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RESEARCH ARTICLE

Differential blood DNA methylation across Lewy body dementias

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Abstract

Introduction: Dementia with Lewy bodies (DLB) and Parkinson's disease dementia (PDD) are characterized by cognitive alterations, visual hallucinations, and motor impairment. Diagnosis is based on type and timing of clinical manifestations; however, determination of clinical subtypes is challenging. The utility of blood DNA methylation as a biomarker for Lewy body disorders (LBD) is mostly unexplored.

Methods: We performed a cross-sectional analysis of blood methylation in 42 DLB and 50 PDD cases applying linear models to compare groups and logistic least absolute shrinkage and selection operator regression to explore the discriminant power of methylation signals.

Results: DLB blood shows differential methylation compared to PDD. Some methylation changes associate with core features of LBD. Sets of probes show high predictive value to discriminate between variants.

Discussion: Our study is the first to explore LBD blood methylation. Despite overlapping clinical presentation, we detected differential epigenetic signatures that, if confirmed in independent cohorts, could be developed into useful biomarkers.

1 | INTRODUCTION

Parkinson's disease dementia (PDD) and dementia with Lewy bodies (DLB) belong to a heterogeneous group of disorders known as Lewy body diseases (LBDs), characterized by the accumulation of α-synuclein in Lewy body structures.[1] Parkinson's disease (PD) is the second most frequent neurodegenerative disease of the elderly and increases the risk for dementia by six-fold.[2] DLB is the most frequent form of dementia after Alzheimer's disease (AD) and is characterized by non-motor cognitive alterations preceding parkinsonism.

Retrospective studies suggest that distribution and severity of Lewy body pathology impacts clinical phenotype.[3] Both DLB and PDD present overlapping clinical manifestations, including fluctuating cognitive impairment, attention deficits, visual hallucinations, and symptoms of REM sleep behavior disorder (RBD).[4]

Differentiation between LBDs is based on the type and timing of clinical manifestations, with predominance of extrapyramidal motor features in PD patients and dementia in DLB cases. However, the appearance of dementia and motor signs in close temporal proximity often confound diagnosis.[3] Although advances in the field have improved diagnostic criteria, *post mortem* pathological examination shows that still there is low sensitivity to detect DLB clinical symptoms among dementia patients.[5] The lack of specific biomarkers and the frequent co-occurrence of multiple neurodegenerative syndromes further hampers determination of clinical subtypes.

Epigenetic mechanisms like DNA methylation modulate the brain transcriptome and have key roles in neurodegeneration. Several studies, including our own, link aberrant DNA methylation with PD pathology.[6-11] Methylation changes in the promoter and intron 1 of *SNCA* gene, encoding α-synuclein, have been observed in brain and blood samples from PD and DLB cases.[7,12-14] We identified genome-wide alterations in brain and blood methylation in PD cases,[9] and more recently, we uncovered longitudinal changes in blood methylation associated with disease progression in PD.[15]

The precise epigenetic changes associated with LBD pathology and progression are not yet defined. Importantly, despite the impact that these alterations could have on disease trajectory, they have been overlooked as potential outcome measures.[16] Due to the characteristic neuropathology of LBD, studies have been focused at identifying biomarkers that reflect altered proteostasis, neuronal decay, altered neurotransmitters, and brain structure. However, effective peripheral biomarkers are not limited to those that mirror brain-localized alterations, but include peripheral changes elicited in response to disease mechanisms. DNA methylation is defined by the underlying genetic sequence and by exposure, including pathology, thus representing a potential peripheral biomarker for brain neurodegeneration.[17]

In this study, we compared the blood methylome of DLB and PDD patients. Despite cases presenting similar clinical and demographic characteristics, we observed significant differences in blood methylation in each group suggesting that clinical variation in LBD may be reflected in the blood epigenome. Interestingly, many sites showing differential methylation (DMPs) in DLB map to genes involved in neuropathology. We also identified DMPs associated with cognitive decline, and others that present decreased methylation in DLB patients experiencing RBD symptoms, thus correlating to cardinal features of disease. Importantly, we identified a set of differentially methylated sites that shows remarkable sensitivity and specificity in discriminating between DLB and PDD cases, thus showing promise as a disease classifier.

2 | METHODS

2.1 | Study cohort

In this study, we analyzed samples from the Harvard Biomarkers Study (HBS) and the Parkinson's Disease Biomarkers Program (PDBP).[18-21] Clinical and demographic characteristics of DLB and PDD patients selected for this study are shown in Table 1. Inclusion criteria is specified in the Results section. Exclusion criteria were diagnosis of a blood or bleeding disorder, known hematocrit < 30, or past medical history of cancer. PD cases carrying a known G2019S LRRK2 mutation were also excluded. Presence of REM sleep behavior symptoms was assigned based on the Mayo Sleep Questionnaire[22] in patients from PDBP; or clinician diagnosis for HBS participants.

We received all the samples de-identified. The use of HBS biosamples and data for the current study was approved by the Institutional Review Board (IRB) of Partners HealthCare. IRB approval was obtained at each study site participating in the PDBP. All participants signed informed consent forms.

2.2 | Array processing

Genomic DNA samples isolated from whole blood were received from HBS or PDBP blind-coded and randomized with respect to disease status. DNA (1 μg) was bisulfite converted (EZ DNA Methylation kits, Zymo Research) per Illumina's recommendation. The samples were processed and hybridized to Infinium MethylationEPIC BeadChip (Illumina) and signal was scanned with Illumina's iScan. Raw IDAT files were exported for processing in R.

