RESEARCH ARTICLE

Epigenetic Association Analyses and Risk Prediction of RLS

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ABSTRACT: Background: As opposed to other neurobehavioral disorders, epigenetic analyses and biomarkers are largely missing in the case of idiopathic restless legs syndrome (RLS).

Objectives: Our aims were to develop a biomarker for RLS based on DNA methylation in blood and to examine DNA methylation in brain tissues for dissecting RLS pathophysiology.

Methods: Methylation of blood DNA from three independent cohorts (n = 2283) and post-mortem brain DNA from two cohorts (n = 61) was assessed by Infinium EPIC 850 K BeadChip. Epigenome-wide association study (EWAS) results of individual cohorts were combined by random-effect meta-analysis. A three-stage selection procedure (discovery, n = 884; testing, n = 520; validation, n = 879) established an epigenetic risk score including 30 CpG sites. Epigenetic age was assessed by Horvath's multi-tissue clock and Shireby's cortical clock.

Results: EWAS meta-analysis revealed 149 CpG sites linked to 136 genes ($P < 0.05$ after Bonferroni correction) in blood and 23 CpG linked to 18 genes in brain (false discovery rate [FDR] < 5%). Gene-set analyses of blood EWAS results suggested enrichments in brain tissue types and in subunits of the kainate-selective glutamate receptor complex. Individual candidate genes of the brain EWAS could be assigned to neurodevelopmental or metabolic traits. The blood epigenetic risk score achieved an area under the curve (AUC) of 0.70 (0.67–0.73) in the validation set, comparable to analogous scores in other neurobehavioral disorders. A significant

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difference in biological age in blood or brain of RLS patients was not detectable.
Conclusions: DNA methylation supports the notion of altered neurodevelopment in RLS. Epigenetic risk scores are reliably associated with RLS but require even higher accuracy to be useful as biomarkers. © 2023 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

Key Words: restless legs syndrome (RLS); epigenome-wide association studies (EWAS); methylation risk score; epigenetic age

Introduction

Restless legs syndrome (RLS) is a circadian movement disorder with impaired sleep, depression, anxiety, and potentially increased cardiovascular risk.[1] Its prevalence is up to 10% in elderly European ancestry. The pathophysiology of RLS still has not been elucidated sufficiently. Long-term treatment is frequently unsatisfactory, indicating the importance of dissecting the pathogenesis of RLS in order to identify new entry points for treatment.[2] Genome-wide association studies (GWAS) identified common genetic risk variants within 19 risk loci accounting for 60% of the single nucleotide polymorphism (SNP)-based heritability.[3] In contrast, large-scale epigenome-wide studies (EWAS) have not been performed yet. The single previously published EWAS involved blood DNA samples of only 15 cases and 15 controls.[4] Using DNA methylation at 49 CpG dinucleotides (CpG sites), that study derived an epigenetic diagnostic score for RLS with high accuracy (area under the curve [AUC] of 87.5%). However, this diagnostic score also predicted iron deficiency anemia with a similar accuracy (83%), thus indicating that the study may have focused on symptomatic RLS resulting from iron deficiency.

We have examined the epigenome-wide DNA methylation in idiopathic RLS, performing EWAS on large sets of blood samples (1133 RLS cases and 1150 population-based controls) and on tissue samples from several brain regions (40 RLS cases and 21 controls). Thereby, we aimed for a deeper understanding of the epigenetic pathophysiology of RLS and for a biomarker that might serve in diagnostics and prediction of the disease.

Patients and Methods

RLS Patients and KORA Controls

Peripheral blood DNA was available from three independent batches (Table 1) of German or Austrian ancestry comprising 1133 mutually unrelated patients with RLS and 1150 mutually unrelated population controls with similar age and sex distribution that participated in the Cooperative Health Research in the Augsburg Region (KORA) study on the Bavarian population.[5] RLS patients were recruited in specialist clinics for movement disorders and in sleep units. RLS was diagnosed in face-to-face interviews by an expert neurologist, based on the International Restless Legs Syndrome Study Group diagnostic criteria.[6] Individuals with secondary RLS were excluded. The overall response rate of RLS patients for study participation was 90%. In KORA, the response rate among the randomly invited individuals was 75%. Shared European ancestry of RLS cases and controls was confirmed by principal component analysis and admixture supervised ancestry composition analysis of pruned genotype data (Supplementary Fig. 1 in Appendix S1). The study protocols have been approved by the responsible ethics committees. All study participants gave informed consent.

