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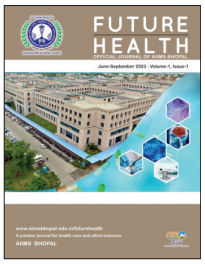

Original Article

Biochemical and genetic analysis of Restless Legs syndrome (RLS): A pilot study

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ABSTRACT

Objectives: Restless Leg Syndrome (RLS) is a neurological disorder characterized by an uncontrollable urge to move the legs, often worsening at night, leading to insomnia and discomfort. RLS is linked to genetic factors, defects in iron metabolism, dopaminergic dysfunction, and disturbances in the central opiate system. Specific genetic variants, including MEIS1, BTBD9, PTPRD, and MAP2K5/SCOR1, affect dopamine synthesis, iron transport, and neuroprotection. Research on RLS prevalence and its underlying causes in India is limited, highlighting the need for more in-depth genetic and biochemical studies to improve diagnosis and treatment.

Material and Methods: A hundred suspected RLS patients were screened at AIIMS Bhopal's Pulmonary Medicine department. After obtaining consent, 36 confirmed RLS cases and age-matched healthy controls were enrolled. Blood samples were collected for fasting blood sugar (FBS), liver function tests (LFT), renal function tests (RFT), serum ferritin, and iron analysis using an auto-analyzer. Genotyping for single nucleotide polymorphisms (SNPs) was performed using ARMS-PCR.

Results: RLS significantly impacts daily life, contributing to insomnia, irritability, and other comorbidities. The study revealed lower iron levels in RLS patients, suggesting a potential secondary form of the condition linked to iron deficiency. Variations in serum iron, total iron-binding capacity, iron saturation, and ferritin further underscore the relationship between iron metabolism and RLS. Genetic analysis identified SNPs in genes such as MEIS1, TOX3, and PTPRD, potentially contributing to RLS. Although no specific at-risk alleles were prevalent, heterozygous mutations, particularly at the T allele, suggested a possible genetic predisposition that may exacerbate RLS.

Conclusion: This study supports the iron-dopamine hypothesis, linking RLS to brain iron deficiency that disrupts dopamine signaling. Genetic factors may further aggravate this disruption. Understanding the interplay between iron levels and dopaminergic function is crucial for effectively managing both primary and secondary RLS, aiding the development of better long-term treatment strategies.

Keywords: ARMS polymerase chain reactions, liver function test, renal function test, restless leg syndrome, and single nucleotide polymorphism.

INTRODUCTION

Restless Leg Syndrome (RLS), also known as Willis-Ekbom disease, is a neurological disorder characterized by an overwhelming urge to move the legs, often accompanied by unpleasant sensations like pulling, burning, tingling, or itching. First identified by Ekbom in 1945, RLS remained underdiagnosed for years due to its diverse symptoms and absence of specific biomarkers. Symptoms typically worsen in the evening or night, interfering with sleep, and while legs are the most commonly affected, it can also involve the arms. Alongside motor symptoms, individuals often experience pain, cognitive disturbances, mood disorders, and sleep disturbances.[1-4] RLS is

classified into two types: primary and secondary. Primary RLS is believed to be hereditary with no known cause, while secondary RLS is linked to other medical conditions such as iron deficiency, kidney disease, and pregnancy.[5]

Diagnosis follows the criteria set by the International Restless Legs Syndrome Study Group (IRLSSG), which includes the urge to move the limbs, discomfort that improves with movement, worsening symptoms at night, and the absence of other underlying causes. The severity is assessed using the International Restless Legs Scale (IRLS), classifying patients as mild, moderate, or severe.[6]

RLS affects 1% to 5% of the population, with higher rates in older adults and women, and pregnancy being a significant risk factor. It is classified into two types: idiopathic (primary), without a clear cause, and secondary, linked to medical conditions like anemia, chronic liver disease, rheumatoid arthritis, multiple sclerosis, and obesity. Renal impairment and iron deficiency are strongly associated with secondary RLS, with iron-deficiency anemia increasing risk.[7-16] Additionally, low brain iron causes hypoxia and demyelination, which further contribute to the disorder's pathophysiology.[3,4]

Managing RLS requires assessing associated medical conditions and symptoms. Treatment options include pharmacotherapy tailored to the patient's medical history, as severe RLS can severely impair quality of life and lead to insomnia.