2.3 | Data normalization and quality control

Analysis was conducted as we described previously.[15] Briefly, we performed careful quality control and pre-processing steps using the R Bioconductor package Minfi v. 1.22.1. Minfi Detection P values were calculated (detP). No samples had mean detP value > 0.05. Sex prediction was performed, and all samples were concordant with prediction. No samples showed ratios of non-methylated/methylated sites

TABLE 1 Clinicodemographic characteristics of the study cohort

Phenotype	Dementia with Lewy bodies (DLB)	Parkinson's disease dementia (PDD)
N (Female/Male)	42 (7/35)	50 (12/38)
Age (years)*	71.4 (7.7)	72.6 (8.6)
Education (years)*	15.1 (1.7)	14.8 (2.2)
Disease duration (years)*	6.02 (6.5)	8.41 (8.3) ***
MMSE†	18.66 (4.5)	20.63 (3.3)*
Number of cases that ever smoked	18	15
Report RBD symptoms‡	19	17
Race (N) White	41	48
American Indian	1	0
Black	0	2
Ethnicity (N) Non-Hispanic	42	46
Hispanic	0	4

*Data are presented as average values and (standard deviation).
†Mini-Mental State Examination.
‡REM-sleep behavior disorder. Significant differences among groups, * $P < 0.05$ and *** $P < 0.001$ as per nonparametric t test.

(uMeth/mMeth) < 10.5 (Figure S1A in supporting information). The call rate was calculated as the proportion of probes in each sample with a detP of < 0.01. We ran technical replicates across batches for control. No samples were removed due to low quality, but four PDD cases were excluded from analysis due to incomplete clinical information. We normalized the data using ssNoob (Figure S1B) and probes that failed in one or more samples (detP > 0.01), were located on sex chromosomes, had SNPs at any CpG sites, or were defined as cross-reactive were removed, leaving 771,475 probes for analysis.

2.4 | Methylation data analysis

All probes were used to build multi-dimensional scaling plots to visualize the variation in the data. Probe-wise differential methylation analysis was performed with the Bioconductor package limma. Beta values were converted to M-values for statistical analysis. The experimental design was modeled as ≈ 0 + condition + age + sex + (disease) duration + batch + smoke + CD8T + CD4T + NK + Bcell + Mono + Neu, where the last six terms represent cell type composition estimations from R Package, FlowSorted.Blood.EPIC.

The corresponding Beta values were used to calculate differential methylation as Delta Beta between the indicated comparisons and for visualization. Differentially methylated regions (DMRs) were analyzed using DMRcate.

2.5 | Best discriminant analysis

Least absolute shrinkage and selection operator (LASSO) penalized logistic regression[23] was implemented using the glmnet package in

R to identify the subset of DMPs that best discriminated PDD from DLB cases. The tuning parameter "lambda" was chosen to minimize 10-fold cross-validated error. Discriminant utility was characterized by receiver operator characteristic (ROC) curves and area under the ROC curve (AUC). [24]

3 | RESULTS

3.1 | Study cohort

Our study included 42 DLB and 50 PDD cases from the HBS[18,19] and the National Institute of Neurological Disorders and Stroke (NINDS) PDBP.[21] Criteria for inclusion as PDD was based on the Movement Disorders Society Task Force Level I Criteria for an operational diagnosis of PDD[25] requiring (1) diagnosis of PD, (2) PD developed 1 year or more prior to the onset of dementia, (3) Mini-Mental State Evaluation (MMSE) < 26, (4) cognitive deficits severe enough to impact daily living, and (5) impairment in at least two cognitive domains (attention, executive dysfunction, visuo-constructive ability, and memory performance).

Inclusion of DLB cases was based on clinical diagnosis of probable or possible DLB according to the DLB Consortium criteria,[26] which requires (1) the presence of dementia and (2) the occurrence of at least two core features, including fluctuation in attention, visual hallucinations, or parkinsonian motor signs. Table 1 summarizes the characteristics of the cohort, mainly composed of White non-Hispanic subjects with a majority of males and high education levels. Disease duration was shorter for the DLB group ($P < 0.001$ as per nonparametric t test). Cognitive decay was more pronounced in DLB cases ($P = 0.036$ as per unpaired t test). Approximately 43% of DLB cases and 36% of PDD patients smoked and 55% of DLB and 30% of PDD cases presented RBD symptoms.

3.2 | Changes in blood cell composition associated with disease type

Alterations in blood cell composition have been reported for different neurodegenerative conditions, suggesting systemic immune alterations.[15,27–29] DNA methylation signals can be used to estimate the proportional abundance of lymphocyte types. We observed that overall blood composition varied between DLB and PDD groups (Figure 1A), a change that appears to be driven by pathology rather than sex distribution (Figure 1B). Compared to PDD cases, DLB patients presented a higher estimated proportion of CD4T cells, natural killer cells (NK), and monocytes ($P = 0.00094$, $P = 0.0024$, and $P = 0.017$, respectively, as per t test with Wilcoxon post hoc); and a smaller proportion of neutrophil levels ($P = 0.00091$), with no significant changes in B cells or CD8T cells (Figure 1A). To evaluate how these changes compare to blood composition alterations in PD cases without cognitive impairment, we ran a secondary analysis on data derived from our recent lon-

