Original Article

Blood-based sex-differential DNA methylation in Alzheimer's disease

Tianmi Yang, Chunyu Li, Qianqian Wei, Yangfan Cheng, Yi Xiao, Huifang Shang[*] iD

Department of Neurology, Laboratory of Neurodegenerative Disorders, Rare Diseases Center, National Clinical Research Center for Geriatrics, West China Hospital, Sichuan University, Chengdu 610041, Sichuan, China.

[*]**Correspondence to:** Prof. Huifang Shang, Department of Neurology, Laboratory of Neurodegenerative Disorders, Rare Diseases Center, National Clinical Research Center for Geriatrics, West China Hospital, Sichuan University, No.37, Guoxue Lane, Chengdu 610041, Sichuan, China, E-mail: hfshang2002@126.com

Abstract

Aim: We aim to explore the blood-based sex-differential DNA methylation profiles in patients with Alzheimer's disease (AD).

Methods: The same analytical pipeline and meta-analysis procedure were applied to 254 patients with AD and 261 matched healthy controls (HC) from four blood-based datasets to identify sex-differentially methylated positions (sex-DMPs) and their biological functions. We further reviewed brain-based sex-DMPs previously reported in AD and intersected with blood-based sex-DMPs.

Results: We identified 134 sex-DMPs in AD, 88 were unique to AD, and 46 were shared with HC. Eleven novel sex-DMPs and 28 sex-DMPs consistent across blood and brain were recognized for the first time. Differentially methylated genes, such as *COL25A1*, *MSUT2*, *ELAVL4*, *SLC17A7*, *EPHA4*, and *PPP2CB*, were closely related to the pathogenesis of AD. In addition, immune-related processes were enriched in the enrichment analysis.

Conclusion: Sex-differential DNA methylation in blood provides potential biomarkers for AD pathogenesis, and also points to potential drug targets for precision medicine.

Keywords: Alzheimer's disease, dementia, DNA methylation, epigenetics, blood, sex characteristics, gender

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease and the leading cause of dementia in older adults. AD is characterized by a gradual decline in memory, behavioral, and social skills, significantly impacting daily life and functioning. With the global population aging, the socioeconomic burden of AD is escalating sharply[1,2]. Notably, pronounced sex differences are evident in AD risk, prevalence, and clinical presentation[3,4]. Women face a higher risk of developing AD, and more women than men are affected by dementia, with an estimated 27.0 million women compared to 16.8 million men living with dementia in 2016 (age-standardized)[2]. Clinical manifestations also vary substantially between males and females, particularly regarding neuropsychiatric symptoms[3,4]. However, the molecular mechanisms underlying these sex differences in AD remain largely unknown.

DNA methylation is an important form of epigenetic modification, which plays a key role in determining genomic structure and function, regulating gene expression and cell differentiation without altering the DNA sequence[5]. Environmental factors throughout life can drive DNA methylation changes, some of which exhibit sex-specific patterns[6-9]. Sex differences in DNA methylation have gained attention across various life stages, from newborns to the elderly, as well as in diseases such as cancers, psychiatric disorders, and neurodegenerative diseases[6-13]. Studies regarding sex-related differential methylation in AD have primarily focused on brain tissue[12,13], while peripheral blood has received relatively little attention, despite the tissue-specific nature of DNA methylation[14-16]. One study approached this gap by using sex-stratified and methylation-by-sex interaction analyses to detect DNA methylation differences in peripheral blood from AD patients, though it did not directly compare male and female AD patients[17].

Therefore, further elucidating blood-based, sex-differential DNA methylation will enhance our understanding of sex differences in AD. Additionally, integrated analyses of sex-related DNA methylation patterns across both brain and blood tissues in AD patients may reveal valuable epigenetic biomarkers, offering insights into the disease's progression and potential therapeutic targets.

METHODS

Study cohorts

To systematically explore blood-based sex-differential DNA methylation profiles in AD, we obtained data from 254 patients with AD and 261 healthy controls (HC) from three independent datasets: the Australian Imaging, Biomarker & Lifestyle (AIBL) cohort (GSE153712, the Nabais dataset containing 161 AD patients)[18], the AddNeuroMed cohort (GSE144858, the Lunnon dataset containing 93 AD patients and 96 HC)[19], and the third cohort was previously described by Li *et al.* (GSE53740, containing 165 HC)[20]. Patients with AD were diagnosed according to the NINCDS-ADRDA criteria[21]. Age and sex for AD and HC were matched individually.

Data pre-processing

DNA methylation in the peripheral blood of all participants was measured using the Infinium Human Methylation 450K Bead-Chip, except for the AIBL cohort[18], where the Infinium Human Methylation EPIC (850K) Bead-Chip was applied. We used the R package ChAMP[22] (version 2.21.1) to process the β-value matrix for each dataset separately. Probes with a β value of 0 were replaced with 1.00×10^{-6}, and probes with missing β values were imputed using the k-nearest neighbor algorithm by impute.knn function in impute package[23]. Quality control (QC) was performed using the champ.filter function[24]. Probes meeting the following criteria were removed: (1) probes with detection $P < 0.01$ in one or more samples; (2) probes with < 3 beads in at least 5% of samples per probe; (3) non-CpG probes as identified by Chen *et al.*[25]; (4) probes located close to single nucleotide polymorphism (SNP) (an SNP with minor allele frequency ≥ 0.01 was

present in the last five base pairs of the probe); (5) multi-hit probes as identified by Zhou *et al.*[24]; (6) probes located on X and Y chromosomes. The number of probes filtered at each QC step is listed in Supplementary Table 1. We then applied the β-mixture quantile normalization (BMIQ) method[26] to normalize the β values of type 1 and type 2 design probes within the Illumina arrays. A quantile-quantile (QQ) plot was generated and the genomic inflation factor (λ) was estimated for each dataset using the R qqman package[27].

