ORIGINAL RESEARCH

Transcriptomic Changes and Pathological Mechanisms in Familial and Sporadic Idiopathic Restless Legs Syndrome: Implications for Inflammation and Cell Adhesion Molecules

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Purpose: Individuals affected by restless legs syndrome (RLS) tend to have familial predispositions without fully explained by genetic variants, and transcriptomic analysis may help elucidate the pathogenic mechanisms of RLS. The study aims to investigate transcriptomic changes and underlying pathological mechanisms in familial and sporadic idiopathic RLS to uncover potential contributors to its pathogenesis.

Patients and Methods: This study included 37 RLS patients, 39 unrelated healthy controls and 19 healthy relatives of RLS patients with a positive family history. Messenger RNA (mRNA) extracted from the peripheral blood mononuclear cells of these participants was analyzed via next-generation sequencing, followed by GO and KEGG pathway analyses. Differentially expressed mRNAs were validated by RT-qPCR in a subset of patients and controls. The relationships between the expression levels and clinical indices were evaluated via correlation analysis.

Results: After comparing with unrelated healthy controls and excluding genes with similar expression patterns in familial healthy controls, we identified nine upregulated and 28 downregulated mRNAs specifically in RLS patients. GO enrichment analysis indicated that these mRNAs are involved in protein binding and catalytic activity. KEGG analysis revealed that inflammation-related signaling pathways and cell adhesion molecules (CAMs) may be associated with RLS. Three specific mRNAs, including SPARCL1, CCL8 and SELE, demonstrated notably downregulated expression in RLS patients and were subsequently validated in a subset of 10 patients and 19 healthy controls.

Conclusion: This study revealed differentially expressed SPARCL1, CCL8 and SELE in RLS patients, indicating the potential involvement of inflammatory pathways and CAMs in RLS pathogenesis. These findings further support the association between RLS, inflammation, and synaptic transmission, providing insights into potential diagnostic and therapeutic strategies targeting these pathways.

Keywords: restless legs syndrome, peripheral blood mononuclear cells, bioinformatics, transcriptomics, inflammation, message-RNA sequencing

Introduction

Restless leg syndrome (RLS) is a neurological condition characterized by an uncontrollable urge to move the legs, typically accompanied by discomfort and unpleasant sensations in the lower extremities.[1] The severity of symptoms typically follows a circadian pattern, worsening in the evening or at night and improving with movement of the lower limbs.[1–4] Although peripheral iron metabolism disorders are common, only 10–20% of RLS patients present with

decreased serum ferritin levels.[5] Therapeutic options for RLS include iron supplements, dopamine agonists, calcium channel ligands and opioids.[6,7] Notably, dopamine agonists can worsen the condition over time, a phenomenon known as augmentation, characterized by a gradual exacerbation of symptoms beyond pretreatment levels and the loss of the circadian pattern typically observed in RLS.[8-10] Dopaminergic dysfunction and brain iron deficiency (BID) are widely believed to play key roles in the pathophysiology of RLS,[11] along with the involvement of adenosinergic, GABAergic, opioid and glutamatergic neurotransmission systems.[12] Additionally, inflammatory mechanisms have been implicated in RLS,[13] as evidenced by elevated levels of inflammatory cytokines[14] and changes in oxidative stress.[15] However, the precise mechanisms remain to be further validated. Elucidating these pathways remains crucial for developing more effective therapies, particularly given RLS's significant impact on sleep quality and quality of life.

Alterations in dopaminergic signaling are considered one of the primary pathological mechanisms of RLS, evidenced by symptom improvement with dopamine D2/3 receptor (D2/3R) agonists,[16] and worsening with dopaminergic antagonists.[17,18] However, the simplistic view of reduced DAergic function in RLS does not fully explain the circadian rhythm of RLS.[19] Genetic studies have revealed significant associations between RLS and cell adhesion molecules (CAMs). Linkage studies in RLS families have identified at least eight susceptibility loci,[20] and genome-wide association studies (GWASs) have so far identified 21 susceptibility genes.[20-22] Among these genes, the myeloid ecotropic viral integration site one homolog (MEIS1), BTB domain containing 9 (BTBD9), and protein tyrosine phosphatase receptor type delta (PTPRD) genes are linked to dopaminergic transmission and iron metabolism.[23] Notably, PTPRD[24] may regulate synaptic states by mediating microcellular connectivity in dopaminergic neurons. Thus, revealing the circuits and synaptic connections involving CAMs is crucial. Over 60% of RLS patients report a positive family history, underscoring the significant role that genetic factors play in the etiology of this condition.[25] Having a first-degree relative increases the risk of developing RLS by 6–7 times.[25] Additionally, pathway analysis from a recent GWAS meta-analysis suggested a potentially pathological role for neurodevelopmental processes,[22] which are essential for properly forming and maintaining functional neural circuits in the nervous system. Neurophysiological studies implicate that the dysfunction of complex cortico-subcortical networks may underlie RLS, highlighting the presence of hyperactive excitatory mechanisms and impaired inhibitory control in RLS.[26,27] Such excitability alterations have been associated with altered sensory processing in RLS. Elena et al[28] reported increased excitability in the primary somatosensory cortex (S1) and impaired somatosensory gating, which play a critical role in aberrant sensory information processing. Supporting this, deficits in cortical plasticity and sensorimotor integration have also been observed in RLS patients.[29]

Chronic inflammatory states and oxidative stress may contribute to the pathophysiology of RLS.[30] Some studies have evaluated peripheral inflammatory markers in RLS patients and healthy controls[31-34] and identified inflammatory cytokines and immune markers as potential biomarkers.[15] Although proteomic studies have reported upregulation or downregulation of proteins involved in inflammatory processes, these findings should be considered preliminary.[15] The potential role of inflammatory factors in RLS pathogenesis or their value as biomarkers remains to be determined.

Next-generation sequencing offers a powerful high-throughput approach to investigate transcriptomic alterations and associated signaling pathways in RLS patients. Several previous transcriptomic studies have provided valuable insights into RLS genetics and pathophysiology.[33,35] Therefore, we hypothesize that differential mRNA expression patterns and associated signaling pathways in RLS patients, independent of familial genetic aggregation, may play a role in the pathogenesis of this disease. This could provide clues for new diagnostic and therapeutic approaches.

Materials and Methods
Subjects
From September 2022 to September 2023, we consecutively enrolled a total of 95 participants, including 37 RLS patients, 39 unrelated healthy controls (HCs) and 19 healthy relatives of RLS patients with a positive family history (familial healthy controls, fHC) from Shanghai General Hospital affiliated to Shanghai Jiao Tong University School of Medicine.

37 RLS patients were diagnosed by movement disorder specialists using International RLS Study Group (IRLSSG) criteria,[36] exhibiting characteristic symptoms (urge to move/worsening at rest with lower-limb sensory disturbances

relieved by movement), with an average disease duration of 13.0 years. As in our previous study,[4] we conducted face–to–face interviews to collect the demographic information, disease duration, symptoms, medications, and findings from general neurological and medical examinations. Exclusion criteria included: (1) sleep disorders other than RLS; (2) psychiatric disorders; (3) neurological complications; or (4) use of any medications that could alter the circadian rhythm within three weeks before the study. All patients had no RLS comorbidities and were not taking dopaminergic or α2δ-ligand drugs.