Post-Mortem Brain Samples

Brain tissue was available from 40 RLS cases and 21 controls, obtained from the Neurobiobank Munich (NBM) in two batches (Table 1) and in accordance with protocols approved by the LMU Munich Ethics Committee. Members of the German Restless Legs Syndrome Foundation were informed of the possibility of donating their brain, but no specific selection was made and no compensation or other preferential treatment was given. Written informed consent was obtained from all donors. All procedures were performed in accordance with the 1964 Declaration of Helsinki or comparable ethical standards. The brains were taken at different German hospitals, immediately frozen, and sent to the NBM for storage. All RLS cases had been diagnosed and confirmed in follow-up visits by clinical experts. Controls were selected from the NBM registry by matching for age, sex, post-mortem interval, and comorbidities. Four different brain regions—cerebellum (CB), parietal cortex (PC), caudate nucleus (NC), and putamen (PU)—were examined in each individual if available. After mechanical disruption and homogenization (Precellys® 24), DNA was extracted by Qiagens AllPrep DNA/RNA/miRNA protocol. Extracted DNA did not show any signs of degradation.

Quality Control, EWAS, and Meta-Analyses

Methylation profiling was performed by Illumina MethylationEPIC BeadChip (Illumina, San Diego, CA) according to the manufacturer's protocol. Determination

TABLE 1 *Sample overview of peripheral blood and brain tissue study cohorts*

Batch		N	Age (years) (mean [range])	Males (n [%])	Females (n [%])	PMI (mean [range])
Combined (blood)	Total	2283	60.45 [6–95]	786 [34.43]	1497 [65.57]	–
	Cases	1133	60.79 [6–95]	388 [34.25]	745 [65.75]	–
	Controls	1150	60.34 [34–88]	398 [34.61]	752 [65.39]	–
Batch 1 (blood)	Total	879	62.87 [35–90]	271 [30.83]	608 [69.17]	–
	Cases	426	63.54 [35–90]	129 [30.28]	297 [69.72]	–
	Controls	453	62.23 [38–88]	142 [31.35]	311 [68.65]	–
Batch 2 (blood)	Total	520	59.51 [38–83]	170 [32.69]	350 [67.31]	–
	Cases	257	60.53 [40–75]	84 [32.68]	173 [67.32]	–
	Controls	263	58.51 [38–83]	86 [32.69]	177 [67.31]	–
Batch 3 (blood)	Total	884	58.59 [6–95]	345 [39.03]	539 [60.97]	–
	Cases	450	58.33 [6–95]	175 [38.89]	275 [61.11]	–
	Controls	434	58.86 [34–83]	170 [39.17]	264 [60.83]	–
Combined (brain)	Total	61	79.16 [19–96]	24 [39.34]	37 [60.66]	48.93 [7.82–168]
	Cases	40	85.24 [63–96]	11 [27.5]	29 [72.5]	52.61 [7.82–133.5]
	Controls	21	68.17 [19–89]	13 [61.9]	8 [38.1]	42.63 [8–168]
Batch 1 (brain)	Total	44	77.86 [19–96]	18 [40.91]	26 [59.09]	53.09 [7.82–168]
	Cases	29	83.5 [63–96]	9 [31.03]	20 [68.97]	55.13 [7.82–133.5]
	Controls	15	67.33 [19–89]	9 [60]	6 [40]	49.57 [8–168]
Batch 2 (brain)	Total	17	82.68 [53.33–95.67]	6 [35.29]	11 [64.71]	38.26 [11.83–88]
	Cases	11	90.13 [81.5–95.67]	2 [18.18]	9 [81.82]	46.06 [11.83–88]
	Controls	6	70.25 [53.33–81.08]	4 [66.67]	2 [33.33%]	25.27 [12–46.7]

Abbreviation: PMI, post-mortem interval.