Sex-differentially methylated positions and regions
The remaining probe data were compared between males and females to identify sex-differentially methylated positions (sex-DMPs) using the limma package[28], as implemented in the champ.DMP function. A multiple-testing adjustment was performed using the Benjamini-Hochberg false discovery rate (FDR) method[29]. We selected probes with FDR under a threshold of 0.1 (adjusted *P* value < 0.10) for further meta-analysis, given the different platforms (450K and 850K microarrays) of the two AD datasets. Differentially methylated regions (DMRs) were extended segments of the genome that showed quantitative alterations in DNA methylation levels between the two groups. Sex-DMRs were detected by applying the Bumphunter algorithm[30] implemented in the champ.DMR function, which clustered the groups containing more than seven continuous probes as DMR (FDR < 0.05).

Meta-analysis
Sample size weighted meta-analysis for common DMPs across individual AD cohorts was conducted in METAL, converting the direction of effect and adjusting the *P* value into a signed Z-score[31]. The same meta-analysis procedure was performed for HC. To further characterize the results, we then intersected the meta-analysis outcomes of AD and HC to identify the unique sex-DMPs in AD and the shared sex-DMPs in both AD and HC.

Gene set enrichment analysis
Before enrichment analysis was performed, significant DMPs in the meta-analysis were annotated according to their locations with respect to genes (TSS1500, TSS200, 5'UTR, 1stExon, body, 3'UTR, and intergenic) or CpG islands (island, shore, shelf, and open sea), using the IlluminaHumanMethylation450kanno.ilmn12.hg19 package. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was conducted for these differentially methylated genes using the KOBAS-i (http://kobas.cbi.pku.edu.cn/, and a mirror site is available at http://bioinfo.org/kobas)[32], an intelligent version with novel machine learning-based method for gene set enrichment analysis (GSEA). Following the recommendations in the GSEA User Guide[33], terms with an FDR of less than 0.25 are considered statistically significant to avoid overlooking potentially interesting results. The Jaccard similarity index and the Infomap algorithm implemented in KOBAS-i were used to visualize the output terms[32].

Comparison to previous brain tissue-based sex-DMPs
Recent studies have suggested sex-specific differences in DNA methylation in the brain tissues of patients with AD. Pellegrini *et al.* identified 77 sex-dependent DMPs consistent across four brain regions (frontal cortex, temporal cortex, entorhinal cortex, and cerebellum)[13]. Additionally, Zhang *et al.* found that 381 CpGs in females and 76 CpGs in males were significantly associated with the AD Braak stage by analyzing more than 1,000 postmortem prefrontal cortex brain samples[12]. To assess the consistency of sex-DMPs in the brain and peripheral blood, we intersected blood-based DMPs identified in our meta-analysis and brain-based DMPs reported by Pellegrini *et al.* and Zhang *et al.*[12,13].