Healthy controls were age-matched, medication-free, and had no history of sleep, physical, neurological, or psychiatric disorders, and primarily were the spouses of patients. 19 fHCs were used as a separate comparison group to explore potential familial or genetic contributions and excluded from the differential expression analysis between RLS patients and HCs. Detailed information on these RLS families is shown in Figure S1.

Peripheral iron status was evaluated by obtaining clinical laboratory data on TfR, ferritin, transferrin saturation (TSAT), and serum iron levels. Written informed consent was obtained from all participants, including those in the pedigree tree. This study was conducted under the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Shanghai General Hospital affiliated to Shanghai Jiao Tong University School of Medicine (Ethics number: 2017–176).

Isolation of Cells, Total RNA Extraction and Quality Control

Total RNA extraction process included blood sample collection after assessments, PBMC isolation within 30 minutes, total RNA extraction and RNA storage. Venous blood was collected in the morning after all clinical assessments after a 12-hour fasting period. Blood samples were drawn into EDTA anticoagulant tubes and promptly transported on ice to the laboratory within 30 minutes to preserve cell viability. PBMCs were isolated via density gradient centrifugation via Ficoll–Paque (GE Healthcare, USA), and the intermediate layer was extracted. Red blood cell lysis (Sigma–Aldrich, USA) buffer was used at room temperature for five minutes, and the mixture was centrifuged at 250 ×g and 4°C for 5 min to remove any residual erythrocytes in the cell pellet. TRIzol reagent and DNase (Cat R401-01, Vazyme, Nanjing, China) were used to extract total RNA and ensure purity and accurate quantification according to the manufacturer's protocol. The same procedures were followed for sample collection, storage, extraction and purification. RNA extraction for PBMC samples in the same group was performed simultaneously and completed within 7 days to ensure meaningful transcriptomic changes. The RNA was stored at −80°C, and its quality was evaluated via a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA). Only samples with a 260/280 absorbance ratio between 1.8 and 2 were considered acceptable for further analysis.

Sequencing Library Construction

MRNA was extracted from total RNA for each group within 7 days using VAHTS® mRNA Capture Beads (N401-02, Human, Vazyme). Double-stranded cDNA was synthesized from purified polyadenylated mRNA templates using the PrimeScript™ Double Strand cDNA Synthesis Kit (TaKaRa), following the manufacturer's protocol. cDNA libraries for Illumina sequencing were prepared using the VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina® (NR604-02, Vazyme). The library concentration was quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA), and library fragment distribution was assessed with an Agilent 4200 TapeStation (Agilent Technologies, China).

Transcriptomic and Functional Enrichment Analysis

Before sequencing, the concentration and size distribution of the cDNA library were assessed using a Qubit 2.0 fluorometer (Thermo Fisher Scientific) and an Agilent 4200 TapeStation (Agilent Technologies) to ensure RNA integrity (RIN ≥ 9). Sequencing was then performed on an Illumina NovaSeq 6000 platform, generating raw image files that were converted into reads. To improve data quality, low-quality reads were filtered out using Seqtk (v1.0) based on the following criteria: (1) removal of adapter sequences, (2) trimming of bases with a quality score below 20 at the 3′ end, (3) exclusion of reads shorter than 25 nucleotides, and (4) removal of ribosomal RNA reads, resulting in a set of clean reads. The spliced mapping algorithm of Hisat2 (v2.0.4) was applied to perform Genome mapping on the preprocessed reads. To ensure comparability of gene expression levels across different genes and samples, read counts were normalized to

fragments per kilobase of transcript per million mapped reads (FPKM) values, following the method described in Mapping and quantifying mammalian transcriptomes by RNA-Seq. Gene fragments were counted using StringTie (v1.3.0) after alignment with HISAT2, and normalization was performed using the trimmed mean of M values (TMM) method, as described in A scaling normalization method for differential expression analysis of RNA-seq data. Sample relationships were assessed through correlation analysis and principal component analysis (PCA), with batch effects corrected accordingly. Differential gene expression analysis was conducted using edgeR (v3.2.0), with age and sex included as covariates. Q value was corrected after false discovery rate (FDR) correction applied to control the P-value threshold, and fold-change values were calculated to identify differentially expressed genes. Differentially expressed mRNAs were defined as those identified with a q value ≤ 0.05 and a fold change (FC) ≥ 1.5. The average level of mRNAs was calculated via the average expression function. The selected differentially expressed genes were mapped to GO and KEGG terms. Terms with a q-value ≤ 0.05 after multiple hypothesis correction (FDR correction) were considered significantly enriched. GO enrichment analysis, KEGG enrichment analysis, and visualization were performed via the ClusterProfiler (v4.0.0) R package.

Preparation of cDNA and Real-Time Quantitative PCR Analysis

cDNA synthesis was conducted with 200 ng of total RNA via a TB Green Premix Ex Taq II (Tli RNaseH Plus) Kit (Cat RR820B, TaKaRa, Dalian, China) following the manufacturer's protocol. Real-time quantitative PCR (RT-qPCR) was conducted via the LightCycler® 480 System (Roche, Basel, Switzerland) to assess the mRNA expression levels. Three technical and biological replicates were performed to verify PCR efficiency (90–110%), and melting curve analysis was checked for non-specific amplification. The primer sequences were as follows: for SPARC-like protein 1 (SPARCL1), forward: 5′- ACGGTAGCACCTGACAACAC-3′ and reverse: 5′- ATGGTGGGAATCGTCTTCTGT-3; for proinflammatory C–C motif chemokine ligand 2 (CCL2), forward: 5′- CAGCCAGATGCAATCAATGCC-3′ and reverse: 5′- TGGAATCCTGAACCCACTTCT-3′; for proinflammatory C–C motif chemokine ligand 8 (CCL8), forward: 5′- TGGAGAGCTACACAAGAATCACC-3′ and reverse: 5′- TGGTCCAGATGCTTCATGGAA-3′; for endothelial adhesion molecule 1 (SELE), forward: 5′- CAGCAAAGGTACACACACCTG-3′ and reverse: 5′- CAGACCCACACATTGTTGACTT-3′; for Homo sapiens apolipoprotein A-II (APOA2), forward: 5′- CTGTGCTACTCCTCACCATCT-3′ and reverse: 5′- CTCTCCACACATGGCTCCTTT-3′. Target gene expression levels were calculated compared to housekeeping gene 18S rRNA (forward: 5′-GCAATTATTCCCCATGAACG-3′ and reverse: 5′-GGGACTTAATCAACGCAAGC-3′). Relative mRNA expression levels were described using the $2 - \Delta\Delta Ct$ method ($\Delta Ct = Ct_{target} - Ct_{reference}$, $-\Delta\Delta Ct = sample\ \Delta CT - 18S\ \Delta CT$).

Statistical Analysis

Quantitative variables are described as either the median with interquartile range (25th–75th percentiles) or the mean ± standard deviation (SD). Group comparisons were performed using Welch's *t*-test or Mann–Whitney *U*-test. For RNA sequencing data, differential gene expression analysis was performed using edgeR, and p-values were adjusted by FDR correction (q-values). Genes with q < 0.05 and fold-change (FPKM-based) were considered significant. Spearman correlation corrected for age was used to test the association between levels of mRNA levels and clinical index. Statistical significance was defined as *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.001$; ns, not significant. SPSS version 20.0 (IBM Corporation, USA) was utilized to conduct statistical analyses, and Prism software (version 8.0; GraphPad Software, USA) was used for data visualization.