of methylation intensities, including background correction and normalization (*preprocessQuantile*), were done using the *minfi* package[7] running on R version 4.0.2 (R Core Team 2020). Probes with detection *P*-value>0.01, on sex chromosomes or at SNPs, with cross-reactivity, or a call rate < 0.95% were excluded, as well as samples with mean detection *P*-value>0.05 or call rate < 95%. The methylation level of each CpG site was assessed as beta-value (β) from which the *M*-value[8] was calculated as log2 (β/(1-β)). For epigenome-wide association study (EWAS) the *limma* package[9] was applied to three sets of peripheral blood samples from cases and controls (Table 1) with adjustment for sex, age, and Houseman-estimates of white blood cells. Covariates in the brain EWAS were post-mortem interval, sex, and age. Further, we corrected for potential technical biases by including the first 30 principal components (PCs) of control probe intensities.[10]

EWAS summary statistics of the three peripheral blood sets were subjected to meta-analysis using the R meta package[11] with a random effects model. The pooled data of the three sets contained 590,431 common CpG sites and N = 2283 individuals. The epigenome-wide significance threshold was 8.47×10^{-8}.

In order to leverage information on the correlation between DNA methylation in blood and in brain we uploaded all CpG sites included in the blood EWAS meta-analysis to BECon (https://redgar598.shinyapps.io/BECon/). Of 28,298 sites above the 75th percentile of correlation, we selected those that reached Bonferroni-corrected significance ($P < 0.05/28{,}298$) in the blood EWAS meta-analysis.

Brain EWASs were performed analogously. Meta-analyses included 763,264 CpGs and 58 individuals for CB, 764,556 and 54 for NC, 761,209 and 54 for PC, and 761,069 and 58 for PU. As there was no serious inflation, we used the less conservative 5% false discovery rate (FDR) significance threshold.

CpG sites were mapped to the nearest genes according to the *IlluminaHumanMethylationEPICanno.ilm10b4.hg19* annotation file.

Phillips et al.[12] reported associations of RLS with alcohol consumption, body mass index (BMI), diabetes,

income, physical activity, and smoking. We excluded these potential confounders as described in the extended online methods in Appendix S1.

Differentially methylated regions (DMRs) were derived from the EWAS meta-analyses on blood and on the four brain regions using the ipdmr software[13] with default settings.

Methylation Risk Score

For constructing a methylation risk score in blood DNA of RLS patients, the largest dataset (batch 3) was used for discovery, batch 2 for testing, and batch 1 for validation (Table 1). Candidate CpG sites for the weighted risk score were selected from the EWAS on the discovery batch (see earlier) if they passed an FDR-threshold of 5% and an abs(logFC)-threshold of 0.3. To avoid redundant information, we calculated the pairwise correlation of all selected CpG sites. If a CpG pair had a correlation larger than 0.3, only the site with the lower *P*-value was kept in the risk score. 308 CpG sites passed the threshold criteria, four of them were excluded due to the correlation criterion, resulting in 304 CpGs for the testing phase. In the testing phase, we performed logistic regression of the disease state in the testing batch, using a model that included CpG sites, age sex, and the Houseman-estimates of white blood cell type composition. In order to optimize the number of CpG sites, we analyzed the receiver operating characteristic (ROC) curve of the prediction of RLS and control status in the testing batch. To do so, we increased the set of CpG sites stepwise by 10, starting with the 10 most significant, each time performing a logistic regression and calculating the AUC of the ROC. The model with the highest AUC value was selected and applied to the validation batch. For comparison, we trained a support vector machine (SVM) classifier with linear kernel (R package e1017, https://CRAN.R-project.org/package=e1071) on the selected model parameters in the testing batch and predicted the disease states in the validation batch. Sensitivity and specificity of this prediction were then entered in the ROC diagram. In order to assess the potential relevance of age, sex, and blood cell composition estimates on the accuracy of the prediction, training and application of the logistic regression model and of the SVM were also done without the CpG sites. For prediction of the brain samples' disease states using blood EWAS results, CpG sites, age, and sex were used, but not the white blood cell type estimates.

Epigenetic Age

We applied Horvath's epigenetic clock,[14] a multi-tissue estimator of DNA methylation age (DNAmAge). We calculated the difference between DNAmAge and chronological age and conducted linear regression analysis of that difference on RLS status, combining batch results by random-effect meta-analysis. In addition, we used Shireby's cortical clock[15] to estimate the DNAmAge in the brain DNA samples. For a detailed description see extended online methods in Appendix S1.

