

Brain and blood DNA methylation profiling in Machado-Joseph disease (MJD)/spinocerebellar ataxia type 3 (SCA3)

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Keywords: Machado-Joseph disease, SCA3, polyQ diseases, DNA methylation, epigenetics, genome-wide, epigenetic clock, episignature, brain, blood

Posted Date: September 9th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10732082/v1>

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Additional Declarations: Competing interest reported. B.S. is a shareholder in EpiSign Inc, involved in commercial uses of EpiSign™ technology. The other authors declare they have no competing interests.

Abstract

Background

Machado–Joseph disease (MJD) is a hereditary neurodegenerative disorder caused by an expanded CAG repeat in the *ATXN3* gene. Despite its monogenic origin, MJD presents heterogeneous clinical and neuropathological features that are only partially explained by genetic factors, suggesting a potential contribution of epigenetic modifiers. In this study, we profiled genome-wide DNA methylation in brain regions and peripheral blood from MJD mutation carriers at both preclinical and symptomatic stages.

Results

DNA methylation alterations in MJD brains were limited at the CpG level, with only three significant DMPs detected. In contrast, 175 DMRs were identified across the two brain regions analyzed, eight of which were shared between the DN and CC. While no specific biological pathways were enriched in the DN, hypermethylated regions in the relatively spared CC showed strong enrichment for myelination-related pathways. In peripheral blood, no genome-wide significant DMPs were detected, consistent with the lack of a distinct MJD episinature. Notably, methylation alterations in blood, detected as DMRs, were more pronounced in preclinical carriers. No blood DMRs were found to overlap with those identified in the brain. In blood samples of MJD patients, methylation levels of 5241 CpGs were associated with disease duration. Age acceleration was not associated with age at onset.

Conclusions

MJD exhibits subtle DNA methylation changes that occur primarily at genomic regions rather than individual CpG sites. In the brain, these alterations are largely region-specific, with the cerebral cortex showing enrichment for myelination-related pathways. In peripheral blood, exploratory analysis suggests that methylation changes are limited and do not mirror those observed in the brain. Together, these findings provide the first integrated analysis of the DNA methylation landscape in MJD across the brain and blood.

Background

Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3 (SCA3), is a late-onset hereditary neurodegenerative disease (1). Because MJD is caused by an expanded polyglutamine (polyQ)-encoding CAG tract in the *ATXN3* gene (2), it is also one of the nine known currently untreatable polyQ diseases (3). *ATXN3* encodes for ataxin-3, an ubiquitously expressed deubiquitinating enzyme (4). Mutant ataxin-3 is thought to alter cellular homeostasis, triggering a cascade of pathogenic events not only in the brain but also in peripheral blood (1, 5, 6). Dysfunction in transcription, autophagy, and mitochondrial pathways, among other processes, has been implicated in MJD pathogenesis (5). The pattern of neurodegeneration in MJD is complex, with the deep cerebellar nuclei among the most severely affected areas; in contrast, the cerebral cortex is relatively spared, showing only slight neuropathological changes (7).

MJD is the most common autosomal dominant ataxia worldwide, with a high prevalence in Portugal, particularly in the Azores Islands (39/100,000) (8). The disease displays marked clinical heterogeneity, including a wide variation in the age at onset (range 4–78 years) (9). Like in patients with other polyQ diseases, the age at onset inversely correlates with the length of the expanded CAG repeat (10). The number of CAG repeats, however, explains only a

partial variance in age at onset (~ 50% to ~ 70%, depending on the cohort analysed) (5), suggesting a role for other genetic modifiers. Because age at onset is the most readily observable and quantifiable trait associated with polyQ mutations, it is the most commonly used feature in studies of disease modifiers for these disorders (11). Several genetic modifiers of the age at onset of MJD have already been identified by us and others, initially through candidate gene approaches (e.g., 11–13) and, more recently, using unbiased strategies (14, 15). While the genetic modifiers identified so far account for only a small portion of the remaining variance in age at onset not explained by the CAG tract, this suggests contributions from epigenetic and/or environmental factors.

DNA methylation, primarily involving the addition of a methyl group to the cytosine of a cytosine-phosphate-guanine dinucleotide (CpG site), is the most extensively studied epigenetic mark and is considered a regulator of chromatin organization and transcriptional competence (16, 17). Due to the reversible nature of DNA methylation and the influence of specific methylation patterns on the pathophysiology and clinical presentation of neurodegenerative disorders, including polyQ disorders (e.g., 18,19) targeting DNA methylation is a promising strategy for developing epigenetic therapies for these diseases (20). Thus, assessing DNA methylation changes in postmortem brains of patients with neurodegenerative diseases, such as MJD, is crucial for improving understanding of the molecular mechanisms underlying their onset and, potentially, for detecting putative disease-related alterations in affected brain regions that could serve as therapeutic targets. However, the limited availability of postmortem brain tissue of sufficient quality for DNA methylation studies remains a current limitation of the field. Moreover, brain-based cross-sectional DNA methylation evaluation is biased toward identifying modifications specific to the end stage of the disease. These limitations underscore the importance of further investigating DNA methylation not only in the brain but also in more accessible tissues, such as blood, while examining potential overlaps between the brain and blood. Genome-wide DNA methylation analysis of peripheral blood has identified disorder-specific epigenatures that enhance molecular diagnosis, variation interpretation, and clinical classification of neurodevelopmental disorders and related conditions affecting the nervous system (21). Using blood samples from MJD carriers, we and others have previously reported: 1) a small effect of the methylation status of specific CpG islands located in the *ATXN3* promoter on the age at onset of MJD patients; however, the direction of this modulation is not consensual (22–24); 2) an association between increased DNA age acceleration (estimated DNA methylation age minus chronological age) and decreased age at onset in MJD patients (25); and 3) no differences in global methylation levels between MJD carriers and control individuals (26). This latter study, however, could not rule out the hypothesis that site-specific methylation changes are present in the blood of MJD patients, as alterations occurring in opposite directions (hyper- and hypomethylation) may counterbalance each other, resulting in the absence of global methylation differences (26). Therefore, despite these previous studies, significant gaps in our understanding of methylation in MJD remain: 1) there is a lack of epigenome-wide data from both brain and blood samples of MJD carriers and controls, precluding a comprehensive understanding of the methylation alterations present in the disease; and 2) correlations between DNA methylation and clinical features of MJD (for example, severity of ataxia or disease duration) have not yet been explored.

In this study, to overcome the aforementioned gaps in the field, we performed a multi-tissue DNA methylation profiling of MJD. This included analysing postmortem samples from the dentate nucleus (DN), a deep cerebellar nucleus significantly affected in MJD, as well as samples from the relatively spared cerebral cortex (CC). We compared these tissues from patients with MJD with those from matched control subjects. Additionally, we examined blood samples from individuals at various stages of the disease, including preclinical and symptomatic MJD mutation carriers. By integrating brain and blood methylation data, we aimed to characterize tissue-specific and shared epigenetic alterations in MJD and their association with clinical features.

Methods

Brain Samples

Postmortem frozen brain samples from six de-identified molecularly confirmed MJD patients and six age- and sex-matched control donors without neurodegenerative diseases and molecularly excluded for the *ATXN3* mutation were used in this work (Supplementary Tables 1 and 2). Given the selective vulnerability of specific brain regions in MJD (7), we analysed two regions per donor: one severely affected, the dentate nucleus of the cerebellum (DN), and the other with minimal pathological changes, the cerebral cortex (CC). The brain samples of MJD patients and control subjects were obtained from the University of Maryland Brain and Tissue Bank of the NIH NeuroBioBank (n = 4 patients; n = 4 controls) and the University of Michigan Brain Bank (n = 2 patients; n = 2 controls). The number of CAG repeats in the *ATXN3* gene in postmortem brain samples was determined by DNA fragment analysis at Laragen Inc. (Culver City, CA, USA) (Supplementary Table 1). Gene expression data in cerebellum and cerebral cortex were retrieved from the GTEx project through the Expression Atlas resource (EMBL-EBI), using TPM as the expression unit and setting the expression cutoff to 0. (27, 28).