Result
Clinical Characteristics of the Participants

During the screening phase, we recruited 37 RLS patients, 39 matched HCs, and 19 familial healthy controls (fHCs) to minimize the impact of familial genetic susceptibility (Figure S1). In the validation phase, a subset of 10 RLS patients and 19 HCs were included and matched by sex and age. The demographic and disease characteristics of all participants are summarized in Table 1. Notably, serum iron levels were significantly lower in RLS patients than in HCs ($P = 0.0198$).

Table 1 Demographic and Clinical Characteristics of Participants

Phase	Demographic and Clinical Characteristics	RLS	fHC	HC	P value (RLS vs HC)
Screening	n	37	19	39	
	Age (years, mean ± SD)	52.24 ± 16.04	37.11 ± 14.58	57.56 ± 16.29	0.1336*
	Sex (male/female)	1.62	1.42	1.56	0.6155#
	Disease duration (years, M (P25, P75))	13.00 (4.75, 23.00)	/	/	/
	IRLSSG (mean ± SD)	16.27 ± 8.61	/	/	/
	TfR (g/L, M (P25, P75))	2.30 (2.00, 2.90)	2.4 (2.23, 2.73)	2.5 (2.25, 2.70)	0.7360*
	Ferritin (ug/L, M (P25, P75))	101.90 (23.70, 217.60)	216.90 (103.20, 441.50)	117.90 (74.65, 269.90)	0.1889*
	TSAT (%, M (P25, P75))	29.30% (19.90%, 38.00%)	39.30% (22.03%, 59.43%)	35.40% (27.20%, 40.30%)	0.3556*
	Iron (umol/L, mean ± SD)	15.61 ± 7.10	24.40 ± 13.93	21.35 ± 9.54	0.0198*
Phase	**Demographic and Clinical Characteristics**	**RLS**		**HC**	**P value**
Validation	n	10		19	RLS vs HC 0.5355*
	Age (years, mean ± SD)	42.80 ± 16.03		46.32 ± 9.50	0.8988#
	Sex (male/female)	1.50		1.47	
	Disease duration (years, M (P25, P75))	17.00 (4.75, 22.50)		/	/
	IRLSSG (mean ± SD)	14.50 ± 8.61		/	/

Notes: *P values for continuous variables were derived from Welch's *t* test or Mann–Whitney *U*-test between the RLS and HC groups. #P values for categorical variables were derived from Fisher's exact test.
Abbreviations: RLS, restless legs syndrome; fHC, healthy relatives of RLS patients with a family history; HC, healthy controls; IRLSS, International RLS Study Group Severity; TfR, transferrin receptor; TSAT, transferrin saturation; SD, standard deviation.
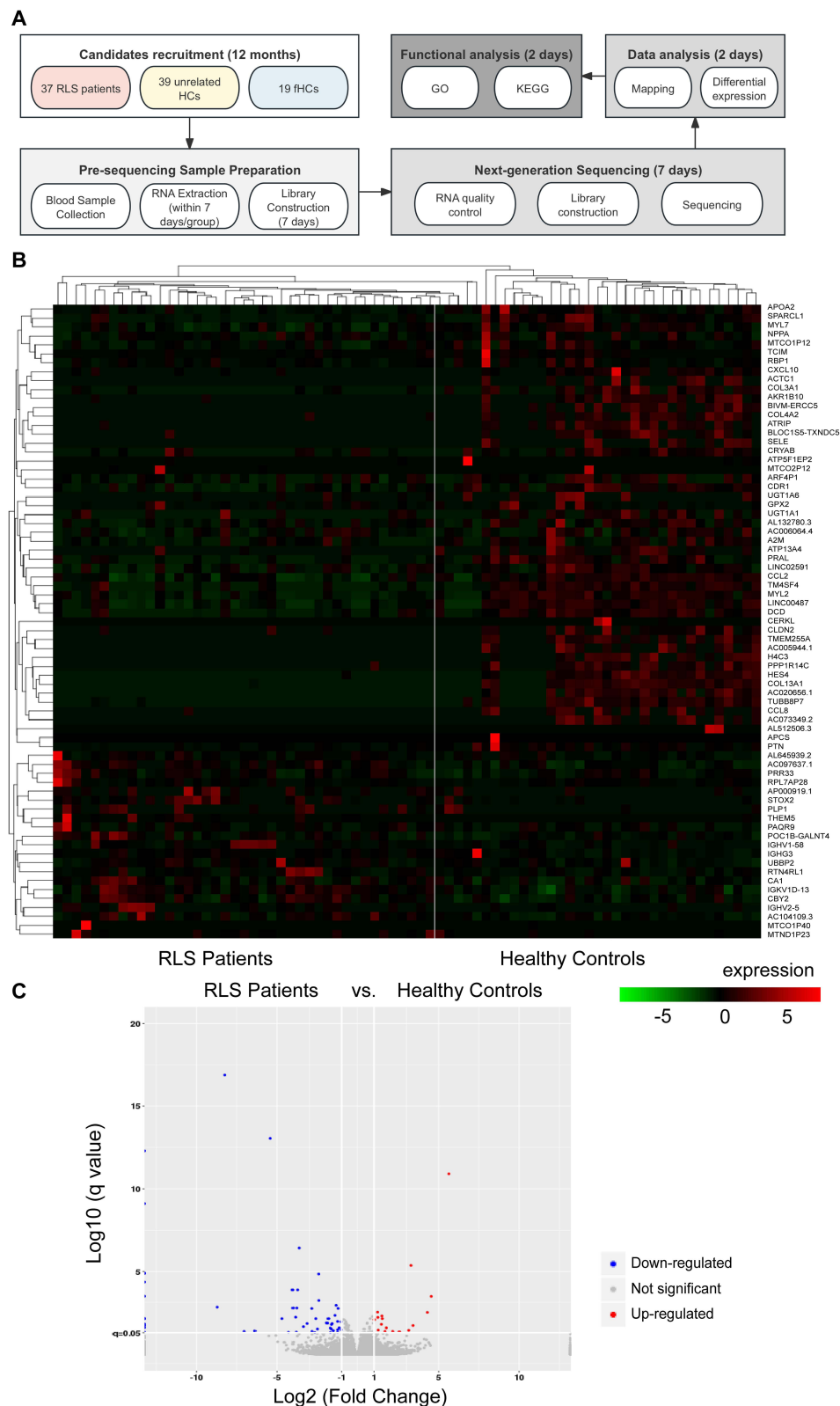
mRNA Expression Profiles of RLS Patients and Healthy Controls

The research design is shown in Figure 1A. Hierarchical cluster analysis performed using the PBMC mRNA results revealed significant differences in mRNA expression between RLS patients and HCs (Figure 1B). In total, 71 differentially expressed mRNAs were identified, with 18 significantly elevated and 53 significantly reduced in RLS patients (q value < 0.05) (Figure 1C; the detailed information is listed in Table S1). Among them, MTND1P23 exhibited the highest upregulation with a fold change of 5.6, while genes such as APOA2 and SPARCL1 showed the most significant downregulation (each with a fold change below −5) (Table S1).