The pathophysiology of RLS involves iron metabolism disturbances, genetic mutations, and dopaminergic dysfunction. Iron is crucial to dopaminergic pathways in regions like the substantia nigra and basal ganglia. Deficiencies contribute to dopaminergic dysfunction, a key factor in RLS. Genetic mutations reported in BTBD9, MEIS1, PTPRD, and MAP2K5/SKOR1 affect iron transport and dopamine synthesis.[17-19]

Brain iron deficiency also influences glutamatergic neurotransmission, which is being explored as a therapeutic target. Medications targeting glutamate receptors or modulating dopamine release show promise. Additionally, iron deficiency alters adenosine receptor function, increasing hyperdopaminergic and hyper glutamatergic states worsening RLS symptoms.[20-26]

RLS is linked to periodic leg movements (PLM), disrupted sleep, and potentially increasing cardiovascular risks. It is common in chronic kidney disease and dialysis patients.[27-48] RLS often coexists with ADHD, sleep disorders, and IBS, requiring a multidisciplinary approach. Treatment should be initiated when symptoms significantly affect daily functioning.[49-56] However, there is limited data on RLS in the Indian population, and no study on SNP analysis from India, making identification and treatment of the condition more challenging.

MATERIAL AND METHODS

Patients coming to the Department of Pulmonary Medicine OPD were screened for RLS as per the clinical proforma (Annexure 1).

Out of 100 patients, 50 were suspected of having RLS and selected. However, cases with co-morbidities such as diabetes mellitus and neurological conditions were excluded as they did not meet the inclusion criteria. After the vetting process, there were 36 RLS cases and 50 healthy adult controls [Table 1].

Inclusion and exclusion criteria

Inclusion criteria for cases were (i) a patient diagnosed according to IRLSSG criteria & (ii) a patient aged between 18-70 years. The exclusion criteria for cases were (i) pregnant women, (ii) patients with an active malignancy, (iii) patients with a history of coronary artery disease, (iv) patients with a history of chronic kidney disease, diabetes, (v) patients who require hemodialysis, and (vi) patients who do not give consent.

Inclusion criteria for the controls were (i) age-matching with the RLS patients, (ii) patients without any chronic diseases, (iii) informed consent. The exclusion criteria for controls were (i) Pregnant women, (ii) patients with pre-existing conditions such as AIDS, Parkinson's disease, and coronary artery disease as per clinician's guidelines, and (iii) patients who do not give consent.

Materials

Participants were included based on the IRLSSG criteria, with only adults over the age of 18 being registered. Individuals with chronic illnesses, diabetes, or kidney disease were excluded from the study. For the control group, adults over 18 years old who did not have any chronic diseases, were not pregnant and did not have diabetes mellitus were included.

A hundred patients were screened, of which 50 suspected RLS cases were selected. However, further examination found that some of them had comorbidities (Diabetes mellitus and some neurological problems) and weren't fulfilling our inclusion criteria and were excluded. After the vetting process there were 36 RLS cases and 50 healthy adult controls.

Biochemical parameters

Serum for LFT (liver function test), RFT (renal function test), and iron profile (Ferritin, TIBC, UIBC, Total Iron, iron saturation) were measured on auto analyzers, (AU680 Beckman Coulter), whereas ferritin was measured using chemiluminescence auto analyzer at the Department of

Biochemistry, All India Institute of Medical Sciences (AIIMS) Bhopal, Madhya Pradesh, India [Table 2].

DNA extraction protocol from blood:

DNA extraction was done using the manual extraction method. Genetic polymorphism testing of genes was detected. The detection of SNPs was carried out by amplification refractory mutation system polymerase chain reaction (ARMS PCR). It is a specialized polymerase chain reaction (PCR) technique used for the detection of specific genetic mutations or SNPs.

Statistical analysis

The data was collected using MS Excel, and the statistical analysis was performed using SPSS software version 21 (IBM, Chicago, IL, US) and GraphPad Prism. The student's t-test was performed to evaluate the differences between age, gender, and biochemical parameters. Analysis of variance (ANOVA) test was employed to evaluate the differences between the polymorphisms and biochemical parameters. Linear regression was performed to evaluate the possible impact and correlation between the IRLSSG score and serum ferritin. Allele frequency was calculated using the chi-square test. All biochemical values were mentioned as mean ± standard deviation (SD). P-values less than 0.05 reflect statistically significant results. Risk analysis between biochemical parameters and genetic factors will be assessed using odds ratio (OR) calculated with 95% confidence interval (CI).

RESULTS

In our study, we initially screened 100 patients and selected 50 potential cases. However, after further evaluation, some patients were found to have co-morbidities like diabetes mellitus (n=3), hemolyzed sample (n=1), and neurological issues (n=10) that did not meet our inclusion criteria and were subsequently excluded. After this vetting process, the final case group consisted of 36 patients, with 50 healthy adult controls. For data analysis, a t-test was used to evaluate demographic and clinical parameters. Linear regression was employed to examine the relationship between RLS scores and serum ferritin levels, ANOVA was conducted to assess the correlation between alleles and clinical parameters, and the OR was calculated to determine the risk of RLS associated with different SNP alleles under study. Additional parameters assessed included LFTs and RFTs. The LFTs conducted were alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), bilirubin (direct/indirect), albumin, globulin, and the albumin-to-globulin (A:G) ratio.