Gene-Set Enrichment Analysis

Tissue and pathway enrichment analyses were carried out using the tools GENE2FUNC within FUMA (v1.3.8, https://fuma.ctglab.nl/gene2func/),[16] PANTHER (v.17.0, http://www.pantherdb.org/),[17] and missMethyl (v.1.32.1, https://bioconductor.org/packages/missMethyl/).[18] With these tools we accessed the databases GO_BP, GTEx (v.8),[19] GWAScatalog, KEGG, and PANTHER-pathways. Enrichment tests compare an input set (eg, the significant CpG sites or the genes associated with them) to the representations of a background set in the tissue and pathway genes sets of the analyzed databases. As background set, we used the CpG sites (or linked genes) covered by the respective EWAS which had generated the input set. In case of the EWAS meta-analysis on blood data, for instance, the background set consisted of 590,431 CpG sites (or 21,084 linked genes with unique Entrez ID). For the enrichment analyses of CpG sites with blood–brain correlation, we restricted the background to the 307,651 sites that could be assessed for such correlation at BECon (see earlier). We set the significance cut-off at FDR of 5% and a required overlap of at least 10 genes in order to match the settings of Czamara et al.[20] However, we also considered smaller overlaps if the analyzed EWAS results comprised fewer than 10 genes with Entrez ID. Analogous gene-based enrichment analyses were performed with the DMR-related gene sets.

Results

RLS EWAS Meta-Analysis of Peripheral Blood DNA

Meta-analysis of the three EWAS on peripheral blood DNA methylation in RLS patients (batches 1–3; Table 1) versus controls resulted in 149 differentially methylated CpG sites (Fig. 1), at a Bonferroni significance level of $P < 8.47 \times 10^{-8}$, that were linked to 136 genes (Supplementary Table 1 in Appendix S1). Effect sizes (logFC) ranged between −0.6 and 0.3 (Fig. 2). 146 (98%) of the significant CpG sites had a negative effect direction. Across all CpG sites, the results of the EWAS were balanced with 50.76% negative association beta-coefficients and 49.24% positive beta-coefficients.

We specifically analyzed 28,298 CpG sites whose correlation between blood DNA methylation and brain DNA methylation is above the 75th percentile according

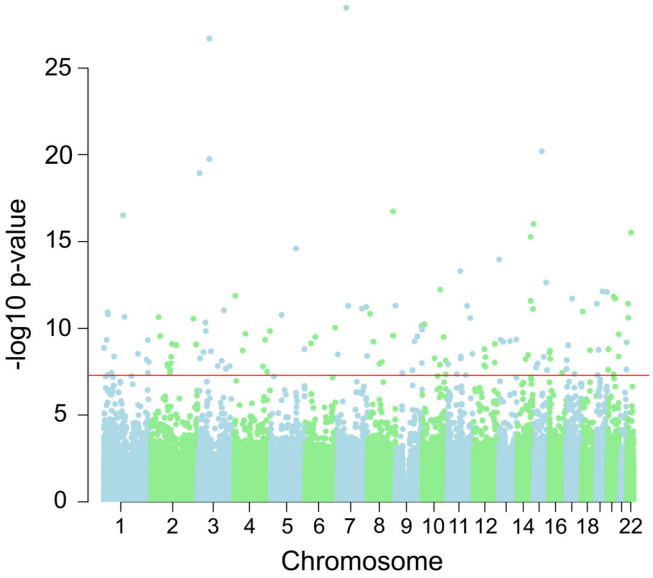

FIG. 1. Manhattan plot of the meta-analysis of restless legs syndrome (RLS) epigenome-wide association study (EWAS) on blood DNA. One hundred and forty-nine differentially methylated CpG sites were associated with RLS at epigenome-wide significance. Linked genes are listed in Supplementary Table 1 in Appendix S1. [Color figure can be viewed at wileyonlinelibrary.com]