## Functional and Pathway Enrichment Analysis of Differentially Expressed mRNAs

To elucidate the biological roles of the differentially expressed mRNAs, GO and KEGG analyses were performed on 71 genes (q value ≤ 0.05 and |FC| ≥ 1.25) (Figure 2A and B). GO enrichment analysis indicated that these mRNAs were associated with various molecular functions, biological processes, and cellular components (Tables S2–S4). Notably, upregulated genes such as MTCO1P40, carbonic anhydrase 1 (CA1), CBY2, and ubiquitin-associated protein 2 (UBBP2) were found to be associated with the following molecular functions: binding, protein binding and catalytic activity. Among the downregulated genes, APOA2 and SPARCL1 were involved in binding, protein binding, and ion binding. KEGG analysis identified 22 signaling pathways, with the q value threshold set at 0.05, among which the interleukin 17 (IL-17) signaling pathway, tumor necrosis factor (TNF) signaling pathway and CAMs were the most prominent. The differentially expressed mRNAs involved in each pathway, along with the distribution of these pathways across various processes, functions, or organs, are presented in Table S5. Additionally, functional enrichment analysis was performed separately for upregulated and downregulated genes. GO analysis revealed that upregulated genes were primarily enriched in immune response pathways (Figure 2C), while downregulated genes were mainly associated with the regulation of leukocyte migration and humoral immune response (Figure 2D). KEGG analysis further showed that downregulated genes were enriched in cytoskeleton-related pathways in muscle cells (Figure 2E and F).

Figure 1 Transcriptomic analysis of RLS patients and healthy controls. (**A**) Study design and timeline. (**B**) Heatmap illustrating the differential expression levels of mRNAs identified in restless legs syndrome (RLS) patients and healthy controls (HCs). The X-axis represents the patient and control groups from the cluster analysis, whereas the Y-axis lists the 71 differentially expressed mRNAs. Red and green indicate upregulation and downregulation, respectively. (**C**) Volcano plot depicting the differences in mRNA expression between patients and HCs. The X-axis represents the log2 (fold change) values, whereas the Y-axis represents the -log10 (q value) values. The gray dots indicate mRNAs without significant expression differences, the red dots represent mRNAs with upregulated expression, and the blue dots indicate mRNAs with downregulated expression. Differentially expressed genes were identified using edgeR, with a false discovery rate (FDR)-adjusted q value ≤ 0.05 and an absolute log2 fold change ($|log_2FC|$) ≥ 1.5.

Abbreviations: RLS, restless legs syndrome; HC, healthy control; FDR, false discovery rate.
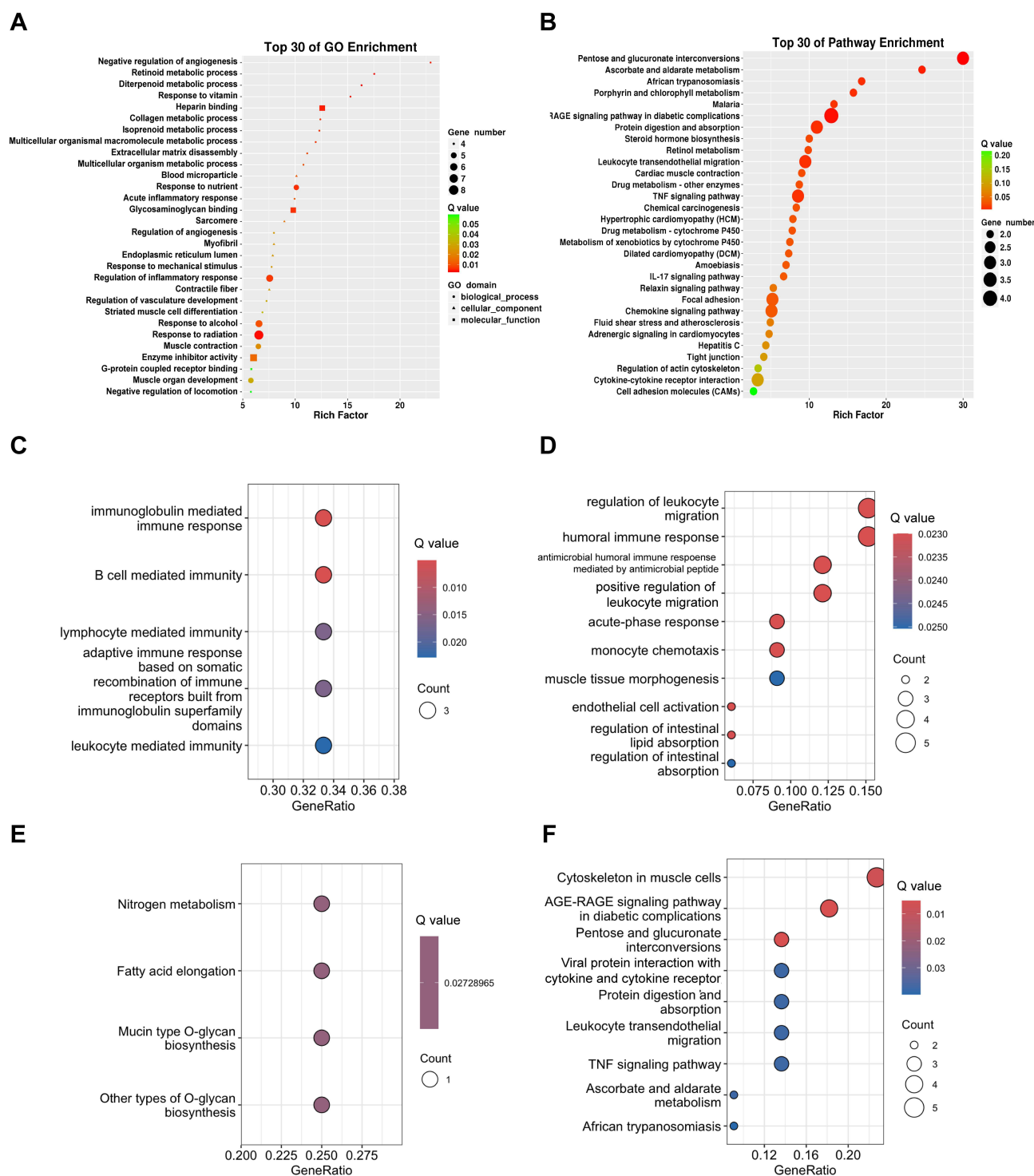
Figure 2 GO and KEGG analysis of differentially expressed mRNAs in RLS patients. (**A**) Bubble plot illustrating selected enriched GO terms. The color gradient represents q values, with red indicating smaller q values. Larger bubbles correspond to a greater number of genes involved in each pathway. Different shapes represent molecular functions (MF), biological processes (BP), and cellular components (CC). (**B**) Bubble plot displaying selected enriched KEGG pathways. The color gradient reflects the q values, with red signifying smaller q values. Larger bubbles indicate that more genes are involved in each pathway. (**C**) GO analyses of upregulated genes. (**D**) GO analyses of downregulated genes. (**E**)KEGG analyses of upregulated genes. (**F**) KEGG analyses of downregulated genes. For (**C–F**), the bubble size and color gradient follow the same conventions as in (**A** and **B**). GO and KEGG pathway enrichment analyses were performed based on differentially expressed genes (DEGs). P-values were corrected for multiple hypothesis testing using the false discovery rate (FDR) method, with a significance threshold set at q-value ≤ 0.05. GO terms and KEGG pathways meeting this criterion were considered significantly enriched.