Statistically significant differences were observed between the case and control groups in AST, albumin, and A:G ratio, each with a p-value of less than 0.05. For the RFTs, serum levels of sodium, chloride, potassium, urea, and creatinine were measured. Significant differences between the case and control groups were found in sodium and creatinine levels, both with p-values less than 0.05 [see Tables 1-3].

Table 1: Demographic information of cases and controls.

Parameters	Case (n=36)	Control (n=50)	p-value
Gender (Male)	27	22	0.000034
Gender (Female)	9(25%)	28(56%)	0.000167
Age	56.80 ± 11.19	39.38 ± 12.54	<0.00001
RLS severity (mean)	14.44 ± 6.46	---	--

For this study, we included 36 cases and 50 controls. There is a statistically significant difference between age and gender for case and control. Mean IRLSSG rating was 14.44 ± 6.46. *p*-value <0.05 is statistically significant. RLS: Restless legs syndrome, IRLSSG: International Restless Legs Syndrome Study Group.

Linear Regression model analysis

Figure 1 shows a linear regression model comparing RLS scoring with serum ferritin levels. We can see that as serum ferritin levels rise, the RLS scoring decreases establishing an inverse relation between the two.

Genotype distribution of SNPs among the healthy and case subjects' analysis:

Supplementary Figure 1a-e presents the genotype distribution of five SNPs among healthy (blue) and affected (orange) individuals. Tables 3-5 outline the RLS-related polymorphisms and their correlations with various biochemical parameters. In this analysis, we examined the differences in clinical parameters across different alleles of polymorphisms and assessed their statistical significance.

For the MEIS1 gene [Table 3], we evaluated both heterozygous and homozygous alleles, but no statistically significant associations were observed. The BTBD9 gene was not analyzed further due to the predominant presence of the heterozygous TG allele in the study population.

In the case of TOX3 [Table 4], we compared the homozygous TT allele, heterozygous TG allele, wild-type GG allele, and the combined (TT+TG) group with the GG allele. However, no statistically significant differences were found in clinical parameters across these comparisons.

For the PTPRD gene [Table 5], the low frequency of the TT homozygous allele led us to combine it with the TG heterozygous group for comparison against the wild-type CC allele. Again, no significant differences were detected.

Table 2: Biochemical parameters of cases and controls.

Parameter	Case (n=36)	Control (n=50)	p-value	Reference range
Iron(µg/dL)	58.23 ± 18.235	111.99 ± 31.262	<0.00001	60-180
TIBC (µg/dL)	363.21 ± 60.147	325.61 ± 58.047	0.004563	250-400
Iron saturation (%)	15.23 ± 5.29	33.89 ± 11.61	<0.00001	15-50
Ferritin (ng/mL)	67.91 ± 42.01	199.23 ± 85.73	<0.00001	10-291
ALT (U/L)	26.48 ± 14.15	26.33 ± 7.94	0.948521	<35
AST (U/L)	26.58 ± 9.39	19.58 ± 5.32	0.000034	<35
ALP (U/L)	89.59 ± 48.68	82.50 ± 20.02	0.355851	30-120
GGT (U/L)	24.93 ± 9.1	22.11 ± 9.26	0.164516	<38
In-direct bilirubin (mg/dL)	0.60 ± 0.31	0.62 ± 0.14	0.689789	0.20-1.0
Direct bilirubin (mg/dL)	0.17 ± 0.10	0.19±0.05	0.187581	<0.2
Total protein (g/dL)	7.14 ± 0.56	7.15 ± 0.55	0.914356	6.6-8.3
Albumin (g/dL)	4.26 ± 0.28	4.4 ± 0.22	0.019478	3.5-5.2
Globulin (g/dL)	2.87 ± 0.42	2.75 ± 0.55	0.288785	1.9-3.7
A:G ratio	1.34 ± 0.35	1.66 ± 0.40	0.000234	1-1.7
Urea (mg/dL)	21.19 ± 6.36	24.32 ± 8.06	0.056575	20.0-40.0
Creatinine (mg/dL)	0.94 ± 0.15	1.05 ± 0.09	0.000029	0.5-0.9
Sodium (mmol/L)	135.94 ± 4.03	133.78 ± 3.54	0.010041	136-145
Chloride (mmol/L)	101.36 ± 4.85	101.74 ± 3.05	0.658636	98-107
Potassium (mmol/L)	4.56 ± 0.50	4.42 ± 0.47	0.176666	3.5-5.1

The differences between the serum iron profile for cases and controls was determined, which includes serum iron (p-value <0.05), TIBC (p-value <0.05), iron Saturation (p-value <0.05) and ferritin (*p*-value <0.05), and all were statistically significant. TIBC: total iron-binding capacity, ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: Alkaline phosphatase, GGT: gamma-glutamyl transferase, A:G: Adenine: Guanine

Table 3: SNPs of MEIS1 gene and their association with clinical parameters. Where, *p*-value<0.05 is statistically significant.