Blood samples and clinical data

Blood samples from 24 molecularly confirmed heterozygous carriers of the MJD mutation, including 17 patients and 7 preclinical carriers of Azorean origin, living in the Azores, were also included in this study (Supplementary Table 2). Preclinical subjects are mutation carriers with no reported complaints and/or a Scale for the Assessment and Rating of Ataxia (SARA) score lower than 3 (29). For patients, the age at onset was defined as the age at which ataxia first appeared, as reported by the patient or a close relative/caregiver. The disease duration was defined as the time elapsed between age at onset and age at neurological evaluation, during which blood was collected. The CAG repeat length in the *ATXN3* gene was determined by DNA fragment analysis in all MJD carriers, as previously described (30).

Twenty-four control subjects without known chronic diseases, paired-matched by age (within a 3-year range), sex, and smoking status (smoker, former smoker, or never smoked) from the same population, and molecularly excluded for the MJD mutation (30) were used in the comparisons with MJD carriers.

DNA isolation and microarray-based DNA methylation profiling

Genomic DNA was extracted from brain and blood tissue samples using the QIAamp DNA Micro Kit (Qiagen, Cat. No. 56304) and the Quick-DNA™ Miniprep Plus Kit (Zymo Research, Cat. No. D4068), respectively, following the manufacturers' instructions.

Bisulfite conversion was performed with 250 ng of genomic DNA isolated from brain tissue and 500 ng from blood samples using the EZ DNA Kit (Zymo Research, Irvine, USA). Genome-wide methylation analysis of brain tissues was conducted at the Epigenomics Core at the University of Michigan, and methylation analysis of blood samples was carried out at UCL Genomics, University College London. Both studies were performed using the Infinium Human Methylation EPIC BeadChip v1.0 (Illumina), which covers more than 850,000 CpG sites at a single-nucleotide resolution and 99% of annotated RefSeq genes. Each methylation measurement was based on the fluorescence signals of two probes: one detecting methylated and the other unmethylated sites. The signal from background noise, identified using negative controls, was subtracted from each measurement.

Beta and M values were used for the representation and calculation of differential methylation, respectively; the Beta value reflects the proportion of methylation at one specific site, calculated as the ratio of the methylated signal to the total signal (methylated + unmethylated), ranging from 0 to 1, with 0 representing 0% methylation and 1 representing

100%. The M values represent the log₂ ratio of the intensities of methylated and unmethylated probes, being more suitable for statistical analyses, as they compensate for heteroscedasticity in Beta values at CpG sites with very high or very low methylation levels (31). Probes (i) failing the detection threshold ($p < 0.01$), (ii) located on the sex chromosomes (X and Y), and (iii) overlapping known SNVs (single-nucleotide variants) were excluded to minimize technical and biological biases. After filtering, the analysis included 814,359 CpGs for brain tissue and 814,338 CpGs for blood-derived DNA.

Differential Methylation analysis

Data normalization was performed sequentially using the Noob and Quantile methods using the shinyÉPICo pipeline (32), which integrates the standard pipelines from the minfi (33) and limma (34) packages. Cell type proportions in the brain and blood were estimated using the estimateCellCounts function from the *minfi* package (33). Correlations between principal components (PCs) and each variable (age, sex, smoking status, and cellular composition) were examined to assess potential confounding effects, using beta values from all CpGs. Neuronal proportions (brain) and granulocyte proportions (blood), which correlated with the main PCs, were included as covariates in the subsequent analysis to adjust for cellular composition. Differential methylation was determined by analysing individual CpGs (differentially methylated positions-DMPs) and regions (differentially methylated regions-DMRs) between MJD carriers and controls. Differential methylation was also estimated between blood samples from patients ($n = 17$) and preclinical carriers ($n = 7$). In this analysis, estimated proportions of all major leukocyte subtypes correlated with methylation and were therefore included as covariates to adjust for cellular heterogeneity. Age, sex, and smoking status were also included as covariates, as patients and preclinical carriers were not pair-matched.

M values were used to perform eBayes-moderated t-tests from the *limma* package (34) to identify DMPs, with an adjusted p-value (Benjamini-Hochberg false discovery rate, FDR) < 0.05 . DMRs were identified using the mCSEA (methylated CpGs Set Enrichment Analysis) package, which applies a gene-set enrichment approach to detect subtle yet consistent methylation changes (35). Probes were ranked by the t-statistics from the *limma* linear model. A region (minimum of 5 CpGs) was considered differentially methylated if the corresponding adjusted p-value (FDR) was lower than 0.05 and the absolute mean beta difference between the groups was greater than 0.05. DMRs are categorized by genomic location (36): promoters, gene bodies, or CpG islands (CGIs). A multidimensional scaling (MDS) analysis based on DMRs, using the mean methylation levels per region, was conducted to evaluate segregation between MJD and control samples.

Gene ontology and transcription factor (TF) binding motif enrichment analysis

Gene Ontology (GO) analysis was conducted using the GREAT online tool (37), with annotation performed using adjusted basal plus extension parameters (5 kb upstream, 5 kb downstream, and 1000 kb distal). GO terms were considered significantly enriched when displaying more than a two-fold change with an FDR p-value below 0.05 in the hypergeometric test. Terms were considered enriched if they contained at least three genes (38).

Transcription factor (TF) binding motif enrichment analysis was performed for DMRs, applying the findMotifsGenome.pl tool from the HOMER motif discovery software (39). A ± 250 bp window was defined around each region. All regions tested in mCSEA were used as the background in both analyses. Given the high correlation among tests, the FDR correction was deemed overly conservative, and a p-value < 0.01 was used for enrichment analysis.

Episignature Discovery

Aiming to detect the presence of a unique genomic DNA methylation pattern detectable in peripheral blood (21) discovery analysis to identify a blood episignature for MJD was performed using array data from a subset of 15 blood samples from MJD patients. For this specific analysis, a total of 169,708 probes were removed for meeting at least one of the following criteria: (a) location on the sex chromosomes; (b) overlap with SNVs; (c) cross-reactivity; (d) high variability in previous Illumina DNAm array versions; or (e) absence from the new EPICv2 array. Following probe removal, 60 matched controls (1:4 ratio) from the EpiSign™ Knowledge Database (EKD) (21) were selected with the 'matchit' package (40), matched for age, sex, and array type. Samples from the EKD are known to exhibit batch effects, and those with > 5% failed probes were excluded. Principal component analysis (PCA) was then applied to assess data structure and detect outliers.

Differential analysis was performed on the discovery cohort using the limma package (34), with beta values logit-transformed into M-values and modelled via multivariate linear regression including estimated blood cell composition as a covariate. For the episignature definition, probes were ranked based on absolute mean methylation difference and the negative log of the FDR values, and further refined using ROC-based filtering with the caret package (41). Highly correlated probes were removed, and the remaining sets were evaluated by unsupervised clustering (Euclidean distance, Ward's method, MDS). Plots were generated with ggplot2 (42) and gplots (43) and the set yielding the best clustering was selected to define the episignature. Leave-one-out cross-validation was applied to the discovery samples to assess the reproducibility and robustness of the profile. Binary classification models were trained with support vector machines (SVM, e1071 package (44)) using the selected signature probes as features. Discovery samples were combined with 75% of the control and EpiSign™ EKD samples for training, and the remaining 25% were used for testing. This 75–25% split was repeated four times, providing a four-fold cross-validation of non-discovery EKD samples. SVM hyperparameters (class weights, regularization parameter, kernel functions) were optimized using grid search.

Associations between methylation levels and clinical data

Associations between DNA methylation levels and clinical measures were evaluated at the CpG-site level using multiple linear regression models in the subset of 17 MJD patients. Before model specification, we assessed the relationships between methylation and all available biological and clinical variables; among these, only granulocyte proportion (GRAN) was correlated with methylation in this subset and was therefore included as a covariate. Chronological age was additionally tested in a separate regression model; however, it showed no association with CpGs and was consequently excluded from subsequent analyses.

Four clinical variables were analysed independently: expanded CAG repeat length, disease duration, age at onset, and SARA score. For expanded CAG repeat length and disease duration, DNA methylation was modelled as the dependent variable, with adjustment for GRAN. For age at onset (log-transformed) and SARA score, DNA methylation was modelled as the independent variable, with adjustment for GRAN and expanded CAG repeat length. Protein-coding genes harbouring CpGs whose methylation levels were associated with disease duration were subsequently analysed using Ingenuity Pathway Analysis (IPA; QIAGEN Inc.) (45) to assess potential interactions with *ATXN3*. The relationship between those genes and *ATXN3* was evaluated with respect to direct connections (absence of intermediate molecules) and indirect connections involving a single intermediate molecule. All analyses were run with default settings, except the confidence level, which was set to include only experimentally observed interactions.