gitudinal study of blood methylomes in PD.[15] Compared to control subjects, DLB cases presented lower proportions of CD8T cells, B lymphocytes, and monocytes ($P = 0.0167$, $P = 0.0003$, and $P = 0.0301$, respectively, as per one-way analysis of variance [ANOVA] with Tukey's corrections for multiple testing; Figure 1C,F,G). PDD cases showed more pronounced changes in blood composition compared to controls; with lower levels of CD8T and CD4T cells, B lymphocytes, and monocytes ($P < 0.0001$, $P < 0.0001$, $P = 0.0017$, and $P < 0.0001$, respectively, as per one-way ANOVA with Tukey's corrections for multiple testing; Figure 1C,D,F,G). In addition, both DLB and PDD cases showed an increased proportion of granulocytes compared to controls ($P = 0.0007$ and $P < 0.0001$, respectively, as per one-way ANOVA with Tukey's corrections for multiple testing; Figure 1H). Notably, PDD cases showed more alterations in blood composition than DLB cases compared to PD patients, with reduced CD8T cells, CD4T cells, and monocytes and increased granulocytes (Figure 1C,D,G,H), while DLB cases only showed significant differences in NK cells, which were higher than PD cases (Figure 1E).

3.3 | Differential blood methylation profiles in DLB and PDD cases

We ran a cross-sectional analysis of blood methylation in DLB and PDD cases, applying linear regression models using age, sex, smoking status, disease duration, and blood cell estimates as covariates. Despite the highly overlapping neuropathology that characterizes LBDs, we identified 26 DMPs in DLB versus PDD ($P < 1.55$ E-6; adjusted $P < 0.05$), six of which reached genome-wide significance at P-value < 9 E-8 (adjusted P-value < 0.012; Table 2A and Figure 2A-G). Overall changes in methylation were modest, with |Delta mean β-values| ≤ 0.03. Only one DMP showed increased methylation in DLB (cg04939302 and not overlapping annotated genes), while the rest showed lower levels of methylation in DLB cases compared to the PDD group. Interestingly, DMP-associated genes *CPNE1*, *RPL31*, *GDNF*, and *TMEM30A* are reported as differentially expressed in a systematic review of gene expression on LBD compiling data from 31 different studies.[30]

To gain biological insight from these epigenetic differences, we applied Ingenuity Pathway Analysis to a larger set of DMPs ($n = 306$; Table S1 in supporting information) using a relaxed-criteria of adjusted P-value≤ 0.25 (corresponding to unadjusted P-value < 9.8 E-05). This selection yielded 140 annotated genes for the core analysis. The three top canonical pathways enriched in DLB included B-cell receptor signaling (z-score = –1.897; P-value = 3.65 E-05); PI3K signaling in B-lymphocytes (z-score = –0.7; P-value = 1.34 E-04), and Senescence pathway (z-score = –1.265; P-value = 2.57 E-04; Figure S2 in supporting information). The top-scoring interaction network included 35 genes (Figure S3 in supporting information), nine of them associated with AD, but none associated with PDD, suggesting specific epigenetic differences between DLB and PDD cases.

We next applied DMRCate[31] to identify DMRs. DMRs are defined as CpG clusters showing concerted changes in methylation and deemed
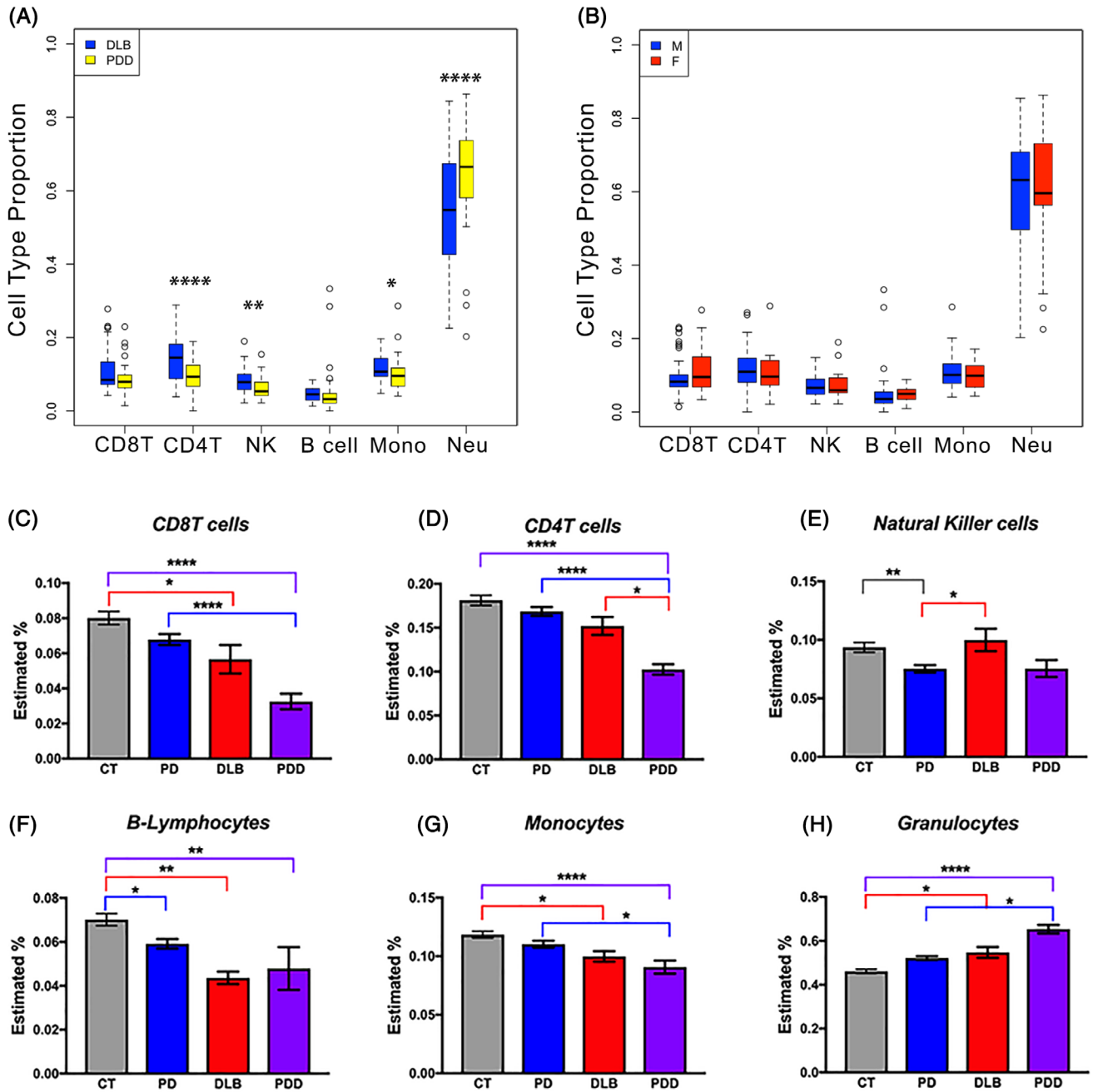
FIGURE 1 Analysis of estimated blood cell type composition in dementia with Lewy bodies versus Parkinson's disease dementia cases. Abundance of specific blood cell types was estimated based on unique methylation markers for cell identity. Estimated proportions of CD4T cells, natural killer cells (NK), monocytes (mono), and granulocytes were significantly different across disease groups (A); but these differences were independent of sex distribution (B). The proportion of each cell type was further compared to the estimated abundance of cell types in Parkinson's disease (PD) cases and healthy control subjects (age and sex matched) from our previous study[15] (C-H). Significant differences across groups are indicated as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ and **** $P < 0.0001$ as per Wilcoxon test after correction for multiple observations (A-B) or one-way analysis of variance with Bonferroni correction for multiple observations (C-H)