MEIS1				
Parameter	**TT**	**TG**	**GG**	**p-value**
Iron	56.25 ± 16.90	59.1 ± 19.05	-	0.672738
TIBC	343.74 ± 48.77	371.77 ± 63.51	-	0.202219
Iron saturation	16.43 ± 5.03	14.70 ± 5.42	-	0.374118
Ferritin	54.54 ± 19.51	73.79 ± 47.93	-	0.209894
RLS rating	14.45 ± 5.95	14.44 ± 6.80	-	0.995149

SNPs: Single nucleotide polymorphisms, TIBC: Total iron binding capacity, RLS: Restless legs syndrome

Table 4: SNPs of TOX3 gene and their association with clinical parameters. Where, *p*-value<0.05 is statistically significant.

TOX3				
Parameter	**TT**	**TG**	**GG**	**p-value**
Iron	57.11 ± 19.04	58.83 ± 11.55	57.94 ± 26.78	0.977203
TIBC	352.62 ± 62.42	366.35 ± 60.90	364.80 ± 62.59	0.878567
Iron saturation	15.34 ± 6.19	14.72 ± 3.11	15.99 ± 7.58	0.826636
Ferritin	64.85 ± 35.75	74.39 ± 53.10	59.25 ± 21.30	0.640598
RLS rating	14 ± 7.50	13.88 ± 5.64	15.63 ± 7.50	0.774026

SNPs: Single nucleotide polymorphisms, TIBC: Total iron binding capacity, RLS: Restless legs syndrome

Similarly, an analysis of the MAP2K5 gene was not conducted due to the predominant distribution of the GA heterozygous allele in the population.

DISCUSSION

This study highlights the critical role of iron in RLS. It shows a marked decline of iron levels in RLS patients compared to healthy controls, suggesting that low iron may be a factor in the onset of secondary RLS. Variations in serum iron, total iron-binding capacity, iron saturation, and ferritin levels suggest a strong connection between iron metabolism and RLS.[20,22] Gender and age differences were observed between RLS cases and controls.[6] Although previous studies suggest

Table 5: SNPs of PTPRD gene and their association with clinical parameters. Where, *p*-value<0.05 is statistically significant.

PTPRD			
Parameter	**TT+TC**	**CC**	**p-value**
Iron	58.58 ± 19.65	56.46 ± 9.07	0.799409
TIBC	358.15 ± 60.12	388.49 ± 58.64	0.265423
Iron saturation	15.22 ± 5.75	15.27 ± 2.00	0.982538
Ferritin	66.54 ± 41.08	74.76 ± 50.02	0.668117
RLS rating	14.43 ± 6.36	14.5 ± 7.63	0.982014

SNPs: Single nucleotide polymorphisms, TIBC: Total iron binding capacity, RLS: Restless legs syndrome

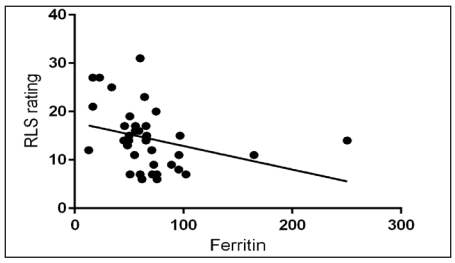

Figure 1: Interrelationship between RLS rating and Ferritin. RLS: Restless legs syndrome.

a higher prevalence of RLS in women, ours found a higher proportion of males in the RLS cohort. However, due to the limited number of confirmed RLS cases, the study could not perform a thorough gender-based analysis.

It was observed that the genotype distribution analysis for specific SNPs highlighted MEIS1, TOX3, and PTPRD as potential genetic factors associated with RLS. Genotype distribution analysis for specific SNPs revealed that genes like MEIS1, TOX3, and PTPRD might contribute to RLS susceptibility. Both cases and controls showed heterozygous mutations, with the T allele predominating [Supplementary Figure 2]. While no distinct at-risk alleles were identified in this cohort, the presence of one copy of the allele in both cases and controls suggests a potential genetic risk factor for RLS. The low iron levels (iron deficiency) may be the primary trigger for RLS, with the identified genetic variants in cases possibly exacerbating the disease. Screening for altered iron profiles, especially in older individuals, may be useful for predicting the likelihood of developing RLS. Iron supplementation could be used as a preventive measure in those with deficiency.[23]

The study also reviewed genetic variants that affect iron transport, dopamine regulation, and neuroprotection of dopaminergic neurons. Key genes involved in these processes include BTBD9 (chromosome 6p21.2), MEIS1 (chromosome 2p14), PTPRD (chromosome 9p24.1-p23), and MAP2K5/SKOR1, all of which play significant roles in iron metabolism and dopamine biosynthesis [Table 6].[4,17-19]