Association between DNA methylation age (epigenetic clock) and clinical data

DNA methylation age, an estimate of an individual's biological age based on methylation patterns at specific CpG sites (46) was calculated for 17 blood samples from MJD patients and for 17 samples of the corresponding paired-matched controls using the online DNA methylation age calculator, based on the original pan-tissue epigenetic clock (46). Of the 353 CpGs included in the original model (46), which was developed based on the 450K array, data for 334 were available in our study and were included in the calculation.

Epigenetic age acceleration was defined as the difference between DNA methylation–predicted age and chronological age (46). Positive values indicate age acceleration, whereby DNA methylation age exceeds chronological age; negative values indicate age deceleration, with DNA methylation age lower than chronological age. We evaluated age acceleration/deceleration in the MJD patient group using the two-sided Wilcoxon signed-rank test, and differences between patients and controls were assessed with the two-sided Mann–Whitney test. Associations between epigenetic age acceleration and clinical variables were analysed using multiple linear regression models. In this analysis, expanded CAG repeat length and disease duration were used as predictors, with epigenetic age acceleration serving as the dependent variable. Conversely, in models in which age at onset (log-transformed) and SARA score were the outcomes, epigenetic age acceleration was included as an independent variable. The age-at-onset model was adjusted for expanded CAG repeat length, whereas the SARA model was adjusted for both expanded CAG repeat length and disease duration.

We thoroughly assessed and verified that the essential assumptions for all regression models were met, including linearity, normality of residuals, homoscedasticity, absence of multicollinearity, and independence of errors.

All analyses and plot generation were conducted using R version 4.4.1.

Results

Methylation changes in the MJD brain are detectable across genomic regions rather than at an individual CpG level.

DNA methylation data from the dentate nucleus (DN) and the cerebral cortex (CC) of six MJD patients were compared with those of six age- and sex-matched control donors. Following multiple testing correction, analysis at the CpG level revealed only three significantly differentially methylated positions (DMPs) in MJD brain regions compared with controls: cg01079515 (hypermethylated in the DN), cg1233316 (hypomethylated in the CC), and cg09167875 (hypomethylated in both the DN and CC) (Supplementary Table 3), which maps to the gene body of *INTS1*.

In the DN we identified 44 differentially methylated regions (DMRs) between MJD patients and controls, including 20 hypermethylated and 24 hypomethylated (Fig. 1A). These DN DMRs comprised 15 promoter-DMRs (4 hypermethylated and 11 hypomethylated), 7 gene-DMRs (6 hypermethylated and 1 hypomethylated), and 22 CGI-DMRs (10 hypermethylated and 12 hypomethylated) (Supplementary Table 4).

In the CC, a total of 131 DMRs were detected between MJD patients and controls, comprising 100 hypermethylated and 31 hypomethylated (Fig. 1A). Among the CC DMRs, we identified 46 promoter-DMRs (32 hypermethylated and 14 hypomethylated), 45 gene-DMRs (40 hypermethylated and 5 hypomethylated), and 40 CGI-DMRs (28 hypermethylated and 12 hypomethylated) (Supplementary Table 4). No DMRs were found in the MJD causative gene (*ATXN3*), in either the MJD DN or CC. To identify core disease methylation signals - methylation alterations with concordant methylation status shared by DN and CC - we intersected DMRs from these two regions. Eight overlapping DMRs, three

hypermethylated and five hypomethylated, were identified (Supplementary Table 4). Four of these DMRs are annotated: *AURKC*, *GREM2*, *NUP210L*, and *TRIM6-TRIM34*. However, no expression of *TRIM6-TRIM34* has been reported in the cerebellum or cerebral cortex, whereas the remaining three genes are expressed in both regions (27). Among these *AURKC* and *NUP210L* harbour hypomethylated promoter-DMRs, whereas *GREM2* contains a hypermethylated gene-DMR (Table 1).

To assess whether samples cluster by biological group based on their methylation profiles, multidimensional scaling (MDS) was performed using the mean methylation values for each DMR. In the DN, MJD samples showed clear separation from control samples across both principal components (Fig. 1B). In the CC, although we did not observe a clear separation between the groups, MJD samples formed a tighter cluster than controls, suggesting a more homogeneous methylation profile in this less-affected region (Fig. 1B).

Functional annotation reveals myelination-related enrichment among hypermethylated DMRs in the CC, in contrast to the lack of enrichment of biological processes in the DN

GO enrichment analysis of DMRs in the DN did not reveal enriched terms. In the CC, analysis of hypermethylated DMRs identified several enriched terms, including regulation of myelination, oligodendrocyte differentiation, structural constituent of myelin sheath, myelin maintenance, and glial cell differentiation (Fig. 1C). The enrichment profile was consistent, with most terms converging on myelin-related functions and oligodendrocyte lineage development. The majority of the hypermethylated DMRs in the CC (72/100) mapped to promoter regions or gene bodies of genes involved in myelination (Supplementary table 4). Enrichment was primarily driven by *MAG*, *MOBP*, *PLLP*, and *MYRF*, as these genes were consistently represented across multiple enriched GO terms (Table 1; Supplementary Table 4). Notably, *MAG* and *MOBP* display hypermethylated DMRs located in promoter regions, where the regulatory implications of DNA methylation are more clearly established. In addition, although not contributing to GO enrichment, *OPALIN* (another myelin-related gene) contains the hypermethylated promoter-DMR with the largest mean methylation difference between patients and controls (Fig. 1D; Supplementary Table 4). GO enrichment analysis of hypomethylated DMRs in the CC did not identify significantly enriched biological processes.

Table 1 lists selected genes harbouring DMRs that are involved in the biological processes identified by GO enrichment analysis, even if they do not contribute to the statistical enrichment. It also includes genes with overlapping DMRs between DN and CC, selected based on their cross-tissue consistency.

To gain insight into regulatory mechanisms potentially affected by differential DNA methylation in MJD brain regions, we performed transcription factor (TF) binding motif enrichment analysis. In the DN: a) analysis of hypermethylated DMRs identified motifs associated with Oct4:Sox17 and PR (progesterone receptor) transcription factors (Fig. 1E); b) analysis of hypomethylated DMRs identified several ETS (Erythroblast Transformation-Specific) family transcription factors (Fig. 1E), including ETV1, which is highly expressed in the cerebellum (27, 28). All transcription factors identified in the DN analysis are reported to be expressed in the cerebellum (27, 28). In the CC, a) hypermethylated DMRs showed enrichment of binding motifs predominantly from the SOX (SRY-box) family (Fig. 1E). Among these, SOX10 encodes a protein (SRY-box transcription factor 10) that plays a key role in oligodendrocyte lineage and myelination (47), consistent with the myelin-related GO enrichment observed in this region; b) analysis of hypomethylated DMRs further identified enrichment for E2F7 (E2F transcription factor 7) (Fig. 1E). All transcription factors identified in the CC analysis are reported to be expressed in the cerebral cortex (27, 28).

Peripheral blood shows less pronounced DNA methylation alterations than brain tissue in MJD, with greater differences observed in the preclinical stage individuals compared to symptomatic patients

To examine whether specific DNA methylation sites are altered in blood samples from MJD mutation carriers across disease stages, and to explore possible associations between DNA methylation and clinical features, we collected cross-sectional DNA methylation data from preclinical and symptomatic mutation carriers and control subjects. Whereas all MJD carriers were pooled for comparisons with controls, comparisons were also conducted separately for patients and preclinical subjects, each compared with its respective control group. At the CpG level, no differences in DNA methylation were detected in any of these comparisons. Comparison of DNA methylation data from pooled MJD mutation carriers (n = 24) and their controls revealed nine DMRs, all hypomethylated in mutation carriers, with no hypermethylated DMRs identified (Fig. 2A and Supplementary Table 5). Similar to the observed in DN and CC, no DMR was identified in the MJD's causative gene, *ATXN3*, in mutation carriers. When comparing blood DNA methylation from MJD patients (n = 17) and their controls, we identified two hypermethylated and 12 hypomethylated DMRs in MJD patients (Fig. 2A and Supplementary Table 5). Notably, comparison of datasets from preclinical MJD carriers (n = 7) and their controls revealed a higher number of DMRs, with 21 hypermethylated and 9 hypomethylated (Fig. 2A and Supplementary Table 5). We next intersected DMRs from comparisons of preclinical individuals and patients with their controls, retaining only regions that showed concordant methylation changes (hypermethylation or hypomethylation) across both comparisons. Only one overlapping DMR was identified: a hypomethylated DMR in the gene body of *CYP2E1*, a gene implicated in oxidative stress and mitochondrial dysfunction (48).