to have a large impact in modulating transcription. Moreover, these larger clusters of epigenetic variation may have a higher utility in the design of a classifier tool that can be deployed into the clinic. We identified 14 DMRs with a CG content ranging between 4 and 29 sites mapping to clusters of 197-1039 bp (Table 2B). DMPs observed at *SRS7*, *SSX2IP*, and *CEP95* (Table 2A) were included in DMRs, hence identifying these sites as part of a coregulated region.

3.4 | Methylation changes associated with cognitive decay and RBD symptoms

We evaluated the relationship between blood methylation and cardinal features of disease, like cognitive decay and RBD symptoms. We tested the correlation between memory impairment (MMSE scores; Table 1) and methylation of significant DMPs (Table 2A). MMSE data was avail-

TABLE 2 Differential methylation in blood from DLB cases compared to PDD cases

A. Differentially methylated probes (DMPs)

Probe	Chr	Genomic Position	Gene name	P.Value	adj.P.Val	DLB AVR. β	PDD AVR. β
cg20461538*	chr1	149858558	HIST2H2BE	4.98E-09	0.00339	0.06554	0.07884
cg01840575	chr2	38977957	SRSF7	8.78E-09	0.00339	0.09445	0.12517
cg00111253*	chr17	62502038	CEP95	1.94E-08	0.00470	0.05422	0.07328
cg19568834	chr20	34252926	CPNE1	2.50E-08	0.00470	0.03371	0.04653
cg26483432	chr1	149815043	HIST2H2AA3	3.05E-08	0.00470	0.06729	0.08414
cg19249461	chr2	101618745	RPL31	6.41E-08	0.00824	0.03649	0.05430
cg07003055*	chr20	3776921	CDC25B	2.16E-07	0.02338	0.02746	0.04065
cg22167839	chr7	35840133	SEPT7	2.55E-07	0.02338	0.03625	0.04603
cg26236440*	chr2	113341947	CHCHD5	2.90E-07	0.02338	0.05384	0.07159
cg04939302	chr2	30213458	n/a	3.38E-07	0.02338	0.78360	0.75396
cg00657871	chr1	85156634	SSX2IP	3.50E-07	0.02338	0.03100	0.04090
cg24968017	chr2	178483516	TTC30A	3.93E-07	0.02338	0.03292	0.04056
cg10203523	chr4	6711606	MRFAP1L1	3.94E-07	0.02338	0.04162	0.04772
cg26473844	chr5	37834909	GDNF	5.15E-07	0.02609	0.06534	0.08482
cg21767703	chr1	236444768	ERO1LB	5.52E-07	0.02609	0.07663	0.09237
cg11499166	chr3	51707791	TEX264	5.69E-07	0.02609	0.71193	0.66018
cg21498459	chr20	49547967	ADNP	5.75E-07	0.02609	0.03602	0.05586
cg21752471*	chr5	133861794	PHF15	6.75E-07	0.02741	0.03415	0.04712
cg08357601*	chr4	71554472	UTP3	6.75E-07	0.02741	0.02295	0.02843
cg04956913*	chr6	30712436	IER3	7.41E-07	0.02815	0.04242	0.07145
cg14634247	chr11	107728952	SLC35F2	7.95E-07	0.02815	0.06802	0.08041
cg22037798*	chr1	231473786	EXOC8	8.03E-07	0.02815	0.04135	0.06026
cg01458961	chr4	102269425	PPP3CA	1.12E-06	0.03765	0.09632	0.12293
cg05143088*	chr6	75994128	TMEM30A	1.29E-06	0.04019	0.05254	0.07136
cg08908148	chr7	39606013	YAE1D1	1.30E-06	0.04019	0.06305	0.07344
cg09310854*	chr4	144106081	USP38	1.64E-06	0.04873	0.02582	0.03879