Table 6: SNP (wild vs. mutant) and their p-value and OR

Gene	SNP	ALLELE	p-value	OR	95% CI
MEIS1	rs2300478	GG* vs. TG	0.9702	1.0784	0.0206 to 56.3943
		GG vs. TT	0.7251	2.0435	0.0381 to 109.6845
		GG vs. TG+TT	0.8718	1.3836	0.0268 to 71.3588
		TG vs. TT	0.1508	1.9360	0.7862 to 4.7677
BTBD9	rs9296249	CC* vs. TC	0.8796	1.3562	0.0263 to 69.9594
		CC* vs. TT	0.6705	3.0000	0.0190 to 473.1018
		CC vs. TC+TT	0.8718	1.3836	0.0268 to 71.3588
		TC vs. TT	0.6299	2.2121	0.0876 to 55.8717
TOX3	rs3104767	TT* vs. TG	0.9121	0.9398	0.3122 to 2.8296
		TT vs. GG	0.2583	0.4773	0.1324 to 1.7210
		TT vs. TG+GG	0.6160	0.7644	0.2675 to 2.1843
		TG vs. GG	0.2099	0.5078	0.1761 to 1.4647
PTPRD	rs1975197	AA* vs. GA	0.3318	3.3571	0.2910 to 38.7359
		AA vs. GG	0.783	0.6667	0.0372 to 11.9362
		AA vs. GA+GG	0.3951	2.884	0.2512 to 33.0706
		GA vs. GG	0.0574	0.1986	0.0375 to 1.0521
MAP2K5	rs1026732	AA* vs. GA	1.0000	1	0.0136 to 73.2695
		AA vs. GG	0.8656	1.4058	0.0272 to 72.5906
		AA vs. GA+GG	0.8718	1.3836	0.0268 to 71.3588
		GA vs. GG	0.7365	1.4118	0.1894 to 10.5221

*Indicate wild type allele of SNP RLS: Restless legs syndrome, SNPs: Single nucleotide polymorphisms, TIBC: Total iron-binding capacity, OR: Odds ratio, CI: Confidence interval.

RLS is particularly prevalent in individuals with end-stage renal disease (ESRD), affecting up to 68% of patients.

Table 7: Odds ratio studies for the alleles of polymorph for their association with RLS.

Name of the gene (SNP ID)	Comparison	OR	p-value	95% CI
MEIS1 (rs2300478)	GG v/s TG	1.0784	0.9702	0.0206 to 56.3943
	GG v/s TT	2.0435	0.7251	0.0381 to 109.6845
	GG v/s TT+TG	1.3836	0.8718	0.0268 to 71.3588
	TG v/s TT	1.9360	0.1508	0.7862 to 4.7677
BTBD9 (rs9296249)	CC v/s TC	1.3562	0.8796	0.0263 to 69.9594
	CC v/s TT	3.0000	0.6705	0.0190 to 473.1018
	CC v/s TC+TT	1.3836	0.8718	0.0268 to 71.3588
	TC v/s TT	2.2121	0.6299	0.0876 to 55.8717
TOX3 (rs3104767)	TT v/s TG	0.9398	0.9121	0.3122 to 2.8296
	TT v/s GG	0.4773	0.2583	0.1324 to 1.7210
	TT v/s TG+GG	0.7644	0.6160	0.2675 to 2.1843
	TG v/s GG	0.5078	0.2099	0.1761 to 1.4647
PTPRD (rs1975197)	AA v/s GA	3.3571	0.3318	0.2910 to 38.7359
	AA v/s to GG	0.6667	0.783	0.0372 to 11.9362
	AA v/s GA+GG	2.884	0.3951	0.2512 to 33.0706
	GA v/s GG	0.1986	0.0574	0.0375 to 1.0521
MAP2K5 (rs1026732)	AA v/s GA	1	1.0000	0.0136 to 73.2695
	AA v/s GG	1.4058	0.8656	0.0272 to 72.5906
	AA v/s GA+GG	1.3836	0.8718	0.0268 to 71.3588
	GA v/s GG	1.4118	0.7365	0.1894 to 10.5221

The presence of different alleles and their overall risk of RLS. RLS: Restless legs syndrome.

Table 8: Allele frequency found in polymorphs studies.