MDS analysis based on the mean beta values of the identified DMRs showed no clear separation between MJD carriers and controls, nor between symptomatic patients and controls. In contrast, analysis focusing solely on preclinical individuals revealed a distinct separation from their respective controls (Fig. 2B). In the direct comparison of DNA methylation data from patients (n = 17) and preclinical carriers (n = 7), 10 hypermethylated and two hypomethylated DMRs were identified in patients (Supplementary Table 6). Among them, a promoter DMR at *PM20D1* showed the highest mean beta difference (Supplementary Table 6). Noteworthy, this DMR exhibited hypermethylation in preclinical carriers compared with controls, whereas it showed hypomethylation in patients relative to controls (Fig. 2C). No significant GO enrichment was observed for blood-derived DMRs across any of the comparisons performed. To assess whether DNA methylation alterations detected in blood reflect alterations in brain regions, we compared blood-derived DMRs with those identified in DN and CC. Across all blood-based comparisons performed, no DMRs showed overlap with those identified in DN or CC.

Absence of a disease-specific methylation episignature in MJD peripheral blood

Blood episignature analysis was performed with 15 patient samples and 60 matched controls, which were used for probe selection and model construction. In comparison, six non-MJD samples from EpiSign™ Knowledge Database (EKD) (21) were employed for testing. Through a rigorous iterative feature selection process, 243 optimal probes were identified based on methylation differences, FDR values, and ROC analysis. The mean methylation difference between cases and controls ranged from – 30% to 44% (median 13.15%), with 91.36% of the selected probes being hypermethylated.

Clustering analysis using the selected probes showed that MJD and control samples did not form distinct groups in either the heatmap (Fig. 2D) or the MDS plot. (Fig. 2E). Furthermore, all six non-MJD samples also clustered with the MJD samples in both analyses (Figs. 2D and 2E), indicating no distinctive MJD methylation signature for the studied cohort.

We developed an SVM-based model to evaluate its effectiveness in diagnosing MJD. The training data consisted of MJD samples and 75% of the non-discovery EKD samples, with selected probes used as features. SVM scores,

referred to as methylation variant pathogenicity (MVP) scores, were computed and averaged across four-fold cross-validation sets to assess the similarity of the methylation profiles of test samples to the selected probe profiles. Higher scores indicate greater similarity to the MJD samples. Using this metric, we found that the model demonstrated high specificity, as almost all EKD test samples obtained scores close to 0. However, it was not sensitive, with five out of the six test samples obtaining an MVP score higher than 0.1, and 4 of them higher than 0.8 (Fig. 2F), confirming a lack of a distinct MJD episinature for the selected cohort samples.

Exploratory analyses suggest that peripheral blood DNA methylation levels and epigenetic age deceleration are associated with disease duration in MJD

To explore the relationship between blood CpG methylation levels and clinical features of MJD, linear regression analyses were conducted in the patient group (n = 17) across all 814,338 CpGs. While no significant associations were detected between CpG methylation and expanded CAG repeat size, age at onset, or SARA score, CpG methylation levels were found to be associated with disease duration (FDR < 0.05): methylation levels of 1,705 CpGs increased with disease duration, whereas levels of 3,536 CpGs decreased with disease duration (Supplementary Table 7). Notably, none of the 26 CpGs within the *ATXN3* locus showed association with any clinical variable.

We next examined whether genes harbouring CpGs associated with disease duration encode proteins known to interact with ataxin-3, as such genes may participate directly in pathogenic processes related to the disease. Among these, only *PSMA5* and *DNAJB2* encode proteins with experimentally supported interactions with ataxin-3. *PSMA5* encodes a proteasomal subunit that has been reported to interact directly with ataxin-3 (49). For *PSMA5*, *cg12464626* located in the 3'UTR showed a decrease in methylation levels of ~ 0.072 per year of disease, indicating progressively lower methylation over the course of the disease. *DNAJB2*, encoding a cochaperone involved in protein quality control, is an indirect interactor of ataxin-3, with a single intermediate molecule (50). In *DNAJB2*, *cg26258152* within TSS1500 showed a ~ 0.047 increase in methylation per disease year, reflecting higher methylation in later stages of the disease.

To explore if the presence of the *ATXN3* mutation alters biological age, we estimated DNA methylation age (biological age) in MJD patients and controls (Supplementary Table 8), calculated age acceleration (DNA methylation age minus chronological age), and analysed, in patients, associations with clinical outcomes. Patients showed positive age acceleration (median 6.81 years [IQR 4.07]; $p = 1.07 \times 10^{-4}$). Controls likewise showed positive age acceleration (median 8.50 years [IQR 4.15]; $p = 6.56 \times 10^{-4}$). Thus, age acceleration in patients was comparable in magnitude to that of controls, with no significant differences ($p = 0.491$) (Fig. 3A).

We found age deceleration associated with disease duration in MJD patients: age acceleration declined by ~ 0.46 years for each additional year of disease ($B = -0.456$, $p = 9.33 \times 10^{-4}$, adjusted $R^2 = 0.498$) (Fig. 3B). No associations were observed between age acceleration and the number of CAGs in the expanded allele. Moreover, no associations were established with age at onset or SARA score.

Table 1

DMR-associated genes driving GO enrichment, involved in enriched pathways, or shared between DN and CC

Gene	Position	bp to TSS	Beta diff	Tissue	Name	Neurobiological relevance
<i>AURKC</i>	DMR chr19:57741325–57742444	-546	-0.05/-0.06	DN/CC	Aurora Kinase C	• Kinase involved in neuronal processes (51)
<i>NUP210L</i>	DMR chr1:154127462–154128443	-361	-0.08/-0.07	DN/CC	Nucleoporin 210 Like	• Nucleoporin implicated in brain disorders (52)
<i>GREM2</i>	DMR chr1:240656342–240656737	+118909	+0.10/+0.12	DN/CC	Gremlin 2, DAN Family BMP Antagonist	• Antagonist of bone morphogenetic proteins (53)
<i>OPALIN</i>	DMR chr10:98116807–98120193	+559	+0.11	CC	Oligodendrocytic Myelin Paranodal And Inner Loop Protein	• Oligodendrocyte differentiation and myelination (54)
<i>MAG</i>	DMR chr19:35781587–35784828	+180	+0.08	CC	Myelin Associated Glycoprotein	• Myelination and oligodendrocyte differentiation (55)
<i>MOBP</i>	DMR chr3:39508059–39543707	+16787	+0.08	CC	Myelin Associated Oligodendrocyte Basic Protein	• Maintenance of the myelin sheath integrity (56)
<i>PLLP</i>	DMR chr16:57294356–57318244	+12299	+0.08	CC	Plasmolipin	• Myelin membrane formation and myelination (57)
<i>MYRF</i>	DMR chr11:61521905–61547669	+14666	+0.06	CC	Myelin Regulatory Factor	• TF that activates expression of myelination genes (58)
<i>CNDP1</i>	DMR chr18:72200626–72201785	-469	+0.07	CC	Carnosine Dipeptidase 1	• Neuroprotective and potentially involved in myelin integrity (59)
<i>ADAMTS4</i>	DMR chr1:161168073–161170110	-246	+0.07	CC	ADAM Metallopeptidase With Thrombospondin Type 1 Motif 4	• Promotes myelination and oligodendrocyte differentiation (60)
<i>ABCA8</i>	DMR chr17:66938261–66951903	+6410	+0.05	CC	ATP Binding Cassette Subfamily A Member 8	• Lipid metabolism and myelination regulation (61)

Gene	Position	bp to TSS	Beta diff	Tissue	Name	Neurobiological relevance
<i>ERMN</i>	DMR chr2:158181100– 158183846	+1752	+0.16	CC	Ermin	• Essential for maintaining the integrity of the myelin sheath (62)
<i>BCAS1</i>	DMR chr20:52565470– 52673626	+67585	+0.08	CC	Brain Enriched Myelin Associated Protein 1	• Participates in myelin formation and response to demyelination (63)
<i>MOG</i>	DMR chr6:29625991– 29638918	+7468	+0.07	CC	Myelin Oligodendrocyte Glycoprotein	• Involved in immune-mediated demyelination (64)
<i>SLC44A1</i>	DMR chr9:108011340– 108148795	+73165	+0.06	CC	Solute Carrier Family 44 Member 1	• Oligodendrocyte differentiation and myelin sheath formation (65)

Beta diff, difference in mean beta values; bp, base pairs; CC, Cerebral cortex; DN, Dentate nucleus; TF, Transcription factor; TSS, Transcription start site; Reference numbers supporting the neurobiological

relevance statements are indicated in parentheses.