RESULTS
Study cohort characteristics
The characteristics of participants from the four datasets are presented in Supplementary Table 2. Our
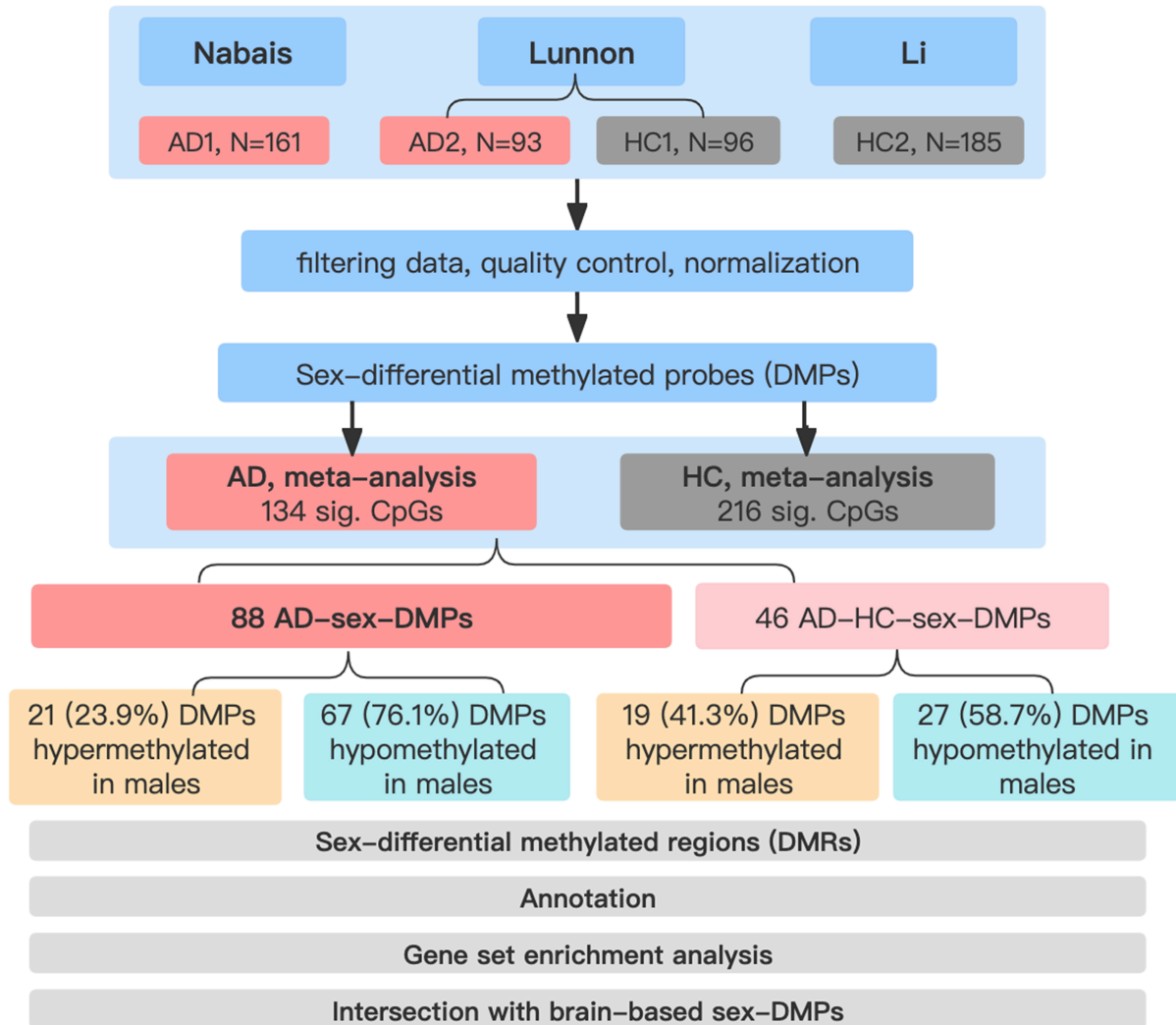
Figure 1. Overview of the study design. AD: Alzheimer's disease; HC: healthy controls.

meta-analysis included 254 AD patients [153 females, 101 males; mean age 74.7 years with a standard deviation (SD) of 6.9 years] and 261 matched HC (161 females, 100 males; mean age 70.1 years with an SD of 8.1 years). The overview of study design is shown in Figure 1.

Sex-DMPs and regions

After data pre-processing, QC and normalization of DNA methylation, the number of autosomal probes used for analyses in each dataset ranged from 262K to 384K, except for the AIBL cohort (EPIC array), for which 747K probes passed QC [Supplementary Table 1]. No evident inflation of the test statistics was observed in the AD datasets [Figure 2]. Significant sex-DMPs at FDR < 0.10 identified in the individual dataset are shown in Supplementary Tables 3-6 and Figure 3A. A total of 216 sex-DMPs were common to both HC datasets, and 134 sex-DMPs were common to both AD datasets. A Venn diagram of the DMPs obtained from the four datasets is shown in Figure 3A.

Six sex-DMRs were significant in both AD datasets, but not in any HC dataset [Supplementary Table 7, Supplementary Figures 1-6]. The average number of CpGs per DMR was 7.7 ± 0.5. Six genes (*PM20D1*,
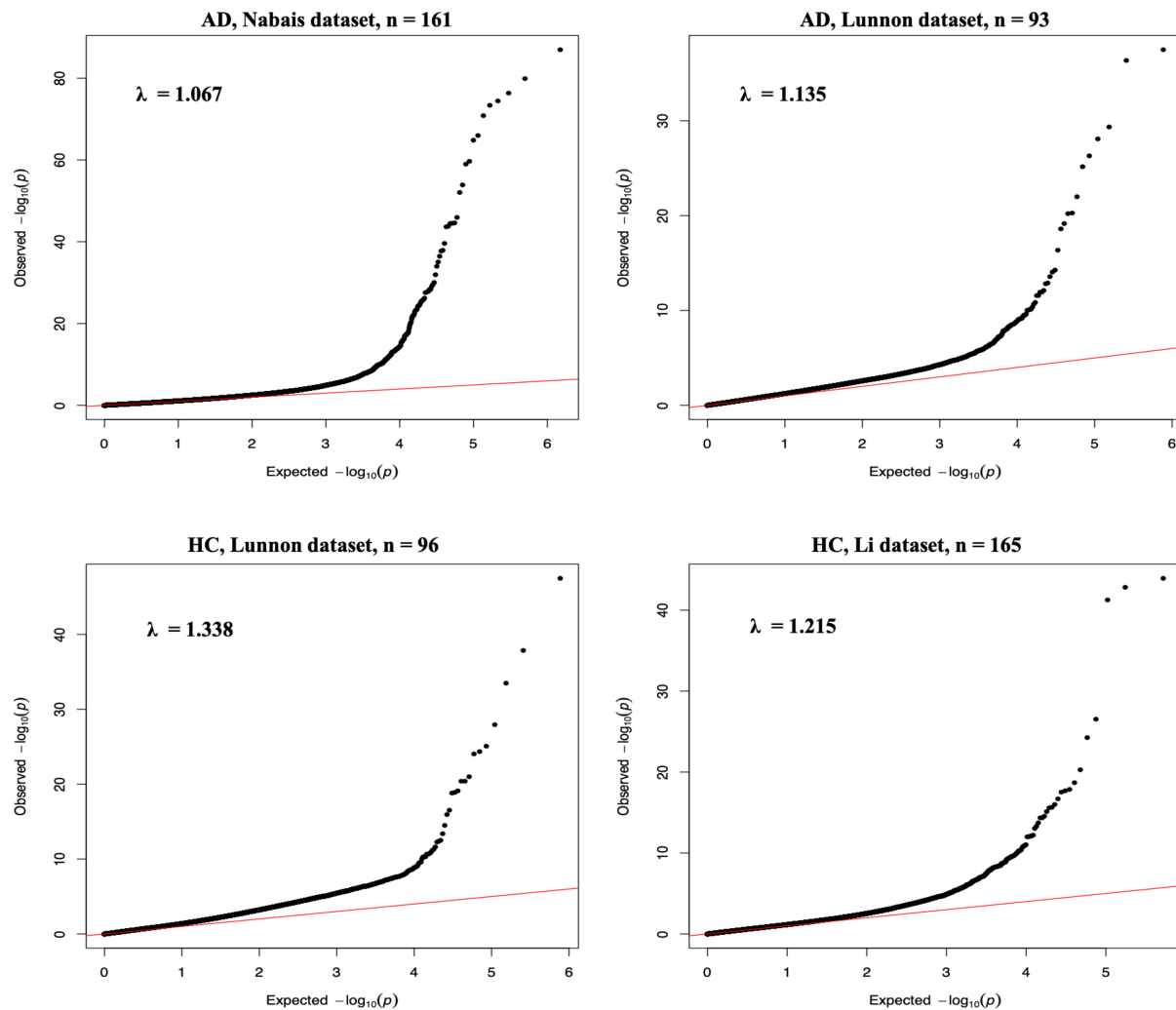
Figure 2. QQ plots of observed and expected distributions of *P*-values for each dataset. λ is the genomic inflation factor. QQ: Quantile-quantile.