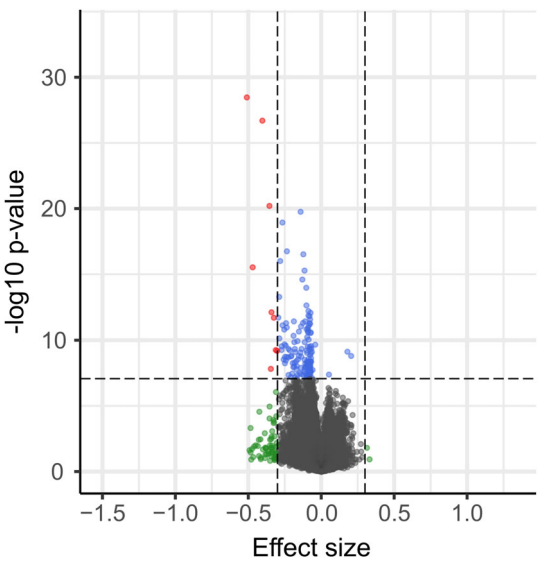

FIG. 2. Volcano plot of restless legs syndrome (RLS) epigenome-wide association study (EWAS) on blood DNA. CpG sites are represented by dots indicating effect sizes E (logFC, *x*-axis) and significances (*y*-axis) of the association between methylation and RLS. Red: Bonferroni-significant, E > 0.3; blue: Bonferroni-significant, E < 0.3; green: non-significant, E > 0.3. [Color figure can be viewed at wileyonlinelibrary.com]

to BECon (https://redgar598.shinyapps.io/BECon/). Nine of them reached Bonferroni-corrected significance ($P < 0.05/28{,}298$), including six that had not already been significant in the general blood EWAS meta-analysis (Supplementary Table 2 in Appendix S1). However, when the nine CpG sites were looked up in the brain EWAS results (see later), none of them reached nominal significance ($P \geq 0.05$).

Analyzing differentially methylated regions, we identified 521 DMRs by the overall blood EWAS which comprised a subset of 11 DMRs that we derived from the set of CpG sites that correlate between blood and brain (Supplementary Table 3 in Appendix S1).

RLS EWAS Meta-Analysis of Brain DNA

Analogous to the blood data, EWAS summary statistics of the two batches of the brain data were combined by random-effect meta-analysis. As compared to the blood data, little inflation was detected. Consequently, we used the less conservative 5% FDR (Benjamini–Hochberg) for multiple-testing correction. For the four different brain regions we obtained four significantly associated CpG sites linked to three genes in the case of NC, 16 significantly associated CpG sites linked to 12 genes in the case of PC, and three significantly associated CpG sites linked to three genes in the case of PU (Supplementary Table 4 in Appendix S1). CB did not yield any significant CpG sites after FDR correction. Significant CpG sites from the blood EWAS did not reach nominal significance ($P > 0.05$) in any of the brain tissues (Supplementary Table 1 and Supplementary Fig. 2 in Appendix S1).

DMRs were found in NC (4), PC (6), and CB (3). They did not overlap with the DMRs derived from the blood EWAS (Supplementary Table 3 in Appendix S1).

Gene-Set Enrichment Analysis of Blood and Brain EWAS Results

Tissue enrichment analyses using the blood EWAS meta-analysis results were significant (adjusted *P*-value $= 3.5 \times 10^{-2}$) for brain tissue when compared to GTEx v8 gene sets with differential overexpression ("up-regulated DEG"; Supplementary Fig. 3 in Appendix S1) by FUMA's GENE2FUNC. The restriction to upregulated DEG was made because the direction of effect was negative for most of the significant CpG sides in blood (Fig. 2), indicating hypomethylation in RLS cases with hypomethylation usually implying increased expression.

Moreover, when considering 54 more specific tissue types available in GTEx, the input genes were overrepresented in multiple CNS tissue types, including cortex ($P = 1.1 \times 10^{-3}$), frontal cortex BA9 ($P = 4.3 \times 10^{-3}$), anterior cingulate cortex BA24 ($P = 6.5 \times 10^{-3}$), substantia nigra ($P = 4.5 \times 10^{-2}$), and spinal cord cervical c-1 ($P = 1.6 \times 10^{-2}$) (Supplementary Fig. 3B in Appendix S1). However, when we tried to replicate this result using the small set of CpG sites that correlate between blood and brain, the enrichment *P*-values of central nervous system (CNS) regions were still on top but did not pass the Bonferroni-corrected threshold anymore, likely due to insufficient power.