Discussion

In this study, we conducted a comprehensive multi-tissue analysis of DNA methylation in MJD encompassing both postmortem brain tissue regions and peripheral blood samples. Given that MJD is characterized by region-specific neuropathology, analysing DNA methylation directly in postmortem samples from a severely affected region (DN) and a relatively spared brain region (CC) may provide biologically relevant insights into disease-associated epigenetic alterations. Moreover, analysis of peripheral blood samples from preclinical and symptomatic MJD mutation carriers should yield complementary information to support the assessment of potentially systemic, broader impact of DNA methylation alterations in this disease.

Following multiple testing corrections, only three CpG sites exhibited significant differential methylation when comparing MJD brains to control brains. This scarcity of DMPs was observed across both severely affected and relatively spared brain regions, suggesting that CpG DNA methylation is globally preserved in MJD. Noteworthy, a similar pattern at the individual CpG level was observed in Huntington's disease, with no DMPs detected in a similarly sized cohort of cortex samples profiled with the 450K array (66). In our study, a single CpG site showed FDR-significant differential methylation in both DN and CC regions. This site was located within the gene body of the *INTS1* gene and was consistently hypomethylated in MJD samples compared with controls. The *INTS1* gene encodes the largest subunit of the Integrator complex, a multiprotein assembly associated with RNA polymerase II. This complex was first described for its role in small nuclear RNA 3' end processing and splicing and is now recognized as an essential regulator of transcription (67). Gene-body DNA methylation remains largely unexplored, and in the absence of expression data from the samples used in this study, the functional implications of *INTS1* gene-body

hypomethylation remain unknown. Importantly, this particular class of DNA methylation has been described as exerting local intragenic effects through transcriptional elongation and alternative splicing (68). To our knowledge, the *INTS1* locus has not been reported as differentially methylated in existing epigenome-wide datasets of other neurodegenerative diseases, suggesting that this hypomethylation signal may be a novel feature of MJD. The extent to which the global transcriptional dysregulation we have reported in blood and brain samples from MJD carriers (6, 69, 70) is linked to the prominent DNA methylation alterations of *INTS1* deserves further investigation.

An analysis of DNA methylation by region revealed several DMRs in the DN and in the CC of MJD brains compared to controls. In contrast to the Huntington's disease study mentioned above, which did not detect significant DMRs at the regional level (66), our regional analysis identified DMRs in MJD brain tissue. These results indicate that DNA methylation changes in MJD brain tissue are primarily subtle, yet consistent across genomic regions. A lower number of bidirectionally methylated DMRs was identified in the DN, whereas a higher number of predominantly hypermethylated DMRs was present in the CC from MJD patients. Although it might seem paradoxical that more DMRs were identified in the less affected region, the observation of more spared regions showing stronger methylation alteration signals than more affected areas has been discussed in prior studies (19, 71) and could be interpreted as reflecting that, in severely affected areas, extensive cell loss dilutes regional methylation signals, leading to fewer robust DMRs; in contrast, in less affected areas, with a mostly preserved cellular architecture, the identification of coordinated methylation patterns is facilitated (17, 68). The intersection of DN and CC DMRs identified three concordant regions annotated to genes, which potentially represent disease-associated methylation changes independent of regional vulnerability to disease toxicity. These DMRs included hypomethylated promoter-associated DMRs in *AURKC* and *NUP210L*, and a hypermethylated gene-body DMR in *GREM2*. *AURKC* encodes a cell-cycle kinase, and in adult brains, it is unlikely to be associated with neuronal cell division. Instead, we can hypothesize that potential implications in MJD could involve non-neuronal cellular processes, such as reactive microgliosis, which has been observed in MJD brains (72, 73). *NUP210L* encodes a paralog of the nucleoporin *NUP210*, a component of the nuclear pore complex. Alterations in nuclear import mechanisms have been implicated in MJD, where nuclear localization of mutant ataxin-3 represents a key pathogenic feature (74). *GREM2* encodes a secreted glycoprotein that functions as an extracellular antagonist of bone morphogenetic proteins (BMPs) (53). Dysregulation of BMP signalling has been linked to altered neuronal homeostasis and enhanced gliosis in neurodegenerative diseases (75). Thus, methylation changes at *GREM2* may be associated with altered regulation of this pathway; however, to our knowledge, no prior epigenome-wide studies in Alzheimer's, Parkinson's, or Huntington's disease have reported differential methylation at *GREM2*, and the functional consequences of the hypermethylated gene-body DMR identified remain unexplored.

In the MJD CC, hypomethylated DMRs were not associated with enrichment of any gene ontology terms. On the other hand, enrichment of myelination-related pathways among hypermethylated DMRs was consistent, with evidence supporting reported oligodendrocyte dysfunction and myelin alterations as central features in MJD pathogenesis. Indeed, oligodendrocyte dysfunction, reduced expression of myelin-related genes, and white matter abnormalities have been reported in MJD across transcriptomic, proteomic, imaging, pathological, and ultrastructural studies in patients and mouse models (76–86). Noteworthy, the previously reported alterations appear early, even before neuronal loss, suggesting a primary toxic effect of mutated *ATXN3* rather than a consequence of neurodegeneration. However, in the study of Schuster et al., analyses of the CC, in the same region examined in our study (frontal cortex), did not show reduced myelin staining or MBP protein levels compared to controls (80). On the other hand, studies analysing the cerebellum have reported reduced myelin staining and lower MBP levels in MJD patients (80, 82). We can speculate that the apparent discrepancy in the CC between our epigenetic findings and the absence of myelin-related alterations in the CC indicate that: (a) regulatory changes do not translate into structural pathology in the CC, at least in the same way as observed in the cerebellum (80, 82), potentially due to region-specific protective

mechanisms; or (b) methylation changes in the CC arise later in the disease course, potentially mirroring regulatory alterations that are initiated earlier in vulnerable regions. Notably, the *MOBP* gene, which we found to be hypermethylated at the promoter in the CC, has been reported to be downregulated in the cerebellum of MJD patients (83). On the other hand, in contrast to consistent reports of myelination-related alterations in the cerebellum, we did not observe myelination-related enrichment in the DN. This discrepancy may reflect the marked vulnerability of the DN region in MJD, where advanced neurodegeneration could influence or mask myelination-related epigenetic profiles. In addition, the DN contains fewer myelinated fibers than the cerebellar cortex (87), which may further limit the detection of myelination-related signals. Importantly, prior reports of myelin alterations came from analyses of the cerebellum as a whole or cerebellar cortex rather than the DN specifically (80, 82).

Transcription factor (TF) motif enrichment analysis, which may reveal a distinct layer of regulation, showed a predominant enrichment of SOX family TF in hypermethylated DMRs in the MJD CC. SOX transcription factors are well-established regulators of neural progenitor identity and lineage specification (88), and this enrichment might be consistent with altered transcriptional programs underlying neural lineage specification and cellular maintenance in this less-affected region. One such TF is SOX10, a key regulator of oligodendrocyte differentiation and myelin gene expression (47), which are also cellular processes identified here through functional enrichment in the CC. In the MJD DN, TF motif enrichment analysis identified PR as an enriched transcription factor in hypermethylated DMRs. PR belongs to the steroid hormone receptor family of transcription factors, whose members, such as the glucocorticoid receptor (GR), recognize highly similar hormone response elements in DNA (89). Interestingly, GR-dependent mechanisms have been implicated in mutant ataxin-3 clearance following treatment with tauroursodeoxycholic acid (TUDCA) (90). Furthermore, enrichment analysis in the DN identified an overrepresentation of ETS family transcription factors in hypomethylated DMRs. Given the established roles of ETS factors in glial activation and neuroinflammatory signalling (91–93), this finding suggests that methylation changes in the DN may preferentially affect regulatory elements of genes involved in inflammatory and stress-responsive pathways. Taken together, considering that the CC is relatively spared whereas the DN is one of the most affected regions in MJD, data from TF binding motif analysis suggest a disease-stage model in which early-stage epigenetic modulation of neural identity (reflected by SOX motifs identified in the CC) may be followed by a later stage in which inflammatory and stress-regulatory alterations predominate (reflected by PR and ETS motifs identified in the DN).