Gene	Allele frequency	Global allele frequency
MEIS1 (rs2300478)	T: 70%, G:30%	T:75.8%, G:24.2%[a]
BTBD9 (rs9296249)	T: 50%, C:50%	T:75%, G:25%[b]
TOX3 (rs3104767)	T:49%, G:51%	T:45%, G:55%[c]
PTPRD (rs1975197)	T: 47%, C:53%	G:8%, A:17%[d]
MAP2K5 (rs1026732)	G: 98%, A:2%	G:64%, A:36%[e]

It shows predominant presence of T allele. a is MEIS1, b is BTBD6, c is TOX3, d is PTPRD and e is MAP2K5 in which allele and Global frequencies are presented

It significantly influences their sleep, quality of life, and cardiovascular health.[57,58] Ethnic and age-related differences are seen in ESRD populations with RLS, especially in those with chronic kidney disease (CKD), particularly among individuals on hemodialysis or peritoneal dialysis.[59] Clinical findings in this study revealed significant differences in iron-related parameters, LFTs, and RFTs between RLS cases and controls.

Although this study found a link between genetic and biochemical markers and RLS, it faced limitations due to the small sample size. Genotypic analysis indicated that certain SNPs might be associated with RLS, but the strength of this association remains unclear. Previous studies have shown that genetic predisposition plays a major role in early-onset RLS, with more than 60% of cases having a positive familial history. There is no information regarding SNP-RLS from India, but variants in MEIS1, BTBD9, and MAP2K5/SKOR1 are known to increase the risk of RLS in some populations [Table 7].[60-62]

The pilot study offers valuable insights into the demographic, clinical, and genetic aspects of RLS in Indian people. Iron deficiency and genetic factors contribute significantly to RLS. Further research with larger subject cohorts is required to better understand the interplay of genetic, environmental, and physiological factors, especially in the Indian population, to enhance early diagnosis and personalized treatment strategies since no genetic study on the Indian population has been reported [Table 8].

CONCLUSION

RLS, a leading cause of insomnia, is often underdiagnosed. This study explores the role of iron metabolism, LFTs, RFTs, and genetic factors (specifically SNPs), in RLS pathophysiology. Iron deficiency, linked to dopaminergic dysfunction, was found to significantly affect RLS patients compared to healthy controls. Genetic analysis revealed SNPs in MEIS1, TOX3, and PTPRD, suggesting a genetic

predisposition to RLS, although no predominant at-risk alleles were found. Screening for iron deficiency and genetic factors, along with iron supplementation, could aid early diagnosis and therapeutic strategies for RLS.

Author contributions: R.C.: Conceptualization, methodology, formal analysis, investigation, resources, writing—original draft preparation, writing—review and editing, visualization, supervision, project administration; A.V.: Methodology, software, validation, data curation, writing—original draft preparation, writing—review and editing; A.K.Y.: Software, validation, data curation, writing—review and editing; J.R.K.: Validation, resources, supervision; S.G.: Formal analysis, writing—review and editing; A.A.J.: Investigation; A.G.: Visualization.

Ethical approval: The research/study approved by the Institutional Review Board at AIIMS Bhopal, number IHEC-PGR/2021/PG/Jan/05, dated 28th August 2024.

Declaration of patient consent: The authors certify that they have obtained all appropriate patient consent.

