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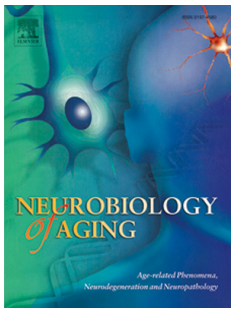

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PII: S0197-4580(16)30138-5

DOI: 10.1016/j.neurobiolaging.2016.07.008

Reference: NBA 9657

To appear in: *Neurobiology of Aging*

Received Date: 6 May 2016

Revised Date: 16 June 2016

Accepted Date: 7 July 2016

Please cite this article as: Smith, A., Smith, R.G., Condliffe, D., Hannon, E., Schalkwyk, L., Mill, J., Lunnon, K., Increased DNA methylation near TREM2 is consistently seen in the superior temporal gyrus in Alzheimer's disease brain, *Neurobiology of Aging* (2016), doi: 10.1016/j.neurobiolaging.2016.07.008.

Increased DNA methylation near TREM2 is consistently seen in the superior temporal gyrus in Alzheimer's disease brain

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Abbreviations: AD (Alzheimer's disease); DMP (Differentially methylated position); EWAS (Epigenome-wide association study); GWAS (Genome-wide association study); PFC (Pre-frontal cortex); SNP (single nucleotide polymorphism); STG (Superior temporal gyrus); TREM2 (Triggering receptor expressed on myeloid cells 2); TSS (Transcription start site)

ABSTRACT

Although mutations within the TREM2 gene have been robustly associated with Alzheimer's disease, it is not known whether alterations in the regulation of this gene are also involved in pathogenesis. Here we present data demonstrating increased DNA methylation in the superior temporal gyrus in AD brain at a CpG site located 289bp upstream of the transcription start site of the TREM2 gene in three independent study cohorts using two different technologies (Illumina Infinium 450K methylation beadchip and pyrosequencing). A meta-analysis across all three cohorts reveals consistent Alzheimer's disease associated hypermethylation (p=3.47E-08). This study highlights that extending genetic studies of TREM2 in Alzheimer's disease to investigate epigenetic changes may nominate additional mechanisms by which disruption to this gene increases risk.

Keywords: TREM2; Alzheimer's Disease; Braak stage; DNA methylation; Epigenetics; AD; brain

1. INTRODUCTION

Alzheimer's disease (AD) is a chronic neurodegenerative disease characterized by the accumulation of amyloid plaques and neurofibrillary tangles within the brain, ultimately leading to neuronal cell loss. This is accompanied by changes in behavior and personality followed by progressive cognitive decline. Although the neuropathology that characterizes the disease has been well-described, little is known about the underlying mechanisms that drive disease onset and progression. As quantitative genetic analyses demonstrated high heritability estimates (58%-79%) for AD (Gatz, et al., 2006), initial approaches to understanding etiology focused on uncovering a genetic contribution to the disorder. In recent years large cohort collections and the relatively inexpensive cost of assessing genetic variation through genome-wide association studies (GWAS) has allowed the identification of numerous common variants that are associated with increased risk of developing AD. Although common sequence variants in a number of genes have been now robustly associated with AD via GWAS and subsequent meta-analyses (Escott-Price, et al., 2015,Harold, et al., 2009,Hollingworth, et al., 2011,Lambert, et al., 2013,Naj, et al., 2011,Sleegers, et al., 2010), collectively common single nucleotide polymorphisms (SNPs) account for only 33% of attributable risk (Ridge, et al., 2013) and the mechanism behind their action remains largely unknown.