B. Differentially methylated regions (DMRs)

| Chr	start	end	Width (bp)	No. CpGs	Min. FDR	Max. β FC	Overlapping promoters
chr12	133065724	133066762	1039	29	2.57E-13	0.02589	FBRSL1
chr6	160210606	160211577	972	17	2.20E-13	−0.02395	TCP1
chr1	231473529	231474257	729	16	1.42E-09	−0.02490	SPRTN
chr17	33759512	33760419	908	13	3.06E-11	−0.12998	SLFN12
chr4	187065336	187065955	620	12	9.63E-11	−0.02707	FAM149A
chr2	11295222	11295833	612	12	3.42E-09	0.03263	PQLC3
chr10	102045829	102046263	435	11	4.64E-10	−0.02024	BLOC1S2
chr18	12377275	12378044	770	10	3.06E-11	0.02990	AFG3L2
chr10	28821963	28822482	520	10	2.70E-09	0.00581	WAC
chr12	109124361	109125017	657	9	7.58E-10	0.01326	CORO1C
chr17	62502038	62502446	409	7	2.68E-13	−0.02343	DDX5-CEP95
chr2	38977756	38978194	439	4	1.18E-10	−0.04126	SRSF7
chr2	20424804	20425000	197	4	1.07E-08	0.01123	SDC1

(Continues)
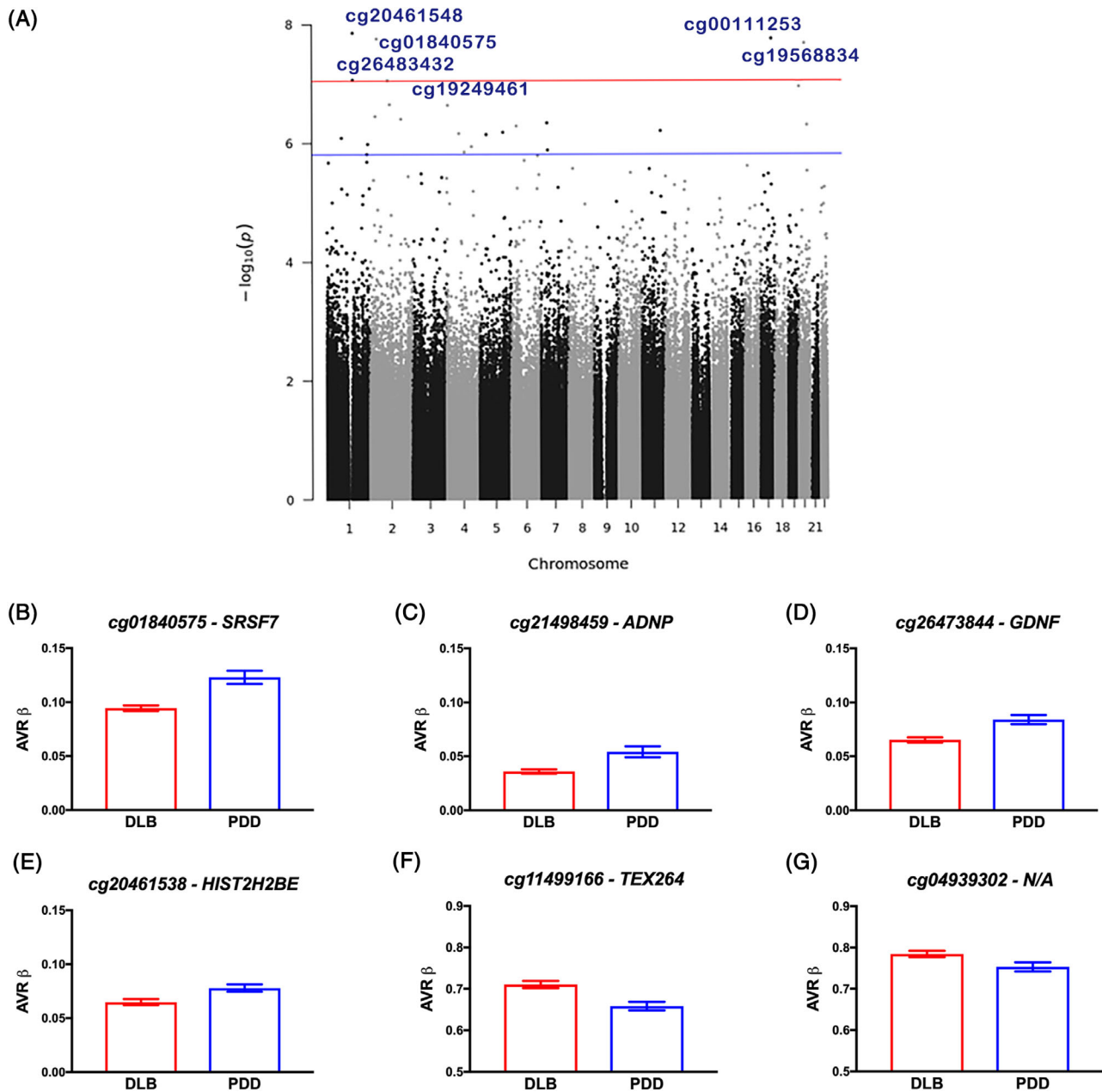
FIGURE 2 Analysis of blood methylation in dementia with Lewy bodies (DLB) versus Parkinson's disease dementia (PDD) cases. A, Manhattan plot compiling genome-wide methylation sites for the comparison of DLB versus PDD. Differentially methylated probes (DMPs) above blue line were significant with false discovery rate < 0.05; DMPs above red line showed genome-wide significance ($P \leq 6.4$ E-08). B-G, Representative box plots of select DMPs showing increased or decreased methylation in DLB cases compared to PDD cases