Abbreviations: RLS, restless legs syndrome; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular function; BP, biological process; CC, cellular component; DEG, differentially expressed gene; FDR, false discovery rate.

mRNA Expression Profiles of Related Familial Healthy Controls

To further investigate the distinct differences in mRNA expression in RLS patients, we compared the mRNA expression profiles of 19 fHC and 39 HCs. In the fHC group, we identified 247 mRNAs with upregulated expression (q < 0.05) and 894 with downregulated expression (q < 0.05) (additional data are listed in Table S6). To minimize the influence of familial genetic aggregation, we filtered out those mRNAs with similar up- or downregulation trends in both the RLS patient and fHC groups. Among them, nine mRNAs were consistently upregulated, and 25 were downregulated in both RLS patients and fHCs (Table S7). Notably, PTN exhibited an increasing trend across the RLS, fHCs, and HCs groups in the sequencing data (Figure S2). The specific and shared DEGs were demonstrated in Figure 3A and B. This process allowed us to focus on the mRNAs demonstrating uniquely upregulated (9 mRNAs) or downregulated (28 mRNAs) expression in RLS patients (q < 0.05, Table 2).

A   **Upregulated DEGs**

9   9   238

RLS Patients   fHC

B   **Downregulated DEGs**

28   25   869
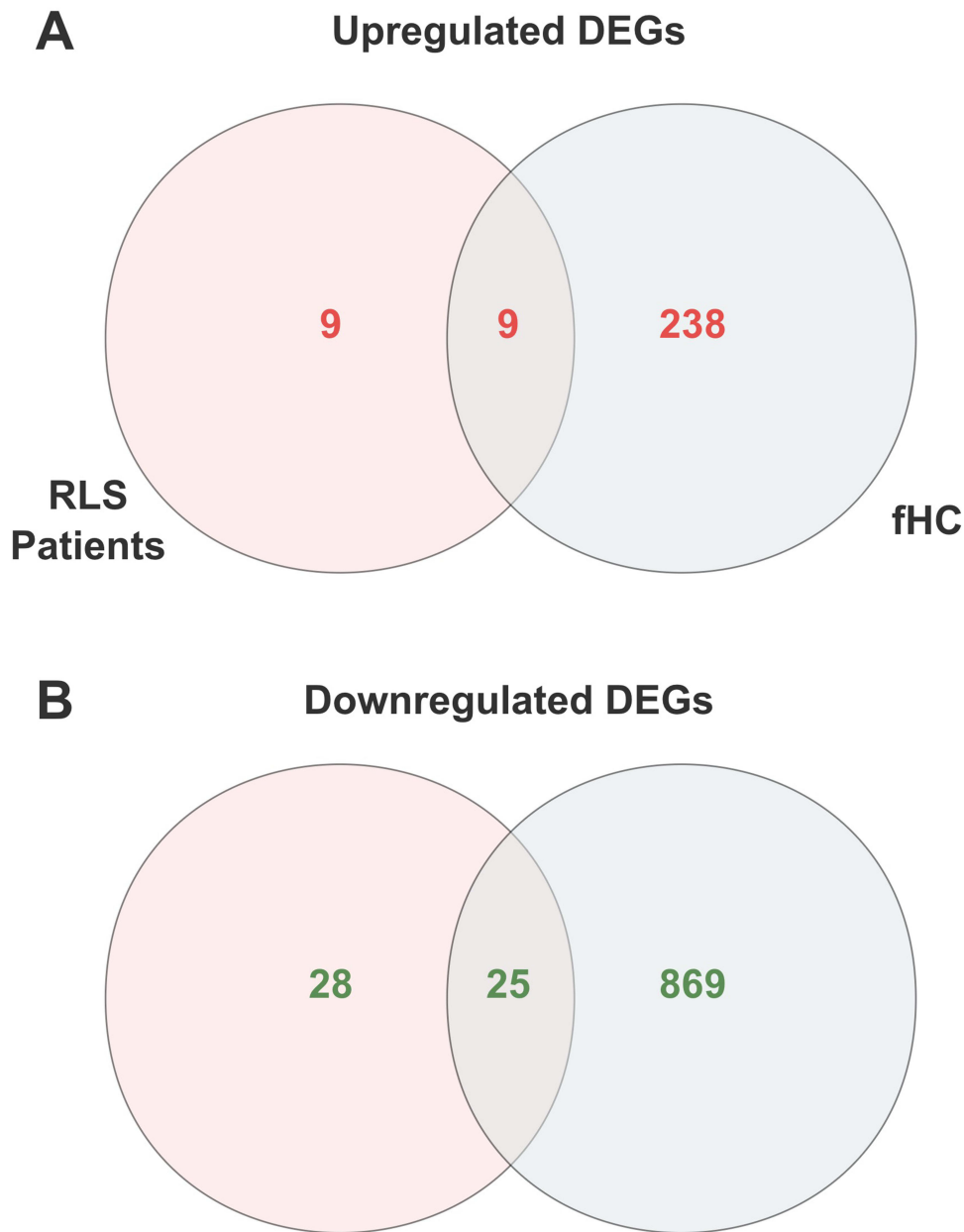
RLS Patients   fHC

Figure 3 Venn diagram of specific and shared DEGs between RLS patient and fHC. (**A**) Upregulated DEGs in RLS patients and fHCs. The numbers indicate unique and shared DEGs between the two groups. (**B**) Downregulated DEGs in RLS patients and fHCs. The numbers in each section represent the DEGs that are specific to RLS patients, specific to fHCs, or shared between the two groups. Statistical significance determined by an adjusted q-value ≤ 0.05 and |log$_2$FC| ≥ 1.
Abbreviations: DEGs, differentially expressed genes; RLS, restless legs syndrome; fHC, familial healthy controls.

Table 2 37 mRNAs Specifically Differentially Expressed in RLS Patients Compared to Both fHCs and HCs

Gene	Locus	Log2 (FoldChange)	Q value	Regulation
MTCO1P40	17:53105734–53106358:+	3.2903	< 0.0001	Up-regulated
CBY2	13:45702311–45714559:+	4.3303	0.0030	Up-regulated
CA1	8:85327608-85379014:−	1.2411	0.0060	Up-regulated
UBBP2	1:217850403–217850633: −	1.0023	0.0081	Up-regulated
IGHV1-58	14:106622357–106622855: −	1.4672	0.0156	Up-regulated
PAQR9	3:142949164–142963682: −	1.7811	0.0255	Up-regulated
THEM5	1:151847263–51853697: −	1.2677	0.0350	Up-regulated
PLP1	X:103773718–103792619:+	3.1508	0.0362	Up-regulated
AP000919.1	18:2948238–2960756:+	1.6931	0.0420	Up-regulated
APOA2	1:161222292–161223631: −	−8.2595	< 0.0001	Down-regulated
SPARCL1	4:87473335–87531061: −	−5.4299	< 0.0001	Down-regulated
MYL7	7:44138864–44141332: −	< −0.00001	< 0.0001	Down-regulated
TCIM	8:40153455–40155308:+	< −0.00001	< 0.0001	Down-regulated
RBP1	3:139517434–139539829: −	< −0.00001	< 0.0001	Down-regulated
AKR1B10	7:134527592–134541408:+	−4.0474	0.0001	Down-regulated
COL4A2	13:110305812–110513027:+	−2.3986	0.0006	Down-regulated
ATRIP	3:48446710–48467645:+	−1.3512	0.0011	Down-regulated
BLOC1S5-TXNDC5	6:7881522–8064364: −	−8.6949	0.0014	Down-regulated
SELE	1:169722641–169764705: −	−4.0126	0.0016	Down-regulated
CDR1	X:140782405–140784871: −	−3.8659	0.0058	Down-regulated
GPX2	14:64939152–64942905: −	−2.5887	0.0071	Down-regulated
UGT1A1	2:233760248–233773299:+	−4.6918	0.0071	Down-regulated
ATP13A4	3:193402077–193593111: −	−1.0353	0.0110	Down-regulated
CCL2	17:34255218–34257203:+	−1.6062	0.0140	Down-regulated
TM4SF4	3:149473974–149503281:+	−3.1420	0.0140	Down-regulated
MYL2	12:110910819–110920722: −	< −0.00001	0.0150	Down-regulated
CLDN2	X:106900164–106930861:+	< −0.00001	0.0247	Down-regulated
TMEM255A	X:120258650–120311556: −	−1.1588	0.0255	Down-regulated
H4C3	6:26103876–26104310:+	−2.4880	0.0301	Down-regulated
HES4	1:998962–1000172: −	−1.0266	0.0334	Down-regulated
COL13A1	10:69801931–69964275:+	−1.2595	0.0334	Down-regulated
TUBB8P7	16:90093154–90096354:+	−6.3741	0.0394	Down-regulated
CCL8	17:34319036–34321402:+	−2.8319	0.0420	Down-regulated
AC073349.2	7:64835280–64836882: −	−7.0378	0.0420	Down-regulated
AL512506.3	13:43883800–44028526:+	−3.8228	0.0460	Down-regulated
APCS	1:159587825–159588865:+	< −0.00001	0.0460	Down-regulated
AC097637.1	3:52221081–52231190: −	−2.6566	0.0486	Down-regulated

