxymethylcytosine (5-hmC), into 5-formylcytosine (5-fC) and 5-carboxycytosine (5-caC).

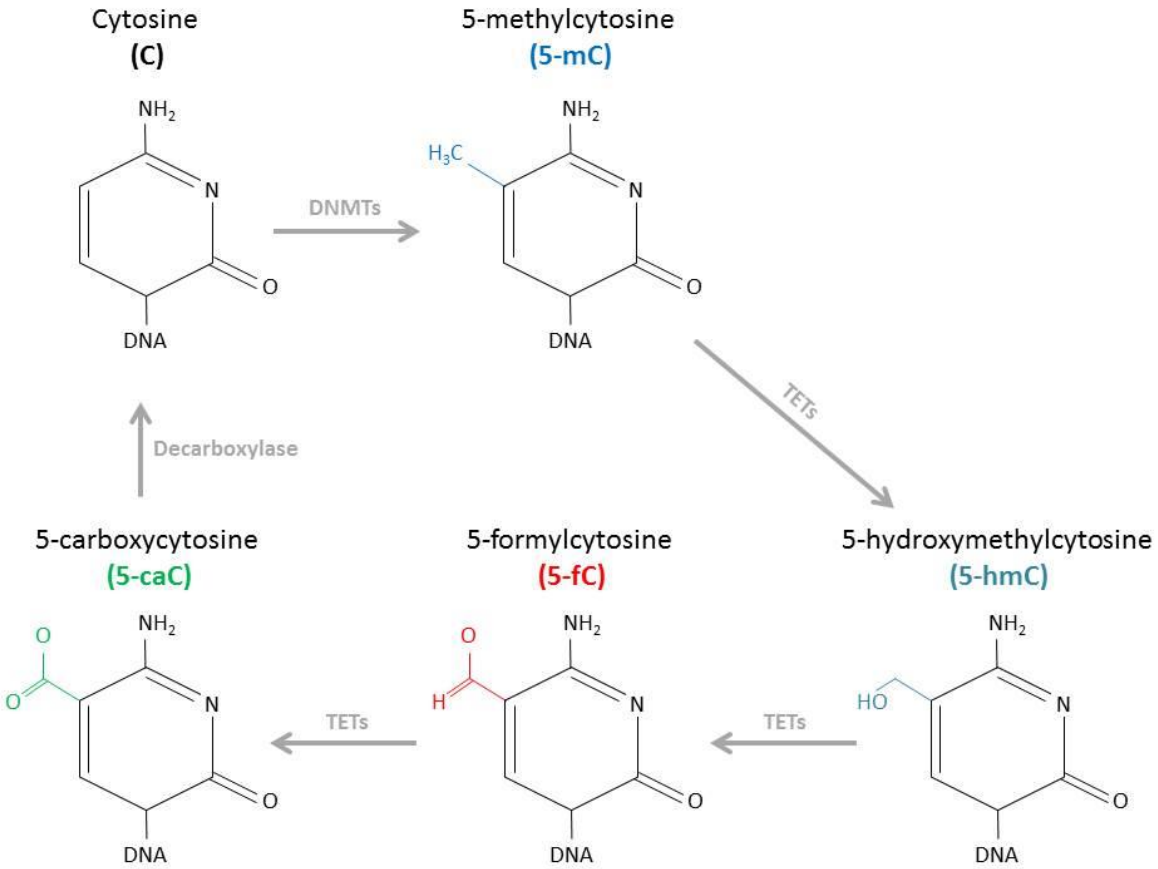

Table 1 – Summary of current EWAS on DNA methylation in AD. A number of studies have examined a role for DNA methylation in Alzheimer's disease, utilising the Illumina Infinium 27K or 450K Beadarrays.

Study	Year	Number of Samples	Tissue(s)	Brain bank	Method	Analysis	Genes identified
Bakulski *et al* (56)	2012	24	Prefrontal cortex	Michigan Alzheimer's Disease Center	27K	Disease Status	948 CpG sites representing 918 genes
Lunnon *et al* (59)	2014	117	Prefrontal cortex Superior temporal gyrus Entorhinal cortex Cerebellum Pre-mortem blood	MRC London Neurodegenerative Disease Brain Bank	450K	Braak	*ANK1, MIR486, PCBD1, SLC15A4, SIRT6, MEST, MLST8, ZNF512, TMX4*
De Jager *et al* (58)	2014	708	Prefrontal cortex	Religious Order Study (ROS) or the Memory and Aging Project (MAP)	450K	Amyloid	*ANK1, FOXK1, RHBDF2, CDH23, SPG7, RHBDF2, KDM2B, WDR81, HMHA1, C10orf54, ITPRIPL2, PCNT, HOXA3*
Sanchez-Mut *et al* (57)	2014	20	Hippocampus	Institute of Neuropathology Brain Bank	27K	Disease Status	*DUSP22, CLDN15 and QSCN6*
Watson *et al* (64)	2016	68	Superior temporal gyrus	Mount Sinai Alzheimer's Disease and Schizophrenia Brain Bank	450K	Disease Status	DMRs identified in 475 genes, enriched for neuron function, development and cellular metabolism.
Smith *et al* (80)	Submitted	147	Prefrontal cortex Superior temporal gyrus	Mount Sinai Alzheimer's Disease and Schizophrenia Brain Bank	450K	Braak	*PROM1, ATG16L2, CDK14, BTBD11, ETS1, HOXA3, ZNF385A*