TABLE 2 (Continued)

B. Differentially methylated regions (DMRs)							
Chr	start	end	Width (bp)	No. CpGs	Min. FDR	Max. β FC	Overlapping promoters
chr1	85156566	85156797	232	4	1.42E-08	−0.01134	SSX2IP

Notes: Genomic position according to Human genomic Build 38. 2. Delta β methylation values calculated as DLB AVR β values − PDD AVR β values. n/a indicates probe not mapping to annotated genes.

*Probe mapping to known SNP. Gray shading indicates significant DMPs at epigenome-wide level. Probe names in **bold** indicate genome-wide significance. *Start* and *end* indicate the genomic coordinates for location of the defined DMR based on Genome Reference Consortium Human Build 38 (GRCh38) hg19. *No. CpGs* indicates how many CpGs were included in the DMR; *Min. FDR* is the minimum adjusted *P*-value from the CpGs constituting the significant region; *Max β FC* is the maximum absolute beta fold change within the region.

able for 36/43 subjects from the HBS group; and Montreal Cognitive Assessment (MoCA) scores were available for 39/43 subjects from the PDBP cohort. MoCA values were converted into MMSE scores according to an equivalency table built using Alzheimer's Disease Neuroimaging Initiative (ADNI)-GO/2 database.[32] Four of the six DMPs tested showed modest, although significant, correlation with cognitive scores, with Pearson's R coefficients ranging between 0.23 and 0.31 and showing that decreased methylation is associated with a poor outcome on cognitive assessments (Figure 3A-D).

In addition, we applied a genome-wide test to determine broader associations between blood methylation and cognitive status. We first analyzed DLB and PDD cases together for increased power and identified 84 probes significantly associated with MMSE scores (adjusted P-value < 0.05; Table S2 in supporting information). We calculated a coefficient describing the percent methylation change per point on the MMSE scale (0–30). MMSE-associated probes showed a coefficient of change ≥ 0.003 (Table S2), which may result in changes between 3% and 7% methylation by the time MMSE scores drop below 20, a level of cognitive impairment consistent with moderate dementia.[33] This range of fluctuation in blood methylation is higher than what we reported earlier for PD cases without dementia.[15]

Because distinct neurocognitive alterations are associated with DLB or PDD, we ran a genome-wide association analysis independently in DLB and PDD groups. We identified a set of unique probes associated with MMSE in each disease group, although none of them survived the correction for multiple observations, which may be partially explained by the substantial reduction of the cohort size (Table S3 in supporting information). Among the MMSE-associated genes in DLB, *HOMER 3* and *BEGAIN* are involved in protein–protein interaction at the synapse according to Reactome Pathway analysis.[34]

We next evaluated the relation between presentation of RBD symptoms and methylation levels at the significant DMPs. Data were available for 40/43 cases from the PDBP cohort and for 32/48 cases from the HBS cohort. RBD symptoms are associated with significantly reduced methylation in 88.5% of the tested DMPs (23/26) only in PDD cases, while no differences were apparent in the DLB group (Figure 3E-H). These results support that blood methylation at least partially reflects disease phenotypes across DLB clinical variants.

3.5 | Discriminating power of blood methylation to classify DLB and PDD cases

After uncovering differential blood methylation in DLB versus PDD samples, we explored whether a subset of these DMPs could discriminate between disease groups. We applied the LASSO method of regularized regression with the 26 significant DMPs (Table 2A). LASSO identified a parsimonious set of 11 loci (cg04956913; cg01458961; cg26473844; cg00111253; cg05143088; cg19568834; cg08908148; cg00657871; cg10203523; cg08357601; cg11499166) with high predictive power (AUC = 0.914; Figure 4 Model 1). The single best predictor was cg11499166 (AUC = 0.755; Figure 4 Model 3); and a discriminant model including cg11499166 and cg04956913 almost completely

recapitulated the discriminant ability of the full LASSO derived model (AUC = 0.897; Figure 4 Model 2), thus supporting the potential use of specific blood DMPs as biomarkers for LBDs.

4 | DISCUSSION

We present here the results of our study of blood methylation across LBDs to evaluate the potential utility of these epigenetic signals as diagnostic biomarkers. Despite DLB and PDD sharing multiple clinical, neurocognitive, neuropathological, and molecular traits, differential diagnosis of LBD is still based on timing of motor and cognitive symptoms. A vast array of markers has been tested to classify LBD clinical variants, including imaging, genetic traits, proteomic biomarkers like α-synuclein in the cerebrospinal fluid, and specific neurocognitive alterations.[35] While combinations of these potential biomarkers often strengthen diagnosis, accurate discrimination between DLB and PDD is still challenging. We identified multiple genomic methylation sites (DMPs) and regions (DMRs) showing significant differences between groups, many associated with genes highly relevant to pathology.