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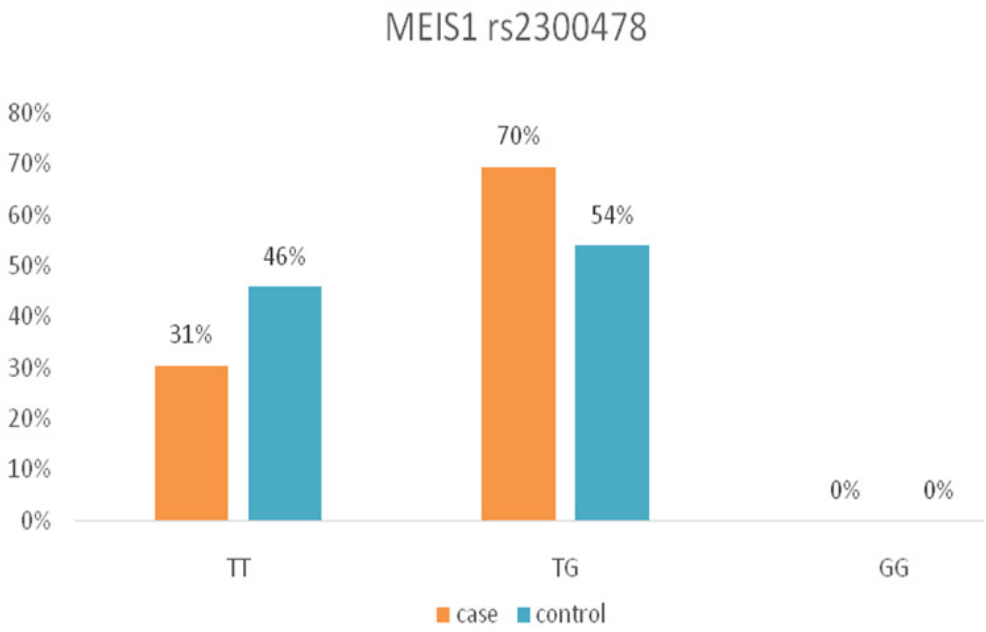
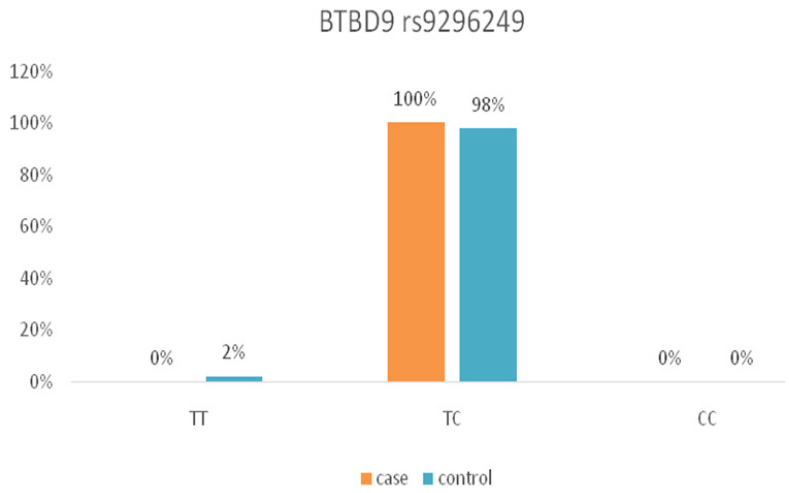
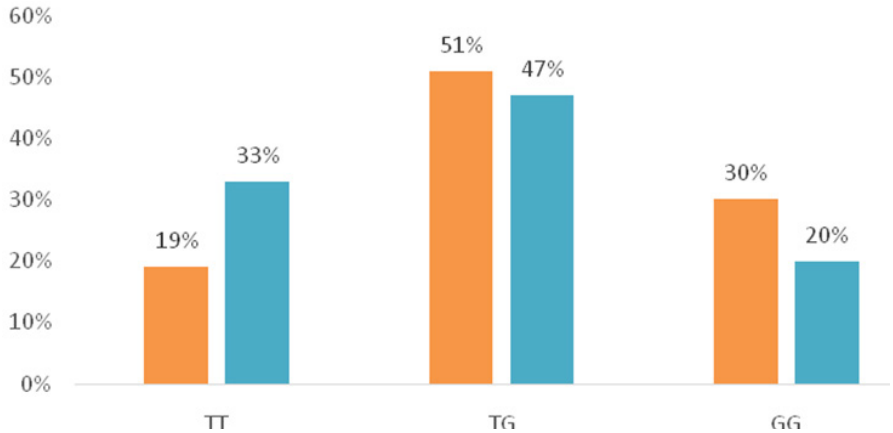


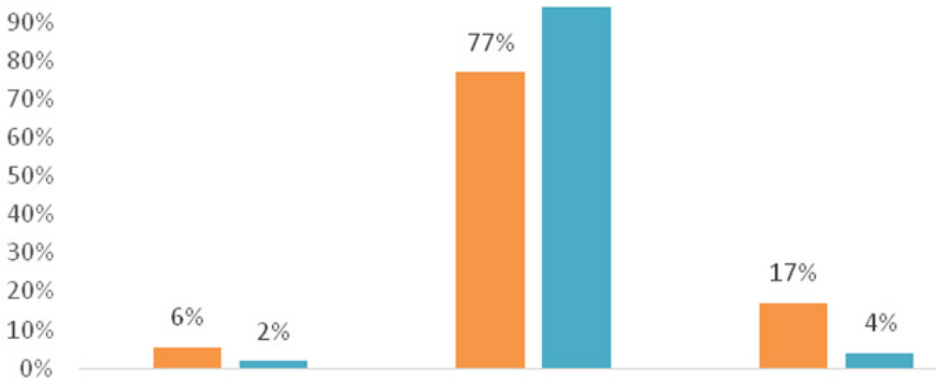
Supplementary Figure 1a: Genotype distribution of MEIS 1 SNP among the healthy and case subjects' analysis. MEIS1: The analysis indicates that the T/G heterozygous mutation was mostly present in cases (70% vs.. 54%) while T/T homozygous mutation was mostly distributed among controls (30% vs.. 46%), whereas no case or control showed any wild type G/G allele.