With the advent of whole genome and exome sequencing, recent efforts have focussed on identifying rare variants for AD with a larger effect size. The most robust locus identified through these studies is the rs75932628 SNP within the TREM2 gene, which leads to a R47H substitution, and has been nominated as a risk loci in numerous case/control studies of AD (Forabosco, et al., 2013,Guerreiro, et al., 2012,Thorlakur Jonsson, et al., 2013,Neumann and Daly, 2013). The minor T allele at this locus significantly increases risk of AD (OR 2.92)

(Thorlakur Jonsson, et al., 2013). Although the genetic epidemiology of TREM2 in AD has now been extensively explored, only one study to date has explored whether alterations in the regulation of this gene, independent of genotype, may be associated with AD (Celarain, et al., 2016). Epigenetic processes occur independently of DNA sequence variation, dynamically regulating gene expression, and are mediated principally through chemical modifications to DNA and nucleosomal histone proteins. DNA methylation is the best characterized modification modulating the transcription of mammalian genomes and the first genome-scale studies to assess DNA methylation (epigenome-wide association studies (EWAS)) in AD brain have recently been published (De Jager, et al., 2014,Lunnon, et al., 2014). One of these studies highlighted hypermethylation at cg25748868, annotated to the TREM2 gene, in the superior temporal gyrus (STG) associated with increased braak stage (Lunnon, et al., 2014). The aim of the present study was to measure DNA methylation in the STG at this locus in a further two independent AD sample cohorts and to perform a meta-analysis on the three cohorts.

2. MATERIALS AND METHODS

2.1 Sample Information

STG brain tissue was obtained from three independent sample cohorts from three different brain banks. Cohort 1 consisted of 95 samples acquired from the MRC London Neurodegenerative Disease Brain Bank (http://www.kcl.ac.uk/iop/depts/cn/research/MRC-London-Neurodegenerative-Diseases-Brain-Bank/MRC-London-Neurodegenerative-Diseases-Brain-Bank.aspx). Cohort 2 consisted of 103 samples acquired from the Mount Sinai Alzheimer's Disease and Schizophrenia Brain Bank (http://icahn.mssm.edu/research/labs/neuropathology-and-brain-banking) (Haroutunian, et al., 1998). Cohort 3 consisted of 192 samples acquired from the Thomas Willis Oxford Brain

Collection (http://www.medsci.ox.ac.uk/optima/information-for-patients-and-the-public/the-thomas-willis-oxford-brain-collection) (Esiri, 1993). For all cohorts samples were classified as either controls (Braak 0-II) or AD cases (Braak V-VI). All samples were dissected by trained specialists, snap-frozen and stored at −80 °C. Further information about the samples is provided in Table 1.

2.2 DNA isolation and sodium bisulfite treatment

Genomic DNA was isolated from ~100 mg of each dissected brain region using a standard phenol-chloroform extraction method, and tested for degradation and purity before analysis. 500ng DNA from each sample was sodium bisulfite-treated using the Zymo EZ-96 DNA methylation kit (Zymo Research) according to the manufacturer's standard protocol.

2.3 Genome-wide DNA methylation analysis

Samples from cohorts 1 and 2 were assessed using the Illumina Infinium 450K methylation beadchip (Illumina) using an Illumina HiScan System (Illumina). All samples were assigned a unique code for the purpose of the experiment and randomized with respect to sex and disease status to avoid batch effects, and processed in batches of four BeadChips. Illumina Genome Studio software was used to extract the raw signal intensities of each probe (without background correction or normalization). All computations and statistical analyses were performed using R 3.2.1 (R Development Core Team, 2015) and Bioconductor 3.1 (Gentleman, et al., 2004). Data was loaded into R using the methylumi package (Davis, et al., 2014) as a methylumi object. Initial quality control checks were performed using functions in the methylumi package to assess concordance between reported and genotyped gender. Data was pre-processed in the R package wateRmelon using the dasen function as previously described (Pidsley, et al., 2013). EWAS data for cohorts 1 and 2 is available on GEO under accession numbers GSE59685 and GSE80970 respectively.

2.3 Bisulfite-pyrosequencing analysis

Bisulfite pyrosequencing was used to quantify DNA methylation at cg25748868 (chr6:41,131,213) (GRCh37 Hg18) upstream of the TREM2 transcription start site (TSS) in cohort 3. A single amplicon (134 bp) was amplified using primers designed using the PyroMark Assay Design software 2.0 (Qiagen) and tested for specificity in our laboratories (Forward Primer = GAGGGTTTTGGTTTTTAAAGGTATAG; Reverse Primer = TACAAAACCTAACCCAAAAATCAC, Sequencing Primer = ATTTTTGTAAGGTTGAAATTAGA). DNA methylation was quantified using the Pyromark Q24 system (Qiagen) following the manufacturer's standard instructions and the Pyro Q24 CpG 2.0.6 software.