In this study we aimed to assess potentially systemic, broader methylation alterations using blood samples from MJD carriers and matched controls. Genome-wide methylation analysis of such samples did not identify any FDR-significant differentially methylated CpG sites. Therefore, in this study the few detectable methylation alterations in individual CpG sites are primarily restricted to the brain. This absence of blood DMPs in MJD mutation carriers is consistent with a model of subtle, localized epigenetic remodelling and does not support widespread epigenetic dysregulation in MJD, confirming our previous findings of the absence of global methylation alterations in blood samples from MJD carriers. Furthermore, no distinct epismature was identified using the epismature discovery pipeline of Aref Eshghi and colleagues (21). Very few DMRs were identified in blood samples from MJD mutation carriers, compared with controls. Notably, DMRs observed in blood samples of MJD patients did not overlap with those identified in DN or CC, indicating that methylation alterations in blood do not serve as proxies for brain epigenetic alterations detected at end-stages of MJD. Interestingly, although the number of DMRs remained very low, more DMRs were identified in preclinical carriers than in symptomatic patients when each group was compared with its respective controls. This pattern appears to mirror results from our transcriptional analyses in peripheral blood samples, in which gene-expression alterations were consistently more pronounced during the preclinical stage than in symptomatic carriers, suggesting that molecular changes across distinct layers may be most detectable early in disease evolution (70, 94). Comparison of each group (MJD patients and preclinical carriers) with its respective controls produced several DMRs, of which only one was shared between the two groups. This DMR, located in the

gene body of *CYP2E1*, a gene involved in oxidative stress and reactive oxygen species (ROS) generation (48), was hypomethylated relative to controls. Although the functional consequences of *CYP2E1* gene-body methylation changes in blood, a peripheral tissue, remain uncertain, it is of note that dysregulated oxidative stress is a known component of MJD pathogenesis and is also observed in the blood (95, 96). A direct comparison of DNA methylation data from blood samples of preclinical carriers and MJD patients revealed very few DMRs. Among the genes containing these DMRs, *PM20D1* showed the strongest methylation difference, with its promoter hypermethylated in MJD preclinical carriers compared with patients. Notably, this DMR was hypermethylated in comparisons between preclinical subjects and controls and hypomethylated in comparisons between patients and their controls. *PM20D1* encodes a secreted enzyme with N-acyl amino acid synthase/hydrolase activity, involved in mitochondrial uncoupling and energy regulation (97), and has been shown to have a protective role against oxidative stress and amyloid-induced toxicity, especially in Alzheimer's disease models (98). Given that we analysed a limited number of blood samples, this finding should be interpreted cautiously and requires confirmation in a longitudinal study. Noteworthy, although blood is not primarily involved in MJD pathogenesis, mitochondrial dysfunction in blood from MJD preclinical carriers has been reported by us (99).

Despite our comprehensive epigenome-wide analyses, performed in disease-relevant brain regions and in peripheral blood, and stringently corrected for multiple comparisons, we did not identify any differentially methylated CpG sites or regions involving the *ATXN3* locus. Such changes have been reported in candidate-gene studies by Emmel et al. (2011) and Wang et al. (2017), which used targeted assays and reported altered methylation at selected CpG sites within the *ATXN3* promoter region, suggesting potential associations with age at onset or phenotypic variability in MJD. These differences likely reflect methodological and conceptual distinctions between targeted candidate-gene approaches and unbiased genome-wide analyses, as the former might detect small, localized effects that are unlikely to survive epigenome-wide FDR correction. Therefore, while we cannot rule out that subtle methylation variation at the *ATXN3* promoter may act as a modifier in specific contexts, the absence of detectable DNA methylation changes at the *ATXN3* locus across all tissues analysed indicates that epigenetic dysregulation in this disease does not primarily involve direct methylation-based regulation of its causative gene.

We found that disease duration was associated with increased methylation at some CpG sites and decreased methylation at others in blood samples from MJD mutation patients. This indicates that DNA methylation changes in peripheral blood exhibit bidirectional and disease duration-dependent effects. These findings should be interpreted with caution due to the cross-sectional study design and the limited sample size and warrant validation in larger, longitudinal cohorts. Among the genes mapping to CpGs whose methylation levels are significantly associated with MJD disease duration, two - *PSMA5* and *DNAJB2* - are known interactors of *ATXN3*, the disease-causative gene. Because the disease-duration-associated CpG at *PSMA5* is in the 3' UTR, this methylation change is unlikely to reflect direct transcriptional activation or repression; instead, it may be compatible with post-transcriptional regulatory mechanisms, given the established role of the 3' UTR in mRNA stability and microRNA-mediated regulation (100). On the other hand, because the disease duration-associated CpG at *DNAJB2* is located at the transcription start site, it may have greater potential to influence gene transcription. Indeed, *DNAJB2*, a member of the Hsp40/DNAJ family, functions as a molecular chaperone that facilitates the proteasomal degradation of misfolded proteins, including ataxin-3, by directing them to the ubiquitin-proteasome system. Overexpression of HSP1/DNAJB2 enhances *ATXN3* degradation in cellular models, reducing its aggregation propensity – a mechanism relevant to MJD pathology (50). However, the functional impact of this methylation change cannot be inferred without corresponding gene-expression data, which is currently lacking.

Analysis of DNA methylation-derived epigenetic age, using the Horvath clock, revealed that both MJD carriers and controls showed significant age acceleration (age estimation by the epigenetic clock is higher than chronological

age), of similar magnitude. Although this could indicate that disease status is not associated with altered epigenetic aging in the peripheral blood of MJD, the lack of detectable differences in age acceleration between patients and controls may also be attributable to limited statistical power, given the relatively small sample sizes in each group. While limited statistical power cannot be excluded, the absence of epigenetic age acceleration differences is consistent with the overall lack of robust peripheral methylation alterations in MJD. Li et al. (2022) reported an association between DNA methylation age acceleration and age at disease onset in MJD, observing the effect in an independent cohort of 40 patients, following adjustment for expanded CAG repeat length. Using the same epigenetic clock, we failed to detect such an effect in a smaller cohort and therefore cannot rule out the possibility that our results are reflecting insufficient power. Although an association with age at onset could not be demonstrated in our data, age acceleration was inversely associated with disease duration, with longer disease duration associated with relative age deceleration (the value of epigenetic age lowering to become closer to the value of chronological age). The inverse association with disease duration results from a pronounced epigenetic age acceleration that occurs early after onset and decreases at later stages. This apparently counterintuitive observation of age deceleration with longer disease duration could further reflect a “survivor” or “selection” effect, suggested for other neurodegenerative disorders (101): faster age acceleration has been associated with shorter survival after diagnosis, implying that individuals with slower epigenetic aging may survive longer, and thus be overrepresented in cohorts with advanced age or longer disease duration. Applied to our results, this would mean that MJD patients who live longer, and have longer disease duration, may exhibit distinct patterns of biological aging as measured by epigenetic clocks, and that those with more prolonged survival are “selected” from a pool of patients whose epigenetic age is more concordant with their chronological age.

While, to our knowledge, this study is the first to provide methylation profiling of MJD, some limitations are worth noting: modest sample size, particularly for brain tissues (which are always limited by brain availability), may have limited power to detect additional genome-wide significant DMPs and DMRs. In addition, findings derive from samples obtained at the end stage of the disease; therefore, it is challenging to infer whether the observed methylation changes are causal or a consequence of the disease process. Moreover, the EPIC methylation array does not distinguish between 5-methylcytosine and 5-hydroxymethylcytosine, thereby limiting the functional interpretation of observed methylation changes. Finally, the lack of functional data, namely gene expression for the same set of samples (brain and blood), longitudinal analyses in blood samples, or experimental models, limits the interpretation of the consequences of observed epigenetic changes and prompts further studies.

Conclusions

MJD exhibits subtle DNA methylation alterations detected primarily at the level of genomic regions rather than at individual CpG sites. In the brain, these changes are largely region-specific, with the relatively spared cerebral cortex (CC) showing enrichment of myelination-related pathways, while a small subset of differentially methylated regions (DMRs) shared between the dentate nucleus (DN) and CC suggests the presence of region-independent disease-associated signals. In peripheral blood, DNA methylation alterations are scarce, do not mirror those observed in the brain, and do not define a peripheral disease episinature. Notably, more methylation alterations were observed in preclinical carriers than in symptomatic patients. Blood methylation levels were associated with disease duration, suggesting stage-related epigenetic changes in peripheral blood that require confirmation in a larger cohort of preclinical carriers and patients. Together, these findings provide the first integrated overview of the DNA methylation landscape in MJD across both brain and blood, offering a framework for future studies to clarify the functional relevance of these epigenetic alterations in MJD pathogenesis.