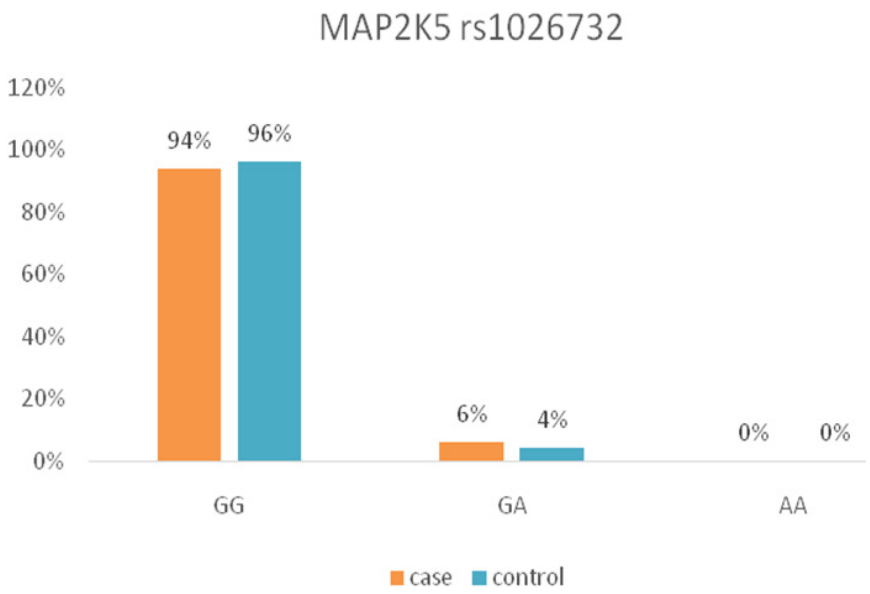
Supplementary Figure 1b: Genotype distribution of BTBD9 SNP among the healthy and case subjects' analysis. BTBD9: The analysis indicates that wild and mutant type alleles were mostly distributed equally among case and control T/C heterozygous allele (100% vs. 98%).



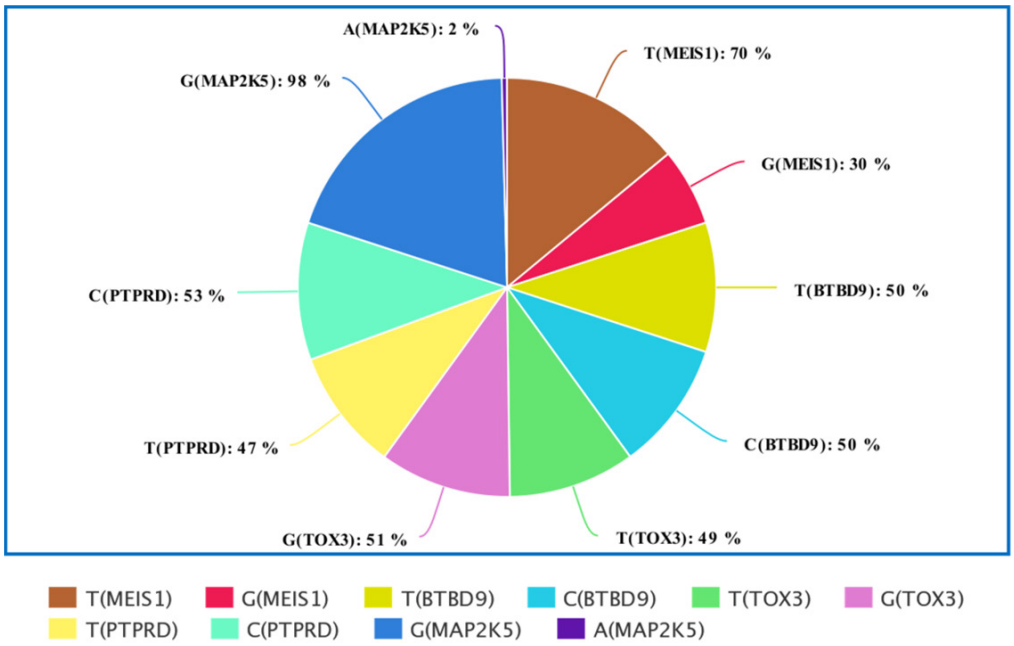
Supplementary Figure 1c: Genotype distribution of TOX3 SNP among the healthy and case subjects' analysis. TOX3: The analysis shows (19% vs. 33%) distribution of case and control in T/T homozygous allele, (51% vs. 47%) distribution of case and control in T/G heterozygous allele and (30% vs. 20%) distribution in wild G/G allele.



Supplementary Figure 1d: Genotype distribution of PTPRD SNP among the healthy and case subjects' analysis PTPRD: Here, the majority distribution of case and control is in T/C heterozygous allele (77% vs. 94%) while T/T homozygous allele has (6% vs. 2%) distribution for case and control and (17% vs. 4%) distribution in C/C wild type allele.



Supplementary Figure 1e: Genotype distribution of MAP2K5 SNP among the healthy and case subjects' analysis. MAP2K5: Here, the distribution is predominantly in G/G homozygous allele (94% vs. 96%) with some distribution in G/A heterozygous allele (6% vs. 4%). The table summarizes the RLS SNP and their correlation with clinical parameters [Table 3a,b,c].



Supplementary Figure 2: Pie chart showing allele frequency of SNP under study