Differential blood methylation has been reported for genes with aberrant transcription in AD and PD brains.[9,36] A recent study in twin-pairs discordant for AD identified 11 differentially methylated genes in blood, many of them associated with neuronal function and therefore supporting that the blood methylome can be an indicator of CNS pathology.[37] In our study, genes mapping to top DMPs include *SRSF7*, which encodes for the Serine and Arginine Rich Splicing Factor 7 (a.k.a. Splicing factor 9G8), a repressor of MAPT/Tau exon 10 splicing and implicated in the abnormal tau isoforms ratios observed in frontotemporal dementia.[38] Another DMP-mapping gene is the activity-dependent neuroprotective protein (ADNP), a master regulator with fundamental roles in brain development. Lower levels of blood-derived *ADNP* transcripts correlate with higher amyloid burden in cortex, and importantly, *ADNP* mRNA isolated from lymphocytes has been used to discriminate between healthy subjects from those with mild cognitive impairment and AD, suggesting this molecule can serve as a serum biomarker for AD.[39] Our results warrant further investigation on whether *ADNP* could discriminate between LBD cases as well. The glial cell line-derived neurotrophic factor (GDNF) is another gene showing differential methylation in our study. Beyond its involvement in PD pathology, a recent study reported a correlation between serum levels of GDNF and other neurotransmitters with the degree of cognitive impairment in PD patients.[40] These findings suggest that DNA methylation may be upstream of transcriptional deregulation of genes associated with cognitive decline and neuropathology, thus supporting the specificity of the signal we captured.

Changes in blood composition have been reported for PD and appear to associate with immune system alterations.[15,27,41,42] In agreement with these previous reports, we observed a decay in CD4 cells for both PDD and DLB, and an increase in NK in DLB cases. Interestingly, a reduction in CD8T cells was associated with intermediate-to-late PD cases;[41] therefore, lower estimated CD8T counts in PDD and DLB may reflect advanced phases of Lewy body disorders. The data we
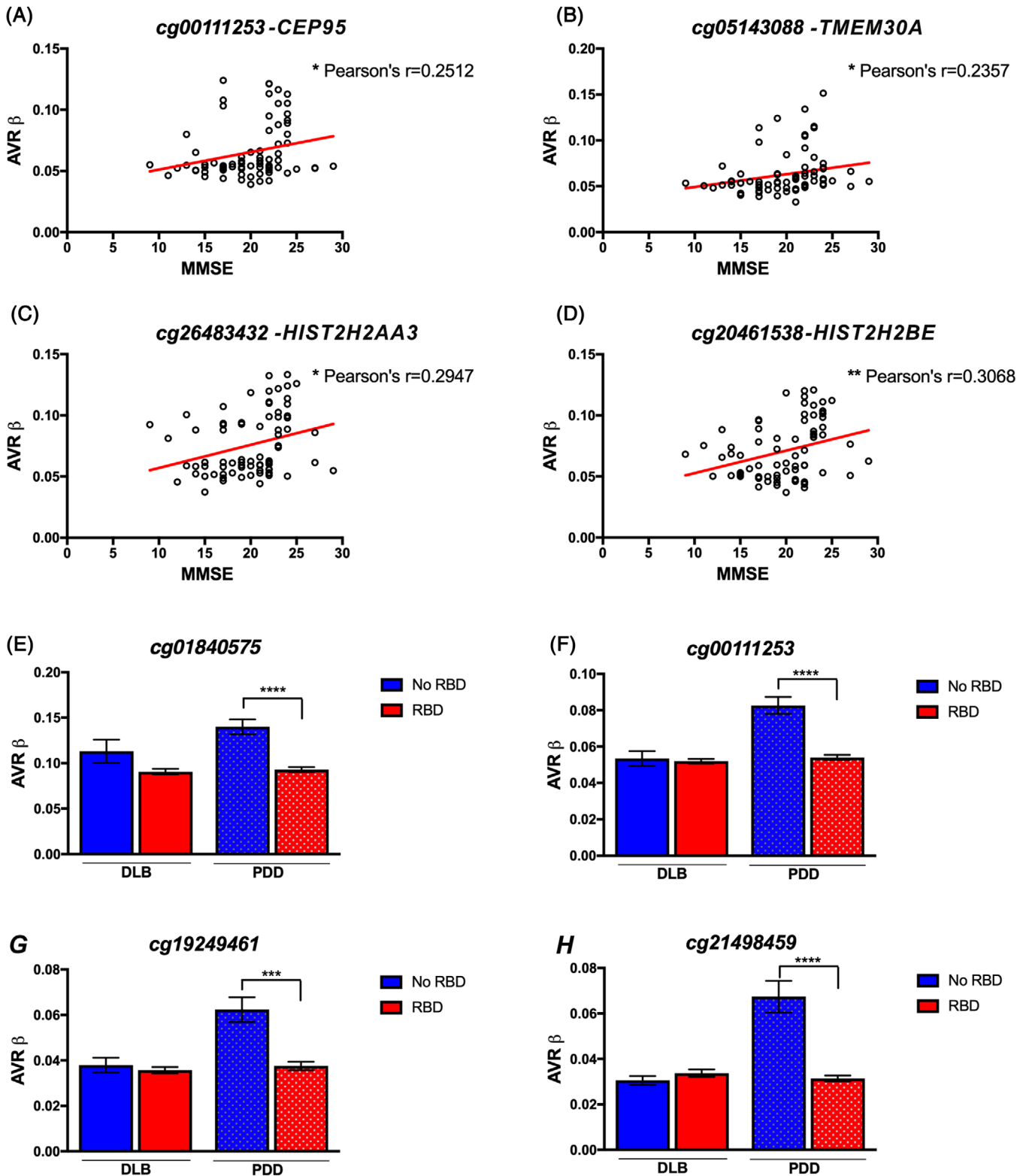
FIGURE 3 Relation of Mini-Mental State Evaluation (MMSE) scores and REM sleep behavior disorder (RBD) symptoms with blood DNA methylation. A-D, Correlation between individual blood methylation levels (in Beta units) for the top differentially methylated probes (DMPs) with cognitive scores on the MMSE. * $P < 0.05$; ** $P < 0.01$ as per Pearson's correlation analysis. E-H, Individual methylation values for each of the significant DMPs were grouped according to presentation of RBD symptoms in each disease group. Graphs illustrate methylation sites were cases presenting RBD symptoms showed different methylation levels. Bars represent average methylation per group ± standard error of the mean. Significant differences across groups are indicated as ** $P < 0.01$; *** $P < 0.001$ and **** $P < 0.0001$ as per one-way analysis of variance with Bonferroni correction for multiple observations
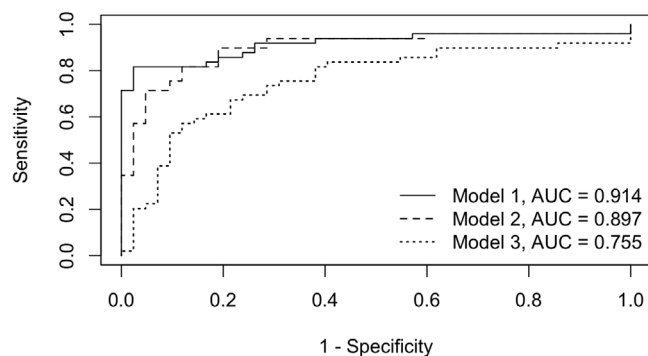
FIGURE 4 Discriminant analysis to identify best predictor models to differentiate dementia with Lewy bodies (DLB) from Parkinson's disease dementia (PDD) cases. Receiver operator characteristic (ROC) curves and area under the ROC curve (AUC) for discriminant models using 11 loci (Model 1: cg04956913; cg01458961; cg26473844; cg00111253; cg05143088; cg19568834; cg08908148; cg00657871; cg10203523; cg08357601; cg11499166); two loci (Model 2: cg11499166 and cg04956913) and the single best loci (Model 3: cg11499166)