Abbreviations

5hmC
5-hydroxymethylcytosine
5mC
5-methylcytosine
CC
Cerebral cortex
CGI
CpG island
CpG
Cytosine-phosphate-guanine
DMRs
Differentially methylated regions
DMPs
Differentially methylated positions
DN
Dentate nucleus
DNA_m
DNA methylation
EPIC
Infinium MethylationEPIC BeadChip array
GO
Gene Ontology
GRAN
Granulocytes
IPA
Ingenuity Pathway Analysis
MDS
Multidimensional scaling
MJD
Machado-Joseph disease
MVP
Methylation variant pathogenicity
PCA
Principal component analysis
PC
Principal component
polyQ
Polyglutamine
SARA
Scale for the Assessment and Rating of Ataxia
SCA3
Spinocerebellar ataxia type 3
SNV
Single-nucleotide variant
SVM

Support vector machine

TF

Transcription factor

Declarations

Ethics approval and consent to participate

Ethical approval for the use of postmortem brain samples was granted by the University of Michigan Biosafety Committee (IBCA00001642) and by the Ethics Committee of the University of the Azores (Parecer 26/2018). The collection and use of blood samples and clinical data were approved by the Ethics Committee of the University of the Azores (Parecer 9/2018, Parecer 1/2021, Parecer 13/2024). All participants provided written informed consent. The study followed the protocols of the Western University Research Ethics Board (REB 106302 and 116108).

Consent for publication

Not applicable.

Competing Interests

B.S. is a shareholder in EpiSign Inc, involved in commercial uses of EpiSign™ technology. The other authors declare they have no competing interests.

Funding

This work was supported by Direção Regional da Ciência e Transição Digital (DRCTD), Governo dos Açores, under the project M1.1.C/C.S./060/2021/01 – ‘Metilação de DNA “genome-wide” na doença de Machado-Joseph (DMJ)/Ataxia Espinocerebelosa Tipo 3 (SCA3). LT and MR were supported by Fundação para a Ciência e a Tecnologia (PhD grant 2021.06389.BD and (<https://doi.org/10.54499/CEECIND/03018/2018/CP1556/CT0009>, respectively. This work was funded in part by the government of Canada through Genome Canada and the Ontario Genomics Institute (OGI-188) awarded to BS.

Author Contribution

Conceptualization: ML, RMF, CL, LT. Resources: ML, MCC. Methodology: LT, MR, RMF, CL, MCC, ML. DNA methylation bioinformatic analysis: LT, RMF. Additional statistical analysis: LT, CL, MR. Episignature analysis: SG, MAL, JK, HMC, BS. Brain sample acquisition: LT, ARVM, AJB, MCC, ML. Investigation: LT, ARVM, AJB, AFF, TK, MR, CL, RMF, MCC, ML. Data interpretation: LT, MR, CL, RMF, MCC, ML. Writing – original draft: LT, ML. Writing – review and editing: LT, ML, MCC, RMF, CL, MR, ARVM, AFF, TK, SG, BS. Supervision: MR, CL, RMF, MCC, ML. All authors read and approved the final manuscript.

Acknowledgement

We thank the University of Maryland Brain and Tissue Bank, a Brain and Tissue Repository of the NIH NeuroBioBank, and the University of Michigan Brain Bank for providing the postmortem brain samples used in this study. We are

grateful to the MJD subjects, their relatives, and the healthy volunteers for participating in this study. We also acknowledge AAADMJ (Associação Atlântica de Apoio ao Doente de Machado-Joseph) for supporting the clinical visits. We further thank the excellent support provided in blood sample collection by Laboratório de Análises Clínicas Dr. Aires Raposo e Dr.^a Teresinha Raposo.

Data Availability

The raw DNA methylation data generated from EPIC array analyses in brain and blood samples are available from the corresponding authors upon reasonable request.

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Figures

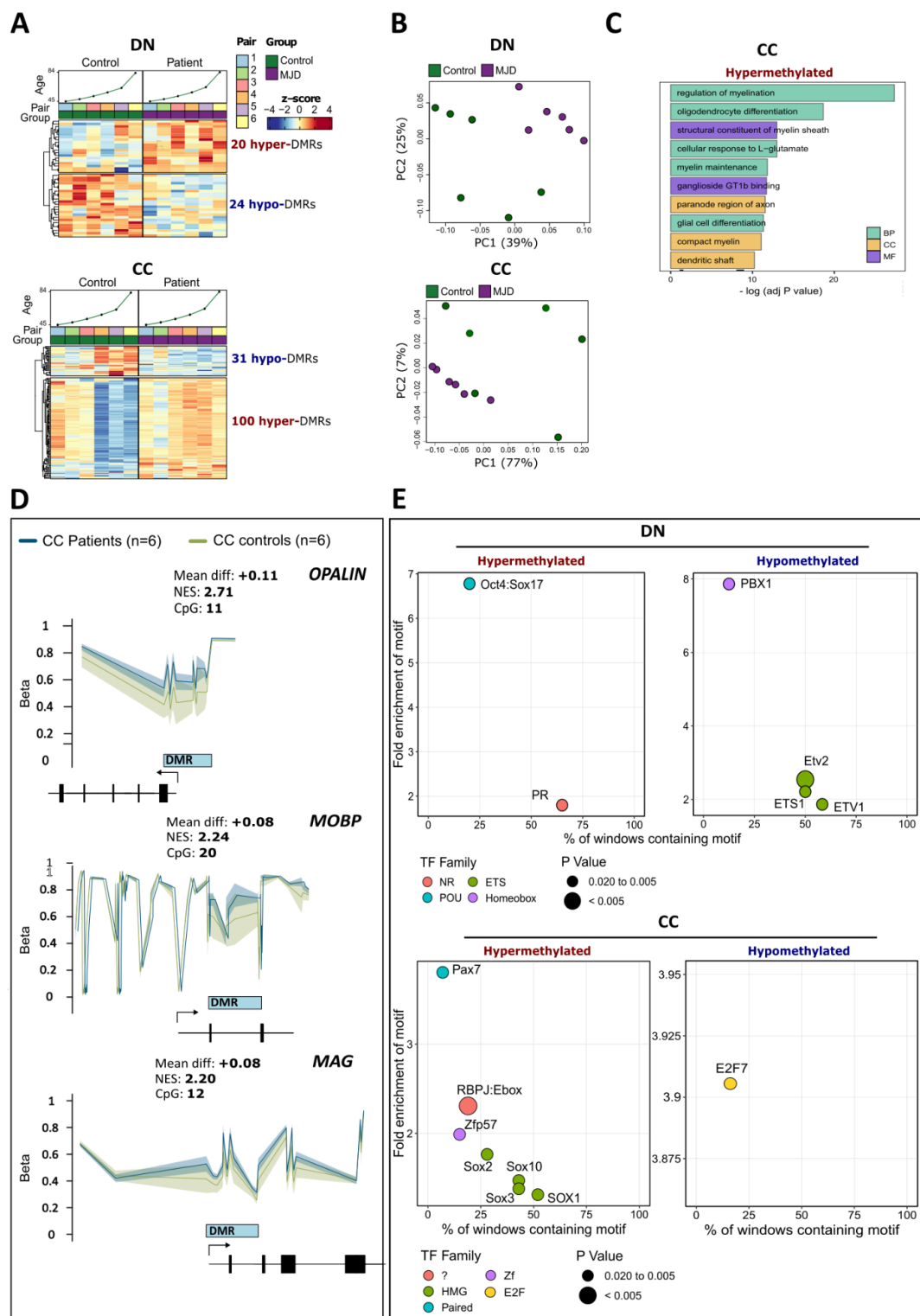


Figure 1

DNA methylation patterns in dentate nucleus (DN) and cerebral cortex (CC) in Machado-Joseph disease (MJD). **A.** Heatmaps showing differentially methylated regions (DMRs) (FDR < 0.05, beta difference > 0.05) in MJD DN (n=6) and MJD CC (n=6) relative to age- and sex-matched controls. On the normalized DNA methylation scale, red and blue indicate high and low methylation levels, respectively. **B.** Cluster analysis of all DMRs in MJD DN and MJD CC, along with their respective control samples, using multidimensional scaling (MDS). **C.** GO analysis of hypermethylated DMRs in MJD CC, showing the most biologically relevant and significantly enriched GO terms identified by GREAT. **D.**

Graphical representation of beta values for CpGs within the identified hypermethylated promoter-associated DMRs in the MJD CC, mapped to biologically relevant genes (black). The gene track shows the DMR's position relative to the gene. Vertical ticks represent CpG sites, the blue box indicates the DMR, and the arrow indicates the direction of transcription. **E.** Transcription factor binding motif enrichment analysis of hyper- and hypomethylated DMRs in MJD DN (left) and CC (right). Colour intensity represents the fold change; bubble size indicates the level of significance ($-\log(p\text{-value})$).