2.4 Genotyping

Genotyping for rs75932628 within exon 2 of TREM2 was carried out by LGC Genomics, Herts, UK.

2.5 Statistical analyses

All three cohorts were analysed independently. Data was adjusted for the effects of age and gender and linear regression models used to compare control brain samples (Braak scores 0-II) to AD brain samples (Braak scores V-VI) using the linear model function in R. A Fisher's meta-analysis of *P* values was performed in the MetaDE package within R (Wang, et al., 2012). We later estimated neuron/glia proportions in data generated on the Illumina Infinium 450K methylation beadchip using the CETS algorithm (Guintivano, et al., 2013), and re-analyzed the data in cohorts 1 and 2 using this estimate as an additional covariate.

3. RESULTS

We recently published an EWAS of AD, demonstrating increased DNA methylation in the STG associated with Braak stage in cohort 1 at cg25748868 (nominal p = 8.81E-05) (Lunnon, et al., 2014), which was observed to a lesser extent in the entorhinal cortex (p=0.042), but not in the prefrontal cortex (p =0.611), nor cerebellum (p=0.067). Cg25748868 is located 289bp upstream of the TSS of the TREM2 gene. Given the interest in this gene in AD because of the recently described novel risk variant we were keen to examine whether we could replicated this differentially methylated position (DMP) in independent AD brain samples. As our previous study in cohort 1 used linear regression models to assess DNA methylation associated with Braak stage in 113 individuals with an even representation of samples across the entire Braak spectrum, and as validation cohort 3 only had samples available with a Braak stage ≤II or ≥V, we initially re-analyzed our data in cohort 1 using a case (Braak≥V)/control (Braak≤II) analysis (N=95 individuals in these groups) to allow a comparable analysis across all three cohorts. In our case-control analysis in cohort 1 we observed a similar association of increased DNA methylation at cg25748868 in the STG (**Figure 1A**; DNA methylation difference = 1.4%, p=1.06E-04) as reported in our published quantitative Braak stage model data. One potential confounder of epigenetic data generated in a heterogeneous tissue such as the brain is that methylomic differences may simply reflect a difference in cellular abundance between cases and controls. As such we utilized a published bioinformatic algorithm that can predict neuron/glia proportions in data generated from the Illumina Infinium 450K methylation beadchip (Guintivano, et al., 2013). When we included these estimates as co-variates in the model, we still observed a significant increase in DNA methylation at this loci in cases (p=6.31E-04). We next assessed this specific probe in a second independent cohort of 103 STG samples (cohort 2), again identifying increased DNA methylation associated with AD at this locus (**Figure 1A**; DNA methylation difference =

0.73%, p = 0.020), which still remained significant when we controlled for neuron/glia proportions (p=0.026).

To independently validate our findings using another technology we quantified DNA methylation at the same site using bisulfite pyrosequencing in a third cohort of 192 STG samples (cohort 3), again finding significantly higher DNA methylation associated with AD (**Figure 1A**; DNA methylation difference = 0.72%, p = 4.01E-06). A meta-analysis of this DMP across the three cohorts showed significant hypermethylation associated with AD (**Figure 1B**; p=3.47E-08). As the rare SNP at rs75932628 has been shown to increase AD risk (Guerreiro, et al., 2013,T. Jonsson, et al., 2013), we were interested to investigate whether this SNP is also associated with DNA methylation at cg25748868. When we compared DNA methylation levels at cg25758868 between carriers and non-carriers of the SNP across all cohorts, we saw no significant difference between the groups (p=0.488). However, as the presence of the T allele is rare within the population (MAF= 0.002 (Genomes Project, et al., 2012)), we only observed heterozygosity in 11 individuals across the study (two individuals in cohort 1, six individuals in cohort 2 and three individuals in cohort 3).