Notes: P values were adjusted for multiple comparisons using the false discovery rate (FDR) correction via the Benjamini-Hochberg method. The adjusted P values are reported as q values.

Validation of Candidate mRNAs Screened by RT-qPCR

We selected SPARCL1, CCL2, CCL8, SELE and APOA2 from the 37 significantly downregulated mRNAs in RLS patients. The expression levels of these genes were validated in a subset of 10 RLS patients and 19 HCs via RT-qPCR (Table 3). The results confirmed significantly lower levels of SPARCL1, CCL8 and SELE in RLS patients compared to HCs (Figure 4A, C and D), while CCL2 and APOA2 showed no significant differences (Figure 4B and E).

Table 3 Differences in the Expression of the 4 Candidate mRNAs in the Validation Cohort (N = 29)

mRNA	RLS	HC	*P* value
SPARCL1	0.29 (0.26, 0.48)	1.09 (0.86, 1.37)	<0.0001****
CCL2	1.01 (0.83, 1.61)	0.93 (0.81, 1.5)	0.7503
CCL8	0.84 (0.76, 1.01)	1.03 (0.90, 1.23)	0.0068**
SELE	0.41 (0.40, 0.84)	0.98 (0.70, 1.25)	0.0056**
APOA2	0.13 (0.08, 1.66)	1.67 (0.47, 2.42)	0.2038

Notes: Data are presented as the median and 25th and 75th percentiles. P values are derived from Welch's *t* test between the RLS and HC groups. **$P < 0.01$; ****$P < 0.0001$.
Abbreviations: RLS, restless legs syndrome; HC, healthy controls; SPARCL1, SPARC-like protein 1; CCL2, proinflammatory C–C motif chemokine ligand 2; CCL8, proinflammatory C–C motif chemokine ligand 8; SELE, endothelial adhesion molecule 1; APOA2, Homo sapiens apolipoprotein A-II.

Relationships between Five Selected mRNAs and Clinical Indices

Correlation analysis of five selected mRNAs, including SPARCL1, CCL2, CCL8, APOA2 and SELE in patients, as presented in Table 4, revealed no significant associations between their downregulated expression and RLS severity, as assessed by IRLSS or peripheral iron deficiency-related laboratory parameters (TfR, ferritin, TSAT, and iron).
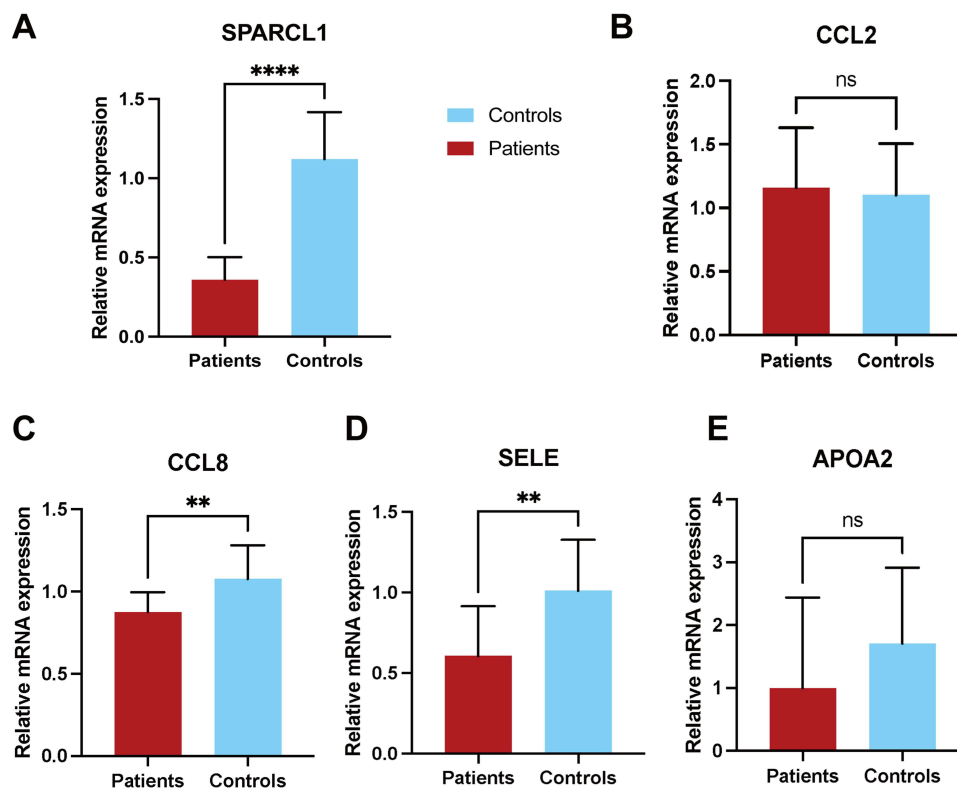
Figure 4 Validation of differential expression of five candidate mRNAs in an independent cohort. (**A**)Relative expression levels of SPARCL1 were compared between RLS patients and HCs. (**B**) Relative expression levels of CCL2 were compared between RLS patients and HCs. (**C**) Relative expression levels of CCL8 were compared between RLS patients and HCs. (**D**) Relative expression levels of SELE were compared between RLS patients and HCs. (**E**) Relative expression levels of APOA2 were compared between RLS patients and HCs. Statistical significance was determined using an unpaired two-tailed *t*-test. Data are presented as mean ± standard deviation. **$P < 0.01$; ****$P < 0.0001$; ns, not significant.
Abbreviations: RLS, restless legs syndrome; HC, healthy controls; SPARCL1, SPARC-like protein 1; CCL2, proinflammatory C–C motif chemokine ligand 2; CCL8, proinflammatory C–C motif chemokine ligand 8; SELE, endothelial adhesion molecule 1; APOA2, Homo sapiens apolipoprotein A-II.