LOC441666, *AURKC*, *PCDHGA4*, *H2AFJ*, *CLDN9*) were mapped to the above six sex-DMRs. Among these, *PCDHGA4* and *H2AFJ* were hypermethylated in males compared to females, and the remaining four genes were hypomethylated in males compared to females. We found no CpGs overlapped between 134 sex-DMPs and six sex-DMRs in AD, suggesting complementary evidence from DMPs and DMRs for sex-associated differences in DNA methylation in AD.

Meta-analysis

To prioritize unique sex-DMPs in AD, we conducted meta-analysis for AD and HC, respectively. For the convenience of classification, we defined the DMP with higher methylation levels in males than in females as "hypermethylated" and "hypomethylated" otherwise. Our meta-analysis identified 216 sex-DMPs [44 (20.4%) hypermethylated and 172 (79.6%) hypomethylated] in HC [Supplementary Table 8] and 134 sex-DMPs [40 (29.9%) hypermethylated and 94 (70.1%) hypomethylated] in AD [Supplementary Table 9]. Compared with HC, 88 sex-DMPs were unique in AD (defined as AD-sex-DMPs), whereas 46 sex-DMPs were shared in both AD and HC (defined as AD-HC-sex-DMPs) [Supplementary Table 10].
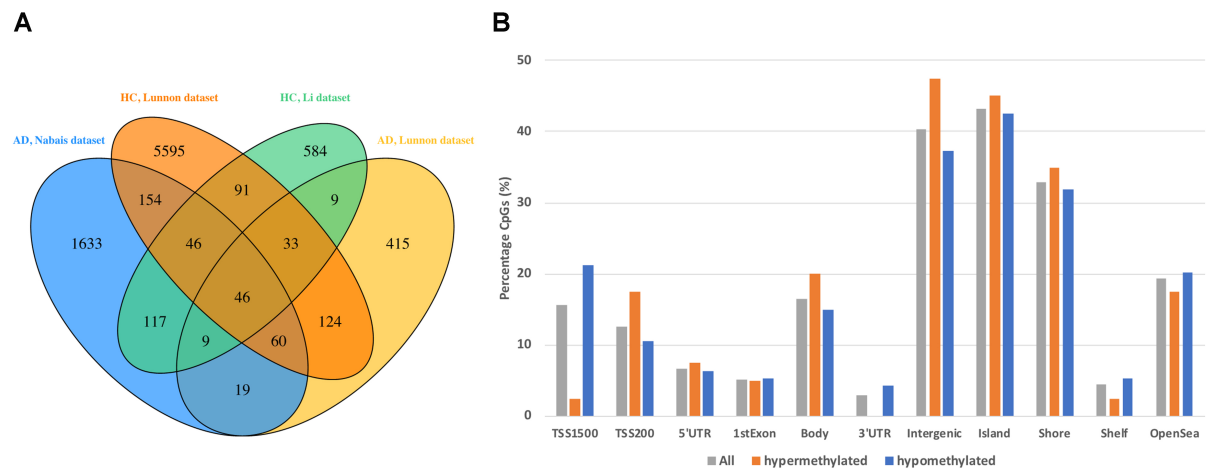
Figure 3. Discovery and annotation of sex-DMPs in AD. (A) Venn diagram of sex-DMPs obtained by four datasets; (B) Annotation in gene features (TSS1500, TSS200, 5'UTR, 1stExon, body, 3'UTR, intergenic) and CpG features (island, shore, shelf, open sea) of 134 sex-DMPs identified in AD meta-analysis. AD: Alzheimer's disease; sex-DMPs: sex-differentially methylated positions; HC: healthy controls.