4. DISCUSSION

Mutations in TREM2 have been robustly associated with AD risk. In addition to the well-described mutation at rs75932628, other rare mutations have also been described; for example D87N (rs142232675) also increases susceptibility to AD (Guerreiro, et al., 2013). More recently common variants across the TREM gene cluster have been associated with AD

pathology or susceptibility, including rs6910730 in TREM1, rs9381040 and rs6916710 in TREML2 and rs7759295 and rs6922617 which are intergenic within the TREM gene cluster (Benitez, et al., 2014,G. Chan, et al., 2015,Cruchaga, et al., 2013,Lambert, et al., 2013,Replogle, et al., 2015). In the present study we observed a small but consistent increase in DNA methylation at a CpG site 289bp upstream of the TSS in the TREM2 gene in the STG in AD brain across all cohorts. The TREM2 gene resides on chromosome 6 and is 4680bp in length (chr6:41,126,244-41,130,924) and contains 5 exons and is known to be expressed as 3 transcript variants. The Illumina Infinium 450K methylation beadchip has only limited coverage of the TREM2 gene, with only seven probes spanning 4280bp across the gene. When we looked for AD-associated differential methylation at the remaining six probes within the gene, we saw no significant difference in either cohort 1 or 2. TREM2 is expressed by macrophages and microglia, and its expression, particularly within the cytoplasm, is dramatically increased in activated microglia (Sessa, et al., 2004). TREM2 is predominantly expressed intracellularly, in the Golgi complex and in cytoplasmic vesicles, but TREM2 must be expressed at the cell surface in order to be functional. In response to cell stimulation, TREM2 is exocytosed to the cell surface, where it is able to act as a functional receptor (Prada, et al., 2006). Cell surface expressed TREM2 associated with its signalling counterpart DAP12 (also called TYROBP) forming a molecular complex that promotes phagocytosis. *In vitro*, TREM2 knockdown microglia have defective clearance of apoptotic neurons and increased synthesis of TNF-α and iNOS, whilst overexpression of microglial TREM2 increases phagocytic activity and reduces the production of the pro-inflammatory mediators TNF-α, IL-1β and iNOS (Takahashi, et al., 2005), indicating that TREM2 may promote a phagocytic, anti-inflammatory phenotype in microglia. Interestingly one recent study highlighted an association of the inflammatory CD33 AD risk allele with increased TREM2 cell surface expression, as well as higher cortical TREM2 RNA expression with

increasing amyloid pathology, supporting a potentially pathogenic role for increased TREM2 expression in AD (Gail Chan, et al., 2015). Another recent study has demonstrated increased expression of TREM2 in the hippocampus in AD (Celarain, et al., 2016). Interestingly this study used bisulfite clonal sequencing and 5-hydroxymethylated DNA immunoprecipitation combined with RT-qPCR (5hMeDIP-RT-qPCR) to additionally assess DNA methylation and DNA hydroxymethylation in TREM2 in the same samples. In line with our findings they reported increased DNA methylation in the TREM2 TSS in AD. Further they reported a significant correlation of TREM2 mRNA expression and 5-hydroxymethylation (5-hmC) levels in exon 2 of TREM2. Although traditionally DNA methylation in CpG islands (CGIs) is associated with gene silencing, recent data suggests that the relationship between DNA methylation and transcription may be more complex, with gene body methylation often being associated with active gene expression and alternative splicing (Smith, et al., 2016). Interestingly the CpG site we investigated resides within a transcription binding site for the transcriptional repressor protein YY1.