Table 4 Correlation Analysis of Four Candidate mRNAs with Clinical Indicators

Clinical index	SPARCL1		SELE		CCL2		CCL8		APOA2	
	r	*P#*	*r*	*P#*	*r*	*P#*	*r*	*P#*	*r*	*P#*
IRLSSG	−0.18	0.45	0.19	0.32	−0.08	0.67	−0.16	0.39	0.16	0.39
TfR	0.44	0.05	0.07	0.73	−0.25	0.21	−0.18	0.38	−0.04	0.85
FER-1	−0.36	0.12	−0.11	0.59	0.32	0.10	0.32	0.10	0.13	0.53
Iron	−0.08	0.73	−0.10	0.63	0.23	0.24	0.35	0.07	−0.18	0.38

Notes: Italicized r represents the Spearman correlation coefficient. #P values are derived from the Spearman rank correlation analysis.
Abbreviations: IRLSS, International RLS Study Group Severity; TfR, transferrin; FER-1, ferritin-1; SPARCL1, SPARC-like protein 1; CCL2, proinflammatory C–C motif chemokine ligand 2; CCL8, proinflammatory C–C motif chemokine ligand 8; SELE, endothelial adhesion molecule 1; APOA2, Homo sapiens apolipoprotein A-II.

Discussion

In this study, we investigated the transcriptomic characteristics of the mRNA expression profiles in RLS patients' PBMCs. A total of 71 candidate mRNAs were identified by comparing RLS patients and HCs. After excluding the influence of familial aggregation, we identified 9 upregulated and 28 downregulated mRNAs specific to RLS patients. RT-qPCR confirmed significant downregulation of SPARCL1, CCL8, and SELE in RLS PBMCs. GO and KEGG analyses indicated potential involvement of the IL-17 and TNF signaling pathways in the pathogenesis of RLS. While these findings suggest that inflammation and immunity may be important contributing factors to the pathophysiology of RLS, further research is needed to confirm these associations and elucidate the underlying mechanisms.

Among genes with a consistent upregulation trend between RLS patients and fHCs, PTN showed an increasing trend across RLS, fHCs, and HCs, after excluding pseudogenes and novel protein-encoding genes. PTN is known for its role in promoting neurite outgrowth.[37] Although this trend was not successfully validated, its dysregulation may still represent a potential genetic risk factor for RLS progression.

Inflammation and Immunity

Our research findings suggest that inflammatory mechanisms are involved in RLS, as indicated by the involvement of the TNF and IL-17 signaling pathways. This aligns with a previous transcriptomic study supporting the involvement of inflammatory network mechanisms and immune processes in RLS. That study identified IL-17, chemokines, and inflammatory mediators of TRP channels, further suggesting that these mediators may be associated with sensory symptoms in RLS patients.[33] Proteomic studies in RLS patients have revealed elevated levels of reactive oxygen species, activation of complement components,[15] and increased levels of inflammatory proteins,[14] further highlighting the role of inflammatory mechanisms. Previous studies have reported significantly higher levels of serum or plasma tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) in RLS patients than in controls, indicating systemic inflammatory changes.[38] Our findings reinforce the involvement of the TNF signaling pathway in the pathogenesis of RLS,[31,38,39] suggesting a potential possible link between RLS symptoms and comorbid psychosomatic disorders.[39] Interestingly, Trotti et al[40] reported that among 137 untreated RLS patients, those with frequent periodic limb movements in sleep (PLMS) were more likely to have elevated levels of hypersensitive C-reactive protein (hs-CRP), although the IL-6 and TNF-α levels were not significantly increased.

Increasing evidence indicates that an abnormal immune regulatory response contributes to RLS, as shown by its higher prevalence in multiple sclerosis (MS)[41] and rheumatoid arthritis (RA).[42] Additionally, an RNA transcriptomic study has implicated the role of inflammatory cytokines in Parkinson's disease (PD) pathogenesis, further supporting a potential immune-RLS link, given RLS is a common comorbidity in patients with PD.[43] We observed an enrichment of differentially expressed mRNAs in the IL-17 pathway in RLS patients, further emphasizing the contribution of immune mechanisms to the pathogenesis of RLS. Autopsy studies on RLS patients showed microvascular T-cell infiltration,[44,45] which may mediate the inflammatory infiltration of microvessels through mechanisms involving leukocyte transendothelial migration. Additionally, alterations in IL-17 may also be associated with RLS by affecting impaired dopamine

synthesis, leading to altered dopaminergic signaling.[46] We also detected the downregulation of CCL2 and CCL8 expression in RLS, which may play a role in the immunomodulatory function of sensory neurons. The anti-inflammatory molecule interleukin 10 (IL-10) has been reported to bind to receptors expressed on somatosensory neurons on the dorsal root ganglion (DRG)[47] and trigeminal ganglion (TG),[48] thereby downregulating CCL2.[49] Given that DRG neuron-derived CCL2 is involved in immune cell infiltration and activation,[50] the restriction of the ability of somatosensory neurons to respond to these alterations through immune modulation is reasonable. Our findings increase the understanding of neuroimmunity in regulating important sensory symptoms in RLS. In conclusion, our findings strengthen the evidence linking inflammation and immunity to RLS. However, the specific functions of inflammatory and immune factors in the pathogenesis and their potential as biomarkers require further investigation in future studies.

Neurotransmission and Synapse

Dopamine dysfunction in RLS is characterized by dysregulation, including increased presynaptic DA synthesis and release, decreased DA uptake, and reduced postsynaptic receptor density.[19] This alteration in the dynamic relationship between presynaptic and postsynaptic functions involves complex changes in multiple elements. One perspective is that differences in microscopic intercellular connectivity among dopaminergic neurons may explain these changes.[51] Pathway analysis of the differentially expressed mRNAs suggests that CAMs play a role. CAMs are described as potential candidates for playing a central role in determining synaptic connectivity and neural development.[52] PTPRD, a CAM, has been identified repeatedly associated with RLS in several GWAS.[53–55] Among these studies, single-nucleotide polymorphisms (SNPs) in PTPRD are associated with reduced mRNA expression.[56,57] Axonal PTPRD is involved in motor neuron axon termination and synapse formation during embryonic development[58] via postsynaptic ligand interactions.[59–65] PTPRD knockout mice exhibit impaired neuronal differentiation and cortical disruption, underscoring its role in neural connectivity.[66] Notably, PTPRD is highly expressed in dopaminergic neurons, suggesting its involvement in dopaminergic neural circuit remodeling. Although whether PTPRD mutations directly impair dopaminergic neurotransmission remains unclear, these findings support its critical role in the synaptic connectivity, potentially leading to altered susceptibility to RLS.[24,67] Dopaminergic network abnormalities in RLS patients and worsening symptoms with prolonged dopamine agonist use may be linked to CAM-mediated circuit dysfunction.[68] A detailed elaboration of CAM-regulated synaptic connections could clarify treatment-related complications and identify new therapeutic targets.

A novel finding in our study was the downregulation of SPARCL1 in RLS patients. SPARC, a matricellular protein (MCP), modulates tissue development and remodeling modulator by influencing key cellular processes such as cell adhesion and proliferation.[69] SPARCL1, expressed primarily in the central nervous system, is reported to be associated with the formation and maturation of excitatory synapses.[70,71] Specifically, SPARCL1 facilitates the development of synaptic N-methyl-d-aspartate (NMDA) receptors, which can be essential for synaptic connectivity.[72] Clinical evidence suggests the involvement of NMDA receptor-mediated alterations in glutamatergic neurotransmission in RLS pathogenesis. Noncompetitive NMDA receptor inhibitors, such as ketamine, have shown efficacy in alleviating RLS symptoms,[73] and another NMDA antagonist, antamethadone, has also proven effective.[74] Although a direct link between SPARCL1 and RLS has not yet been established, our results may suggest the potential role of SPARCL1 in NMDA receptor-mediated glutamatergic dysregulation in RLS. A recent optogenetic study in rats with BID demonstrated hypersensitivity in corticostriatal glutamatergic terminals.[75] This hyperglutamatergic state may be related to the hyperarousal often observed in RLS patients.[76] Given that BID is prevalent in the majority of RLS patients, our results could provide valuable insights into SPARCL1's role in synaptic adaptation and its potential contribution to the hyperarousal state in RLS.