present here suggest that LBD patients may present with distinctive alterations in blood composition, which in turn may contribute to differential methylation signals in blood.

LBDs are characterized by severe cognitive impairment. Differences in neurocognitive performance are apparent between DLB and PDD patients, with DLB cases usually presenting greater deficiencies in attention and verbal memory processing.[35,43] Currently, there is very limited data on the utility of peripheral DNA methylation as a marker of cognitive decline. Blood DMRs associate with decreased cognition in the Whitehall II cohort,[44] and epigenome-wide meta-analysis shows association of altered methylation profiles and global cognitive function and phonemic verbal fluency, suggesting a link between blood-based DNA methylation and cognitive ability.[45] Furthermore, a recent study showed that differential methylation in genes associated with synaptic function, cognitive impairment, and mitochondrial dysfunction is associated with cognitive and motor decline in PD.[46] In agreement with these studies, our analysis of the correlation between genome-wide methylation in blood and MMSE scores uncovered more than 80 DMPs with significant association. Among the top genes, *SYN3* encodes Synapsin III, which modulates α-synuclein aggregation and is an important component of Lewy bodies and Lewy neurites in the substantia nigra of PD patients and the hippocampus of DLB subjects.[47] In addition, blood transcript levels of *CPLX1*, the gene with the highest correlation score in our analysis, are inversely correlated to the expression of *SNCA* (encoding α-synuclein) in familial PD cases;[48] and higher levels of CPLX1 in cortex are associated with cognitive resilience in older adults.[49]

This study has limitations. Validation studies with larger sample size will be required to establish the clinical utility of blood methylation patterns for discriminating DLB from PDD. The effect sizes observed in our study are relatively small, but still in the range of changes observed in other studies of neurodegenerative disorders, including our own in

PD blood[15] and a study of AD blood that reported 2% to 5% methylation change at *TREM2* intronic sites in AD cases. This change appears to be sufficient to trigger transcriptional effects, as methylation showed a significant correlation with TREM2 expression in lymphocytes.[50]

Life-long environmental exposure can affect the blood methylome, thus increasing background signal and interindividual variation. We previously reported the effects of dopaminergic therapy altering blood methylation.[15] Future studies will address whether medication can also affect methylation signals in LBD.

To the best of our knowledge, this is the first exploration of blood methylation profiles as a potential classifier tool for LBDs. Despite the study limitations, our results support further investigation in the use of specific methylation changes as neuropathological signatures to discriminate closely related disorders under the LBD umbrella. Development of a biomarker that could improve early diagnosis of LBD variants could ultimately improve disease management and therapeutic outcomes.

AUTHOR CONTRIBUTIONS

C.N. and K.F. performed methylation data analysis. PDBP provided DNA samples and associated clinical and demographic data. C.R.S. and Y.I.K. designed, conducted, characterized HBS; collected DNA and characterized clinical phenotypes for HBS participants; reviewed and edited the manuscript. E.R. and K.J. performed array hybridization and processing. A.N.S.S. and S.E. performed best discriminant analysis. K.F. contributed to manuscript preparation. P.D. designed the study, supervised methylation analysis, performed data analysis, and prepared the manuscript.

CONFLICTS OF INTEREST

DATA AVAILABILITY STATEMENT

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.