With annotation, the AD-sex-DMPs were mapped to 52 genes that contained 12 (23.1%) hypermethylated and 40 (76.9%) hypomethylated genes [Supplementary Table 9]. The AD-HC-sex-DMPs were mapped to 28 genes: 9 (32.1%) were hypermethylated, and 19 (67.9%) were hypomethylated, which also showed a similar pattern of lower rates of hypermethylated genes in males than in females. In terms of gene features, the hypermethylated CpGs in males were enriched in intergenic regions, gene body, and TSS200 but under-represented in 3'UTR, and the hypomethylated CpGs were enriched in intergenic regions, TSS1500, and gene body [Figure 3B and Supplementary Table 9]. Regarding CpG features, both hypermethylated and hypomethylated CpGs were overrepresented in CpG island and shore [Figure 3B and Supplementary Table 9]. Similar distribution characteristics were observed in the AD-sex-DMPs and AD-HC-sex-DMPs [Supplementary Figures 7 and 8].

Specifically, 11 novel CpGs (cg18355337, hypomethylated, $P = 5.08 \times 10^{-5}$; cg24592500, hypomethylated, $P = 1.25 \times 10^{-4}$; cg22364668, hypermethylated, $P = 1.60 \times 10^{-4}$; cg19653417, hypomethylated, $P = 4.63 \times 10^{-4}$; cg17394530, hypomethylated, $P = 5.84 \times 10^{-4}$; cg05697849, hypermethylated, $P = 0.002$; cg19893929, hypermethylated, $P = 0.004$; cg12873476, hypomethylated, $P = 0.006$; cg12964741, hypermethylated, $P = 0.006$; cg07592361, hypermethylated, $P = 0.008$; cg13772414, hypermethylated, $P = 0.015$) with sex specificity were identified for the first time [Table 1], based on trait records in the epigenome-wide association studies (EWAS) Atlas (https://ngdc.cncb.ac.cn/ewas/atlas), an updated public EWAS knowledgebase covering more than 617 018 high-quality EWAS associations[34]. The other 91.8% of CpGs (77/88 in AD-sex-DMPs and 46/46 in AD-HC-sex-DMPs) [Supplementary Table 10] were consistently replicated in direction with previous sex-related EWAS results of health population[6-9,35]. Impressively, among genes mapped to these 11 novel CpGs, *ELAVL4*, *SLC17A7*, and *EPHA4* were all hypermethylated in males and were closely related to AD based on search records in the PubMed [Alzheimer's disease (All Fields) AND "Gene Name" (All Fields)]. The details are developed in the discussion section.

Enrichment and pathway analysis implicates immune-related processes
To investigate the potential biological pathways enriched with significant sex-differentially methylated genes unique to AD, KEGG pathway enrichment analysis was conducted. For 52 genes mapped from 88 AD-sex-DMPs, we identified 53 significant terms (at FDR < 0.25[33]), which were grouped into six clusters based on the correlation among gene sets [Figure 4 and Supplementary Table 11]. The immune-related cluster

Table 1. Eleven novel AD-sex-DMPs

CpG	Chr	Position	Gene	Gene features	CpG features	Direction	P value
cg18355337	19	55549722	GP6	TSS200	Opensea	--	5.08E-05
cg24592500	1	2863801		IGR	Opensea	--	0.0001248
cg22364668	19	49944826	SLC17A7	TSS200	Shore	++	0.0001598
cg19653417	12	132654924		IGR	Shore	--	0.0004633
cg17394530	6	106434623		IGR	Shore	--	0.0005838
cg05697849	1	50513766	ELAVL4	1stExon	Island	++	0.00232
cg19893929	2	16105823		IGR	Opensea	++	0.004802
cg12873476	8	142402728		IGR	Shore	--	0.005545
cg12964741	18	77160221	NFATC1	Body	Island	++	0.006405
cg07592361	2	241496830	ANKMY1	5'UTR	Island	++	0.00872
cg13772414	2	222383060	EPHA4	Body	Opensea	++	0.01497