Additionally, NMDA receptor-mediated glutamatergic neurotransmission may also be associated with maladaptive synaptic plasticity in the brain,[77,78] which could explain RLS-related sensory symptoms such as chronic pain. A study of neuropathic pain in SPARCL1 knockout mice suggested that SPARCL1-mediated enhancement of NMDA currents plays a key role in chronic pain maintenance.[75] In particular, sensory neurons expressing SPARCL1 have been identified in human sensory ganglia, indicating the participation of SPARCL1 in the transmission of nociceptive signals.[79] Therefore, one hypothesis is that SPARCL1 may modulate synaptic modifications to regulate the transsynaptic transmission of nociceptive signals, potentially alleviating the discomfort associated with sensory symptoms in RLS. The possible role of

SPARCL1 in sensory adaptations is a perspective that has not been previously addressed. However, given the scarcity of relevant studies, the role of SPARCL1 in RLS needs to be further investigated. These observations are clinically significant, as inhibitors targeting inflammatory pathways and synaptic regulation may be promising therapeutic options for RLS.

Although we implemented strict inclusion and exclusion criteria, certain conditions have not been extensively explored concerning RLS, such as nonanemic iron deficiency[80] and undiagnosed cardiovascular abnormalities.[81] Residual effects of prior medications (eg, immunosuppressants/antidepressants) may have influenced the observed gene expression variability, although none of the participants were actively taking dopaminergic or α2δ-ligand medications. While our findings provide transcriptional insights, limitations include the lack of protein-level validation, a relatively small sample size, and potential discrepancies between peripheral blood transcriptomics and CNS pathology. Expanding the cohort size and incorporating more comprehensive clinical assessments, such as sleep parameters, disease severity, and peripheral iron status, are needed to clarify potential correlations and enhance the observations of our findings.

Conclusion

To summarize, the pathophysiological mechanisms of RLS are intricate and multifaceted. Our bioinformatics analysis identified 37 differentially expressed mRNAs (9 upregulated, 28 downregulated) in RLS patients after accounting for familial genetic aggregation. Notably, we validated significant downregulation of SPARCL1, CCL8 and SELE, whereas no significant difference was observed in CCL2 and APOA2 levels. Correlation analysis did not reveal significant associations between the expression of these five mRNAs and RLS severity or peripheral iron status. Functional analyses implicated the IL-17 and TNF signaling pathways as well as CAMs in RLS pathogenesis, suggesting two potential therapeutic avenues: anti-inflammatory therapies and synaptic intervention targeting connectivity in RLS management. These findings reinforce the growing evidence for inflammatory and synaptic adhesion mechanisms in RLS while highlighting the need for functional validation of identified pathways, RLS subtyping based on transcriptomic profiles and development of precision medicine strategies.

Abbreviations

RLS, restless legs syndrome; mRNA, Messenger RNA; PBMCs, peripheral blood mononuclear cells; NGS, next-generation sequencing; KEGG, Kyoto Encyclopedia of Genes and Genomes; RT-qPCR, real-time quantitative polymerase chain reaction; CAMs, cell adhesion molecules; BID, brain iron deficiency; PTPRD, protein tyrosine phosphatase receptor type delta; TfR, transferrin receptors; GWAS, genome-wide association studies; MEIS1, myeloid ecotropic viral integration site 1 homolog; BTBD9, BTB domain containing 9; HC, healthy controls; fHC, healthy relatives of RLS patients with a family history; TSAT, transferrin saturation; SPARCL1, SPARC-like protein 1; CCL2, pro-inflammatory C–C motif chemokine ligand 2; CCL8, pro-inflammatory C–C motif chemokine ligand 8; SELE, endothelial adhesion molecule 1; MTND1P23, Mitochondrially Encoded NADH Dehydrogenase 1 Pseudogene 23; MTCO1P40, Mitochondrially Encoded Cytochrome C Oxidase I Pseudogene 40; CBY2, chibby family member 2; POC1B-GALNT4, polypeptide N-acetylgalactosaminyltransferase 4; PLP1, proteolipid protein 1; STOX2, storkhead box 2; RPL7AP28, Ribosomal Protein L7/L12 Family Member AP28; APOA2, apolipoprotein A-II; BLOC1S5-TXNDC5, BLOC1S5-TXNDC5 readthrough, NMD candidate; TUBB8P7, Tubulin beta 8 class VIII; CA1, Carbonic anhydrase 1; UBBP2, Ubiquitin Associated Protein 2; IL-17, interleukin 17; TNF, tumor necrosis factor; TNF-α, tumor necrosis factor α; IL-1β, interleukin-1β; IL-6, interleukin-6; PLMS, periodic limb movements in sleep; Hs-CRP, hypersensitive C-reactive protein; MS, multiple sclerosis; RA, rheumatoid arthritis; IL-10, interleukin 10; DRG, dorsal root ganglion; TG, trigeminal ganglion; SNPs, single nucleotide polymorphisms; MCP, matricellular protein; NMDA, N-methyl-d-aspartate.

Data Sharing Statement

The data that support the findings of this study are available on request from the corresponding author, Xiaoying Zhu (Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, China). The data are not publicly available due to information that could compromise the privacy of research participants.

Ethics Statement and Informed Consent

The research was approved by the Ethics Committee of Shanghai General Hospital affiliated to Shanghai Jiao Tong University School of Medicine according to the "1964 Declaration of Helsinki", and all participants provided written informed consent to participate in the study. In addition, written informed consent for publication was obtained from all individuals included in the pedigree tree and other relevant participants.

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Author Contributions

All authors contributed substantially to the research, including the conceptualization, design, implementation, data collection, analysis, and interpretation of the study. They were involved in drafting, revising, and critically reviewing the manuscript. Each author provided final approval for the version to be published, agreed on the journal for submission, and accepted responsibility for the article's content. The individual contributions of the authors, based on the CRediT taxonomy, are as follows:

Xin-Rong He: Conceptualization, Methodology, Data Curation, Writing - Original Draft Preparation, Writing - Review & Editing, Supervision, Software, Visualization. Jia-Min Song: Writing - Review & Editing, Methodology, Investigation, Data Collection, Software, Formal Analysis. Jia-Peng Zhao: Data Collection, Formal Analysis, Visualization, Writing - Review & Editing. Jing Zhang: Visualization, Investigation, Writing - Review & Editing. Jing-Tao Feng: Software, Validation, Writing - Review & Editing. Shu-Qin Chen: Resources, Writing - Review & Editing. Zhi-Yuan Zhou: Resources, Investigation, Writing - Review & Editing. Hong-Ming Wang: Data Curation, Investigation, Writing - Review & Editing. Yue Zhang: Supervision, Project Administration, Writing - Review & Editing. Ya Feng: Supervision, Resources, Writing - Review & Editing. Yun-Cheng Wu: Formal Analysis, Visualization, Writing - Review & Editing. Xiao-Ying Zhu: Conceptualization, Supervision, Funding Acquisition, Writing - Review & Editing.

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Disclosure

We have no conflicts of interest to disclose.

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