In the pathway enrichment analyses, GENE2FUNC enrichment analyses of the DMRs derived from CpG sites that correlate between blood and brain identified the kainate-selective glutamate receptor complex among GO-cellular components as well as GO-molecular functions (adjusted *P*-value<0.004). This was due to differential methylation of the genes of subunits GluK2 (*GRIK2*) and GluK4 (*GRIK4*). In *GRIK4* we determined hypomethylation in patients while the DMR in *GRIK2* was hypermethylated, potentially indicating a shift in the subunit expression spectrum.

All further enrichment analyses in blood and brain EWAS did not provide significant results. However, literature-based analyses of the specific functions of individual candidate genes that came up in the brain EWASs revealed that almost all of the candidates could be assigned to neurodevelopmental or metabolic traits (Supplementary Table 5 in Appendix S1).

Methylation Risk Score

As a discovery cohort for RLS methylation risk score construction we used the largest batch (ie, batch 3) (Table 1). With thresholds on significance, effect size, and correlation of $q = 0.05$, abs(logFC) = 0.3, and $r = 0.3$, respectively, as described in the Methods section, we selected 304 CpG sites. From them we derived an optimized subset of 30 CpG sites to be used in the risk score by stepwise adding CpG sites and performing ROC analysis in the test set (batch 2). This set of 30 CpG-sites (Supplementary Table 6 in Appendix S1) predicted RLS with the maximal area under the curve (AUC) of 70% (90% confidence interval [CI] 67%–73%; Fig. 3A) in the blood validation set (batch 1). In comparison, a model with just age, sex, and cell composition reached an AUC of only 51% (47%–54%; Fig. 3B) and did not significantly differ from chance prediction.

The RLS methylation risk score did not reliably distinguish between RLS and control status when applied to post-mortem brain tissues. Here, the 90% CIs of the AUCs always included the 50% level (Fig. 3C). Sensitivities and specificities of the predictions by an SVM resided within or at the upper border of the ROCs' 90% CI areas (Fig. 3).

Epigenetic Age

Horvath's biological age (DNAmAge) estimates in peripheral blood samples were highly correlated with