For each CpG, annotations include the location of the CpG based on hg19/GRCh37 genomic annotation (chr, position), Illumina gene annotations, gene features, and CpG features. The sample size weighted meta-analysis results include the direction of effect, where CpGs that are hypermethylated in males have positive values, and their associated *P*-value. AD: Alzheimer's disease; sex-DMPs: sex-differentially methylated positions.
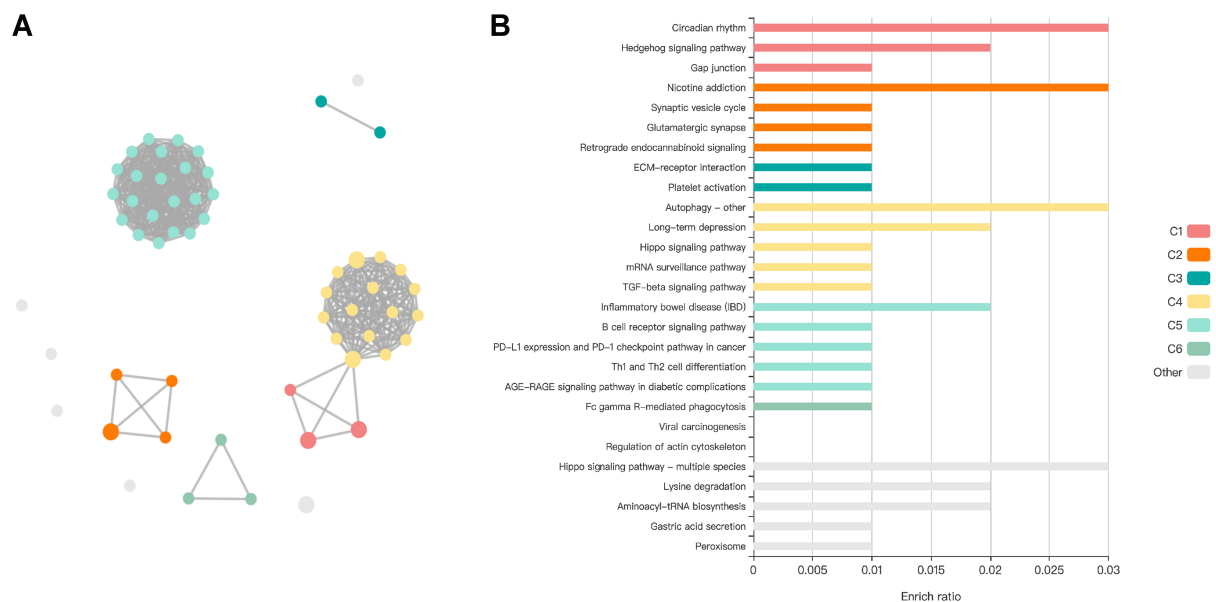

Figure 4. cirFunMap visualization of KEGG pathway enrichment analysis for 52 genes mapped from 88 AD-sex-DMPs. (A) The circular network view (the node color represents different clusters and the node size represents six levels of *P*-value) showed the immune-related cluster (cluster 5) was enriched and independent from the other clusters; (B) The barplot of the *P*-value for terms in different clusters. AD: Alzheimer's disease; sex-DMPs: sex-differentially methylated positions; KEGG: Encyclopedia of Genes and Genomes.

(cluster 5) was independent of the other clusters [Figure 4] and contained multiple aspects of immunity, such as inflammatory bowel disease (*P* = 0.062, FDR = 0.187), B cell receptor signaling pathway (*P* = 0.077, FDR = 0.187), Th1 and Th2 cell differentiation (*P* = 0.086, FDR = 0.187), T cell receptor signaling pathway (*P* = 0.096, FDR = 0.187), Th17 cell differentiation (*P* = 0.099, FDR = 0.187), and natural killer cell-mediated cytotoxicity (*P* = 0.120, FDR = 0.187) [Supplementary Table 11]. These immune pathways were mainly contributed by the *NFATC1* gene (cg12964741, hypermethylated in males), whose proteins belong to the transcription factor family and play a central role in inducible gene transcription during the immune response[36]. Consistent enrichment patterns of immune-related processes were replicated in genes with

hypermethylated levels in male patients [Supplementary Table 12 and Supplementary Figure 9] but were absent in hypomethylated genes [Supplementary Table 13 and Supplementary Figure 10]. In addition, the *PPP2CB* gene (cg26631144, hypomethylated in males) accounted for terms such as hippo signaling pathway (P = 0.010, FDR = 0.187) and autophagy (P = 0.031, FDR = 0.187) in the cluster 4 [Figure 4 and Supplementary Table 11]. The above evidence implies that genes with sex-dependent methylation patterns might be involved in different biological functions in AD, especially immune-related processes.

Comparison to previous brain tissue-based sex-DMPs
We intersected the 134 blood-based sex-DMPs of AD in our meta-analysis and the 77 brain-based sex-dependent DMPs of AD reported by Pellegrini *et al.*[13], identifying 28 common probes with the same sex-independent direction of changes (hyper or hypomethylated) across the blood and brain [Table 2]. Among these, 14 were classified as the AD-sex-DMPs, and the other 14 were classified as the AD-HC-sex-DMPs [Supplementary Table 10]. A locus at cg22266749, which mapped to the TSS200 of the *COL25A1* gene, was significantly hypermethylated in male AD compared to females, in both the blood and brain. *COL25A1* (collagen XXV alpha 1) gene encodes a secreted collagenous protein, a type II transmembrane protein specifically expressed in neurons[37]. The proteolytic fragment of this encoded protein, called the collagenous Alzheimer amyloid plaque component, was found to colocalize with amyloid beta (Aβ) in AD patients[37,38] and promote amyloid plaque formation in transgenic mice[39]. Another important AD-specific sex-DMP, cg22794378, hypomethylated in both the blood and brain of male patients compared with females, is located in the promoter of gene *ZC3H14* (also known as *MSUT2*), which is considered a promising drug target for prevention of tau pathology in AD and other tauopathies[40,41]. *MSUT2* was involved in determining susceptibility to pathological tau, and loss of *MSUT2* decreased tau aggregation and protected against neurodegeneration in mouse brain[40,41]. Recently, the sex differences in plasma p-tau181 levels have been examined in patients with AD[42]. Together, these results revealed that crucial genes such as *COL25A1* and *MSUT2* involved in the formation and regulation of Aβ pathology or tau pathology of AD, exhibited sex-dependent methylation patterns consistent across the brain and blood. However, further systematic investigation is recommended to perceive the regulation of gene expression and pathogenesis in AD by DNA methylation and whether the methylation levels in the brain can be surrogate from blood.