5. CONCLUSIONS

This study further explored a DMP in TREM2 identified in our recent EWAS in AD brain, and demonstrated consistent hypermethylation in AD across three independent cohorts. This opens up the possibility that TREM2 gene regulation may also be important in the etiology of AD and further studies to examine other epigenetic mechanisms along the entire length of the TREM2 gene should be undertaken. As noted earlier the Illumina Infinium 450K methylation beadchip only contains seven probes spanning 4280bp of the TREM2 gene and thus it would be of interest to bisulfite sequence the entire gene to nominate additional differentially methylated loci that could not be identified in the current study. It will also be of considerable interest to correlate DNA methylation changes along the entire gene with levels of gene expression, given that the recent study by Celarain and colleagues identified increased

TREM2 gene expression and DNA methylation in AD hippocampus (Celarain, et al., 2016). Although we investigated whether the DMP we identified may be influenced by genotype at rs759232628, which has been strongly associated with increased AD risk, the rarity of this SNP in the population meant we were not sufficiently powered to make any firm conclusions and future work should be undertaken to examine DNA methylation in individuals harboring the risk allele in larger sample cohorts. Finally although we have shown consistent hypermethylation at this DMP in AD brain, our experiments were performed in tissue homogenates, and as such it is possible that this may represent cell proportion differences in people with AD, given the widespread neuronal loss at late stage disease. Although we were able to control for neuron/glia proportions in cohorts 1 and 2, in the future further studies could be undertaken to measure DNA methylation in pure microglia isolated from AD brain, once these methods are available.

ACKNOWLEGMENTS

This work was funded by a grant from BRACE (Bristol Research into Alzheimer's and Care of the Elderly) and the Alzheimer's Society (grant number AS-PG-14-038) to KL, and NIH grant R01 AG036039 and an Equipment Grant from Alzheimer's Research UK to JM. We thank Carolyn Sloan for technical support and Istvan Bodi and Andrew King for neuropathological diagnosis of cases. We also thank the Oxford Project to Investigate Memory and Ageing (OPTIMA), the National Institute for Health (NIHR) Biomedical Research Unit in Dementia in the South London and Maudsley NHS Foundation Trust (SLaM), Brains for Dementia Research (Alzheimer Brain Bank UK) and the donors and families who made this research possible. The Oxford Brain Bank is supported in part by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre based at

Oxford University Hospitals NHS Trust and University of Oxford. Brain banking and neuropathology assessments for the Mount Sinai cohort was supported by NIH grants AG02219, AG05138 and MH064673 and the Department of Veterans Affairs VISN3 MIRECC.

TABLES

TABLE 1: Sample demographics for the study.

	Cohort 1 (London)		Cohort 2 (Mount Sinai)		Cohort 3 (Oxford)	
	Controls (Braak 0-II)	**AD cases (Braak V-VI)**	**Controls (Braak 0-II)**	**AD cases (Braak V-VI)**	**Controls (Braak 0-II)**	**AD cases (Braak V-VI)**
Number of Individuals	29	66	59	44	75	117
Gender (M/F)	13/16	26/40	31/28	12/32	44/31	50/67
Age at Death (± SD)	77.6 (12.80)	85.4 (8.13)	82.1 (7.56)	88.0 (7.53)	84.1 (7.72)	78.4 (9.27)
Method used to assess DNA methylation	Illumina 450K		Illumina 450K		Pyrosequencing	