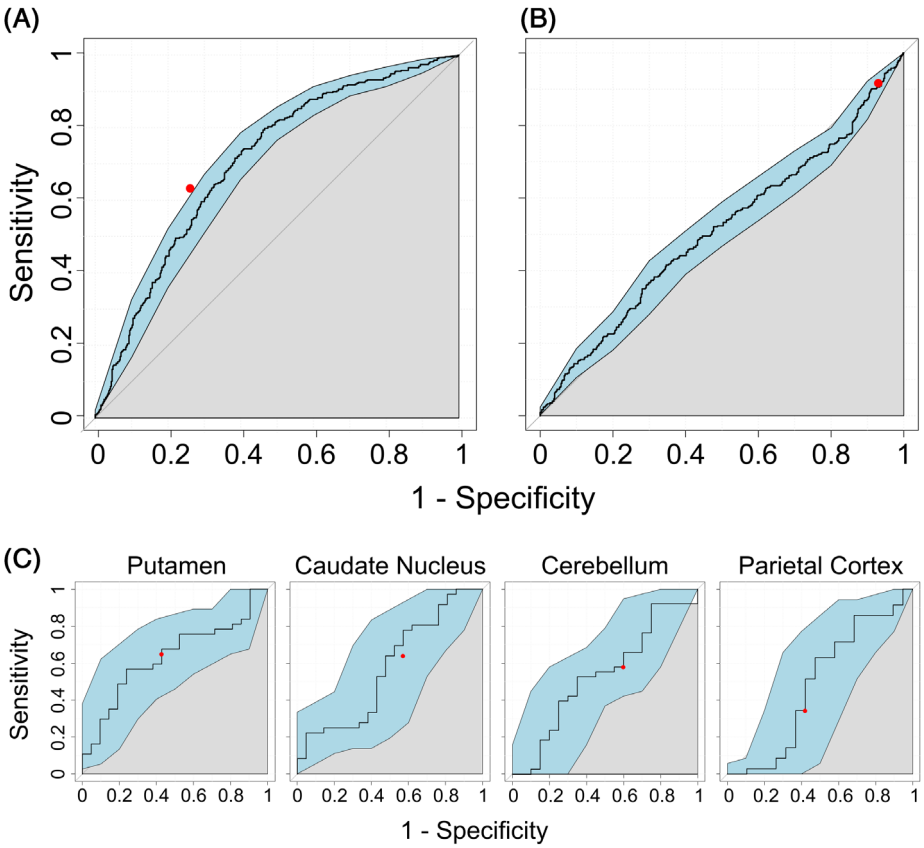

FIG. 3. Receiver operating characteristic (ROC) curves of risk score in the blood validation batch (**A**, area under the curve [AUC] = 0.70, 90% confidence interval [CI] = 0. 67–0.73) compared to a score trained without CpGs (**B**, AUC = 0.51, 90% CI = 0.47–0.54) and applied to brain tissues (**C**, AUCs not significantly different from 0.5). Red dots indicate support vector machine (SVM) prediction. [Color figure can be viewed at wileyonlinelibrary.com]

the chronological age of the individuals with $r = 0.86$ (95% CI = 0.85–0.87). Correcting for sex and chronological age, DNAmAge appeared to be 2.0 years advanced in RLS patients ($P = 2 \times 10^{-9}$) of batch 1. However, this did not replicate in batch 2 (0.3 years, $P = 0.5$) and seemingly was inverted in batch 3 (−0.5 years, $P = 0.09$). Accordingly, random-effect meta-analysis of the three batches did not produce a significant result ($P = 0.4$). Analogous analyses of the brain samples also did not produce significant results, neither in individual batches, nor in meta-analyses of the batches of each of the brain regions ($P > 0.1$), nor in meta-analysis across the two batches and the four regions (1.1 years, $P = 0.6$), nor in regression of the average difference of the four brain regions and meta-analysis of the two batches (1.4 years, $P = 0.36$). Performing the same individual batch- and meta-analyses with Shireby's cortical clock, which previously was shown to have the highest prediction accuracy for brain age assessment,[15,21] also no significant evidence of advanced age in RLS brain tissue was found ($P > 0.1$).

Discussion

To elucidate the underlying pathophysiology and for deriving diagnostic biomarkers, EWASs have been performed on several common diseases during the last decade.[22] Recent studies aimed to establish DNA methylation signatures for different neurobehavioral disorders such as depression[23] or schizophrenia[24,25] to provide supportive diagnostic tools.

The tissue-specific analysis of significant candidate genes of our blood EWAS showed an enrichment of genes differentially overexpressed in the brain. This involvement in neurological processes is in line with genetic[3] and imaging[26,27] studies which identified various CNS structures as relevant for the pathogenesis of RLS. However, we could not replicate the significant blood EWAS results by brain EWAS. This non-replication likely relates to the unavoidably low number of brain samples, that is, to the low replication power, and to the fact that blood methylation is not a good predictor of brain methylation for the vast majority of CpG sites. For less than 6.5% of the CpG sites blood DNA methylation can explain more than 20% of their methylation variance in the brain, and for less than 1.5% more than 50%.[28]

Current concepts on RLS pathophysiology focus on dopaminergic, GABAergic, and glutamatergic neurotransmitter systems[29] and brain iron metabolism.[30,31] Several genes highlighted by our EWAS on cortex, putamen, and caudate nucleus fit into these concepts, such as *BRD2* (Bromodomain-containing protein 2), which is assumed to influence the neurogenesis of GABAergic neurons in the basal ganglia,[32] or *SLC25A28* (Mitoferrin 2), a mediator of mitochondrial iron ion uptake.[33] Notably, enrichment analysis of DMRs derived from CpG sites that correlate between blood and brain identified the kainate-selective glutamate receptor complex driven by the genes of the subunits GluK2 (*GRIK2*) and GluK4 (*GRIK4*). *GRIK4* was hypomethylated in patients while *GRIK2* was hypermethylated, suggesting opposed changes in expression. GluK2 can form homomeric or heteromeric receptors, while GluK4 is always associated with any of the GluK1-GluK3 subunits.[34] Therefore, the opposed changes in expression would change the spectrum of these receptors, potentially alter the balance of inhibitory and excitatory activity, and thus affect behavior.[35] The genes described here may be considered as candidates for functional studies. Some of them have previously been related to other neurobehavioral disorders such as schizophrenia, depression, or bipolar disorder, matching the increased rate of depression and anxiety in RLS.[36]