There was no overlap between our 134 sex-DMPs and 457 sex-stratified CpGs of the brain reported by Zhang *et al.*[12] The former was DMPs between male and female patients, while the latter offered AD Braak stage-associated differentially methylated CpGs in male and female patients, respectively. Not surprisingly, no overlapped DMPs were identified due to the focus on different aspects of sex on AD and the use of different analysis methods.

DISCUSSION

Blood-based epigenetic biomarkers for AD have gained significant attention in recent years[43-45]. Although sex differences have been of interest in AD-related studies, they have often been overlooked as key factors influencing patient responses to treatments in clinical trials, typically treated as covariates or subgroups[46]. In this study, we utilized DNA methylation datasets of AD and HC to identify sex-differential DNA methylation profiles in AD, using a three-step approach. Overall, 88 AD-sex-DMPs and 46 AD-HC-sex-DMPs were identified. Of these, 91.8% had been previously reported in healthy populations[6-9,35], while 11 were identified for the first time in AD. Notably, 28 sex-DMPs showed consistent methylation patterns across both brain and blood. Our findings highlight specific genes, genomic regions, and immune-related processes with sex-differential DNA methylation, which may serve as potential modifiers of AD progression.

Table 2. Twenty-eight sex-DMPs exhibiting consistent methylation changes in both blood and brain

CpG	Chr	Position	Gene	Gene features	CpG features	Direction	P value
cg23719534	15	101099284		IGR	Island	--	1.13E-110
cg22345911	17	80231263	CSNK1D	5'UTR	Island	--	2.80E-33
cg13346869	8	37605517	LOC728024	Body	Opensea	--	1.59E-28
cg22266749	4	110223959	COL25A1	TSS200	Island	++	7.06E-25
cg23001456	17	2615074	KIAA0664	TSS200	Island	--	7.30E-20
cg22794378	14	89029563	ZC3H14	TSS200	Island	--	4.28E-18
cg15817705	1	209406063		IGR	Shore	++	1.57E-15
cg27645294	17	21795257		IGR	Opensea	--	1.14E-14
cg10546176	5	34929404	DNAJC21	TSS1500	Island	--	1.52E-11
cg05020125	8	37605552	LOC728024	TSS200	Opensea	--	9.52E-10
cg26516287	7	12629275	SCIN	Body	Opensea	--	3.88E-08
cg17561891	7	86849173	C7orf23	TSS200	Island	--	2.41E-07
cg22105158	19	3480672	C19orf77	TSS200	Shore	++	1.51E-05
cg19311244	4	77341912		IGR	Shore	--	0.0002931
cg04946709	16	59789030	LOC644649	Body	Island	++	1.19E-85
cg05100634	18	45457604	SMAD2	TSS200	Island	--	1.16E-72
cg06710937	13	23489940		IGR	Island	--	9.23E-53
cg09971754	16	89557657	ANKRD11	TSS1500	Island	++	1.23E-32
cg09725915	2	70369583		IGR	Island	--	5.02E-28
cg14030268	10	119135296	PDZD8	TSS1500	Island	--	1.58E-22
cg03894796	8	144361315		IGR	Island	++	5.37E-12
cg15148078	19	3480561	C19orf77	TSS200	Shore	++	6.01E-07
cg02530860	8	144371537		IGR	Island	++	7.30E-07
cg11841231	2	205543309	PARD3B	Body	Opensea	++	1.01E-06
cg06666376	19	3480596	C19orf77	TSS200	Shore	++	1.25E-05
cg11174255	4	1513259		IGR	Shore	++	0.0002916
cg11565911	12	72233249	TBC1D15	TSS1500	Shore	--	0.007048
cg07645761	16	2892518	TMPRSS8	Body	Shore	++	0.01495

For each CpG, annotations include the location of the CpG based on hg19/GRCh37 genomic annotation (chr, position), Illumina gene annotations, gene features, and CpG features. The sample size weighted meta-analysis results include the direction of effect, where CpGs that are hypermethylated in males have positive values, and their associated *P*-value. AD: Alzheimer's disease; sex-DMPs: sex-differentially methylated positions.

To minimize the influence of age on DNA methylation[7], we included age-matched controls to identify sex-differential methylation patterns in AD. We observed higher methylation levels in females than in males, both in AD (70.1%) and in HC (79.6%), consistent with prior blood-based studies in the healthy population[7,8]. Similarly, higher methylation levels in females (73%) were noted in brain tissues of AD patients[13]. These findings underscore the substantial sex differences in DNA methylation observed in both blood and brain, highlighting the need for further investigations and emphasizing the importance of considering sex differences in future research designs and analyses.