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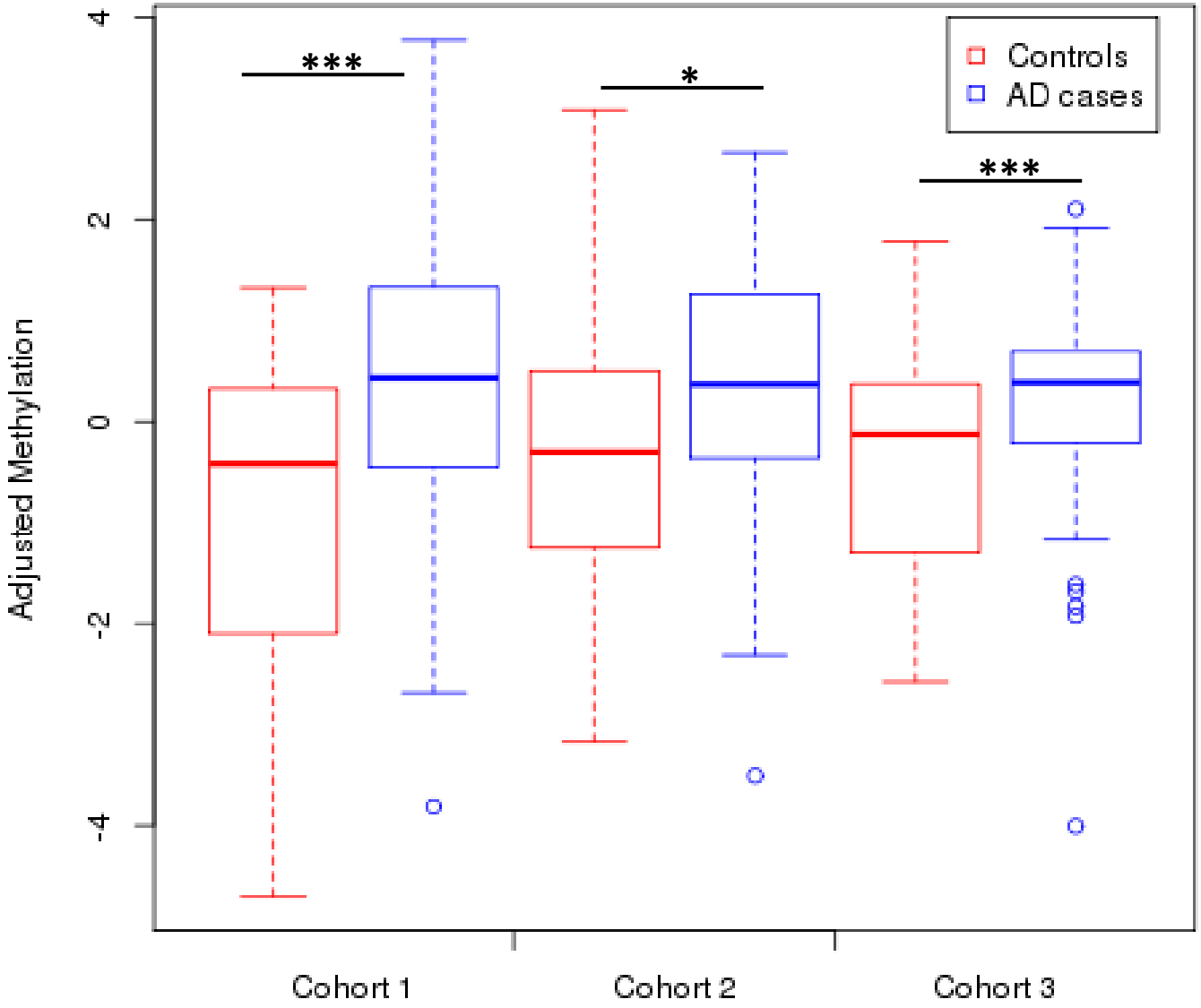
Figure 1: DNA methylation is consistently increased in AD samples compared to controls in the STG at chr6:41,131,213. (**A**) Adjusted methylation levels of cg25748868 in the STG in non-demented controls (Braak stage 0-II) and AD (Braak stage V-VI) samples from three cohorts. In all three cohorts DNA methylation at this loci is increased in AD cases relative to controls. (**B**) Forest plot of meta-analysis of effect size of cg25748868 across the three cohorts. Key: * = P<0.05, ** = P<0.01, *** = P<0.005

A


Adjusted Methylation
4
2
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Controls
AD cases
Cohort 1
Cohort 2
Cohort 3