In order to derive a biomarker for clinical diagnosis and prediction of RLS we constructed a blood DNA methylation signature of 30 CpG sites which we then tested and validated in two independent batches. The signature was significantly associated with RLS with an AUC of 0.70 (0.67–0.73) in the validation set. Similar accuracies have been achieved by methylation risk scores in other neurobehavioral disorders such as depression (validation AUC of 0.68)[23] or schizophrenia (validation AUC of 0.69).[25] Although being significant, scores of that level of prediction accuracy need to be improved to enable clinical application. Combination with other omics levels may help to achieve that goal.[23]

Recently, Roy et al.[4] published an RLS methylation score based on 49 CpG sites. Although it was derived from only 15 cases and 15 controls, this score achieved an AUC of 87.5% in a validation set of 20 blood samples and of 73.4% in neural tissue. 39 of the 49 CpG sites could be retrieved in our blood DNA methylation dataset after quality control. Only four sites passed the nominal significance threshold of 0.05 and after Bonferroni correction for 39 tests none were significant. Besides differences in discovery sample size (15/15 vs. 450/434) and in the method of risk score construction, Roy et al.[4] may have addressed specifically iron-deficient RLS since their score also identified iron-deficient anemia with an AUC of 83% whereas the present study addressed idiopathic RLS in a large heterogeneous set of individuals (n = 2283).

RLS is an age-related disease. Therefore, we also studied the epigenetic age in our RLS patients. Epigenetic age has been proven to be valuable as a biomarker of biological aging and for prediction of morbidity and mortality.[37] Associations with markers of physical and mental fitness, and with age-accelerating effects of diet/obesity, infections, and diseases have been identified.[38]

Using Horvath's multi-tissue clock[14] we did not find any evidence of advanced epigenetic age in blood or brain samples of RLS patients. Horvarth's multi-tissue clock may not be optimally suited for assessing brain aging, however. Therefore, we applied Shireby's cortical clock[15] which has been trained on brain tissues and found to indicate advanced epigenetic aging in cortical tissue of brain samples with neurodegeneration[21] while other clocks have not been proven to detect dementia and cognitive aging sufficiently.[39] Shireby's cortical clock also did not show advanced epigenetic aging in RLS. Indeed, there also is no other evidence for an increased risk of RLS patients to develop neurodegenerative diseases.[40-42] Thus, in line with the GWAS and EWAS results, the epigenetic age analysis also underlines the concept that RLS is rather a neurodevelopmental disorder than a neurodegenerative disease.

Two relevant limitations of our study need to be considered. The design is cross-sectional and thus susceptible to bias which we cannot entirely exclude although we focused on idiopathic RLS and matched cases and controls for age, sex, and ancestry. Once population cohorts such as the UK Biobank and the German NAKO provide a sufficient number of samples with both RLS phenotypes and epigenome-wide methylation data, RLS EWAS should be conducted in these datasets. Moreover, our major results originated in the EWAS on blood DNA samples although blood most likely is not the tissue of origin of RLS. They did not replicate in the brain samples available to us, but the number of brain samples was small and they were selected from regions which may not be relevant for RLS. Nevertheless, we consider our blood results valuable. As other groups who work on the epigenetics of brain diseases such as schizophrenia[24,25] or depression,[23] we were able to derive functional epigenetic risk scores from the blood data. Blood is the tissue of choice for clinical diagnosis and prediction while brain will never be used for this purpose. Moreover, genetic causes of neurological disease may leave epigenetic traces in blood cell DNA as has been shown for various monogenic neurodevelopmental disorders.[43]

In summary, our EWAS and epigenetic aging results support the notion of altered neurodevelopment but not neurodegeneration playing a role in RLS. The epigenetic risk scores derived in blood were reliably associated with RLS but require even higher accuracy to be useful as biomarkers. ●

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Data Availability Statement

Data available on request due to privacy/ethical restrictions.

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.