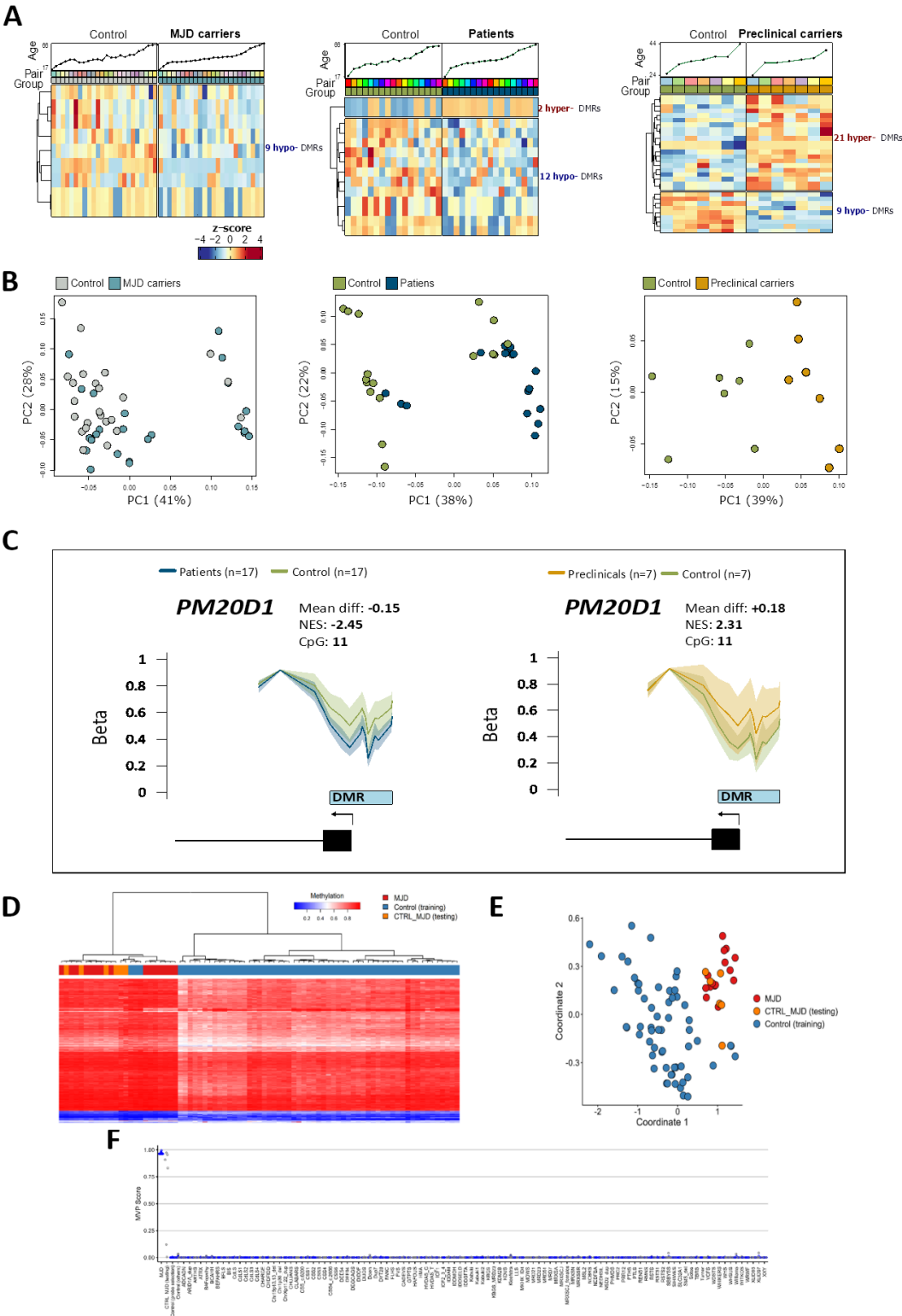


Figure 2

Blood DNA methylation analysis in Machado-Joseph disease (MJD) carriers, including patients and preclinical subjects. **A.** Heatmaps showing differentially methylated regions (DMRs) (FDR < 0.05, beta difference > 0.05) in three comparisons: all MJD carriers (n=24), patients (n=17), and preclinical subjects (n=7), relative to age-, sex-, and smoking status-matched controls. On the normalized DNA methylation scale, red and blue indicate high and low methylation levels, respectively. **B.** Cluster analysis of blood MJD carriers, patients, preclinical subjects, and their respective control samples based on all DMRs using multidimensional scaling (MDS). **C.** Graphical representation of beta values for CpGs within the promoter-DMR of the *PM20D1* gene in patients and preclinical blood analysis. The gene track shows the DMR's position relative to the annotated gene. Vertical ticks represent CpG sites, the blue box indicates the DMR, and the arrow indicates the direction of transcription. **D.** Hierarchical clustering heatmap based on selected methylation probes, including MJD samples, non-MJD samples, and matched control samples. Rows represent probes and columns represent samples; methylation levels range from low (blue) to high (red). **E.** MDS analysis using the selected probe set, illustrating the spatial distribution and overlap between MJD samples, non-MJD samples, and matched control samples. **F.** Methylation variant pathogenicity (MVP) scores derived from a support vector machine (SVM) classifier, averaged across four-fold cross-validation, comparing EKD test samples and non-MJD samples to discovery cases.

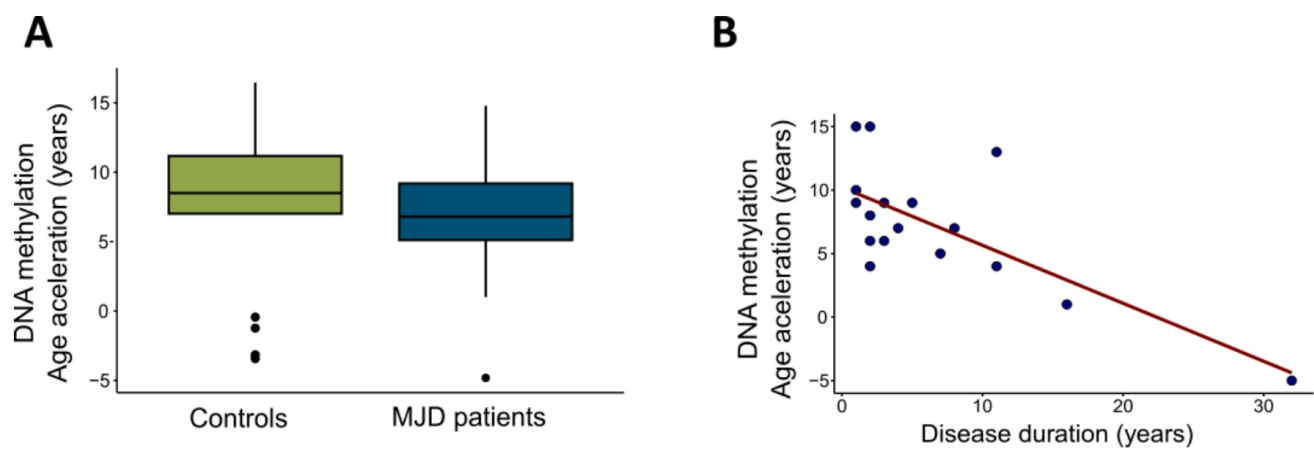


Figure 3

DNA methylation age acceleration in blood samples of Machado-Joseph disease (MJD). **A.** Boxplot showing DNA methylation age acceleration in MJD patients and matched controls. **B.** Association between DNA methylation age acceleration and disease duration in MJD patients.

Supplementary Files

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