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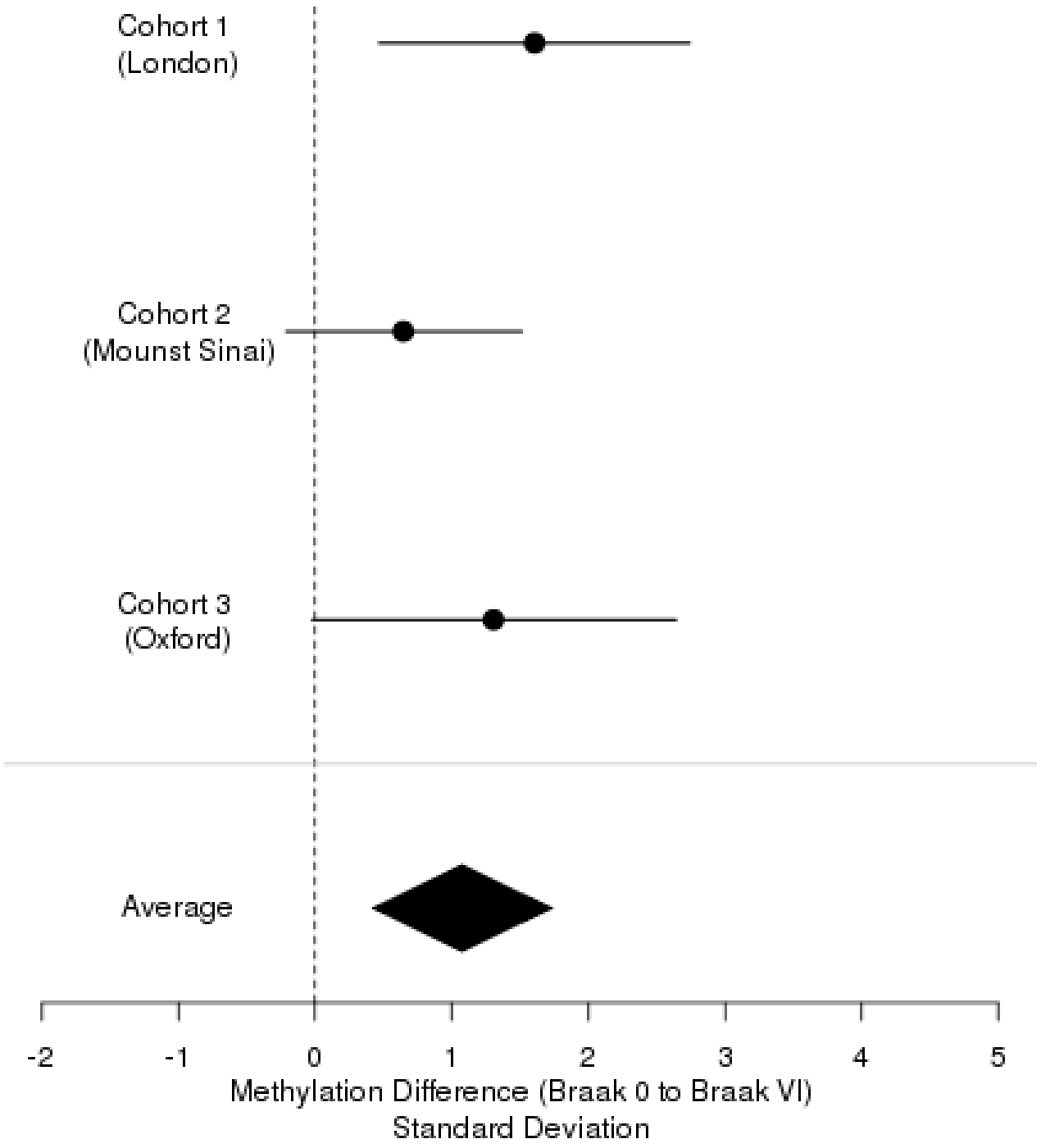
Cohort 1
(London)
Cohort 2
(Mounst Sinai)
Cohort 3
(Oxford)
Average
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-1
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3
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Methylation Difference (Braak 0 to Braak VI)
Standard Deviation

HIGHLIGHTS

- We analysed DNA methylation levels at Chr6:41,131,213, 289 bp upstream of the TREM2 gene, in the superior temporal gyrus in three independent cohorts of individuals with Alzheimer's disease and non-demented controls.
- We used two different technologies (Illumina Infinium 450K Methylation Array and Pyrosequencing) to assess DNA methylation at this locus.
- We demonstrated increased DNA methylation at this locus in all three cohorts.
- A meta-analysis across all three cohorts showed consistent hypermethylation at this locus (p=3.47E-08) associated with Alzheimer's disease
- This study highlights that extending genetic studies of TREM2 in Alzheimer's disease to investigate epigenetic mechanisms may nominate additional mechanisms by which this gene infers disease risk.