In addition to corroborating previous findings of sex-related differences in DNA methylation, our meta-analysis identified several novel sex-differential methylated genes in AD. For example, *ELAVL4* (cg05697849 at gene promoter, hypermethylated in males), which encodes a neuronal-specific RNA binding protein involved in neural development and glutamate regulation, was linked to AD pathogenesis[47,48]. *ELAVL4* has also been implicated in accelerating tau pathology and glutamatergic neuron maturation through aberrant splicing[49]. Furthermore, the expression of the *ELAVL4* gene was different between males and females in the

brain tissue of AD patients[47]. Another notable gene, *SLC17A7/VGLUT1* (cg22364668 at gene promoter, hypermethylated in males), regulates synaptic glutamate transport and long-term potentiation[50,51], and interacts with the Aβ precursor[52]. *EPHA4* (cg13772414 at gene body, hypermethylated in males) modulates Aβ production[53,54], with its downregulation leading to increased Aβ and *BACE1* expression[54]. *PPP2CB* (cg26631144 at gene promoter, hypomethylated in males), encoding the protein phosphatase 2A catalytic subunit, has been identified as a key protein in the protective effect of apolipoprotein E ε2 against AD[55]. These findings are promising; however, further investigation is needed to elucidate the functional roles of these methylated genes in AD progression. It is also essential to determine whether these novel DMPs can serve as biomarkers for early diagnosis or prognosis in AD. Additionally, analyzing the relationship between DNA methylation changes and gene expression will help better understand the clinical relevance of these epigenetic modifications.

These sex-differential methylations may reveal molecular mechanisms underlying sex disparities in AD. Our enrichment analysis highlighted immune processes modified by DNA methylation showed sex differences. Many studies have strongly implicated a critical role played by immune dysregulation and neuroinflammation in the pathogenesis of AD[56]. In fact, evidence from clinical[57] and basic[58] studies also supports sex-specific differences contributing to this complexity. A gene expression meta-analysis revealed consistent immune signatures in the blood and brain in female AD patients but was absent in males[57]. Longitudinal studies showed that compared with male 5xFAD mice, female 5xFAD mice displayed an earlier and more robust induction of multiple inflammatory mediators such as CXCL10, CCL2, IL-1β, and TNFα[58]. These findings underscore the need for integrating omics analyses to better understand sex differences in AD[46].

Our results, which compare blood-brain methylation and previous cross-tissue DNA methylation analyses[16,17], indicated that DNA methylation changes are not entirely consistent between blood and brain tissue. Considering the tissue-specific nature of DNA methylation, future studies simultaneously analyzing blood and brain tissue methylation changes will help to comprehensively evaluate the potential of blood-based DNA methylation as an AD biomarker, and uncover the association of DNA methylation alterations between blood and brain tissue in AD.

This study has several limitations. First, although we applied the same analytical pipeline and meta-analysis procedure for matched AD and HC groups, we were unable to fully adjust for potential confounders, such as lifestyle factors and chronic diseases (e.g., diabetes), which may affect DNA methylation patterns. Therefore, our findings should be interpreted with caution. Validation in future studies with larger sample sizes and more rigorous control for these confounders is needed. Second, insufficient clinical data (disease duration and degree of dementia at the time of blood collection) of patients with AD were extracted, impeding further interpretation of the results. Third, DNA methylation exhibits racial differences[59,60], and our study primarily included subjects from Caucasian populations, which may introduce racial bias and limit the generalizability of the findings to other populations, such as Asians. Future studies should aim to include more diverse populations to validate and broaden the applicability of our findings. Fourth, the lack of a non-AD dementia control group limits our ability to distinguish whether the identified sex-DMPs are specific to AD or may be associated with other forms of dementia. We recognize that including non-AD dementia controls in future studies would help clarify whether these methylation patterns are uniquely associated with AD or are more broadly linked to dementia pathology.

In conclusion, our findings highlight significant sex differences in blood DNA methylation in patients with AD and address the DNA methylation associations between blood and brain tissues. These blood-based sex-

differential DNA methylation profiles not only provide valuable insights into the epigenetic mechanisms underlying sex bias in AD, but also bring new opportunities to identify potential sex-specific biomarkers which could help in personalized medicine for AD.

DECLARATIONS

Acknowledgments

Authors' contributions
Contributed to the conception and design of the study: Yang T, Li C, Shang H
Organized the datasets: Yang T, Li C, Wei Q, Cheng Y, Xiao Y
Performed the statistical analysis: Yang T, Li C, Wei Q, Cheng Y
Wrote the manuscript: Yang T, Shang H
All authors contributed to the manuscript revision and have read and approved the submitted version.

Availability of data and materials
All datasets analyzed in this study are publicly available, as described in the "Methods" section. The Australian Imaging, Biomarker & Lifestyle (AIBL) cohort (GSE153712), the AddNeuroMed cohort (GSE144858), and the third cohort were previously described by Li *et al.* (GSE53740).

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Conflicts of interest
All authors declared that there are no conflicts of interest.

Ethics approval and consent to participate
Ethics approval for the Nabais dataset, the Lunnon dataset, and the Li dataset is based on the corresponding original studies that have been published. The Nabais dataset was approved by the University of Queensland Human Research Ethics Committee (HREC): HREC/14/QRBW/ 495 (RBWH). The Lunnon dataset, which is part of the AddNeuroMed cohort, was approved by the AddNeuroMed/DCR (SLaM/IOP 30/07/2006/ SLaM/IOP 30/04/2008). The Li dataset was approved by the Institutional Review Boards for human research at the University of California, San Francisco (UCSF) and the University of California, Los Angeles (UCLA).

Consent for publication
Not applicable.

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