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Original Article

ORIGINAL ARTICLE

Reassessment of candidate gene studies for idiopathic restless legs syndrome in a large genome-wide association study dataset of European ancestry

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Abstract

Study Objectives: Several candidate gene studies have been published for idiopathic restless legs syndrome (RLS) in populations of European ancestry, but the reported associations have not been confirmed in independent samples. Our aim was to reassess these findings in a large case–control dataset in order to evaluate their validity.

Methods: We screened PubMed for RLS candidate gene studies. We used the genome-wide association study (GWAS) dataset of the International EU-RLS-GENE Consortium as our replication sample, which provided genome-wide single-variant association data based on at most 17 220 individuals of European ancestry. We performed additional gene-based tests using the software MAGMA and assessed the power of our study using the genpwr R package.

Results: We identified 14 studies conducted in European samples which assessed 45 variants in 27 genes of which 5 variants had been reported as significantly associated. None of these individual variants were replicated in our GWAS-based reassessment (nominal $p > 0.05$) and gene-based tests for the respective five genes *ADH1B*, *GABRR3*, *HMOX1*, *MAOA*, and *VDR*, were also nonsignificant (nominal $p > 0.05$). Our replication dataset was well powered to detect the reported effects, even when adjusting for effect size overestimation due to winner's curse. Power estimates were close to 100% for all variants.

Conclusion: In summary, none of the significant single-variant associations from candidate gene studies were confirmed in our GWAS dataset. Therefore, these associations were likely false positive. Our observations emphasize the need for large sample sizes and stringent significance thresholds in future association studies for RLS.

Statement of Significance

This study reports the first detailed reevaluation of genetic associations reported in individual candidate gene or candidate genetic variant case–control studies for idiopathic restless legs syndrome. Confirmation of discovered associations in independent samples (replication) is a key approach to discern true-positive and false-positive signals. Reassessment in our large and well-powered case–control genome-wide association study dataset showed that none of the reported candidate gene associations were replicated. Our results add to the evidence that genetic association studies require large sample sizes and stringent significance thresholds in order to ensure reliable results.

Key words: restless legs syndrome; genome-wide association study; genetic association studies; movement disorders

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Introduction

Restless legs syndrome (RLS) is a sleep-related movement disorder, especially common in populations of European ancestry. Heritability estimates of 50%–60% in twin and family studies early on indicated a significant impact of genetic factors on RLS susceptibility [1, 2]. While monogenic forms may exist in individual families, the majority of idiopathic RLS cases appears to have a multifactorial form for which common small-effect size variants determine the genetic risk. Knowing the genetic underpinnings of a disease facilitates research and patient care on multiple levels. It guides and improves the understanding of the underlying molecular and cellular processes involved in disease risk, development, and progression. Moreover, it can provide hypotheses for new therapeutic approaches as well as for prevention and prediction strategies. Ultimately, genetic risk profiles will be important for implementing precision medicine.

Accordingly, genetic studies were and are an important pillar of RLS research. For supposedly monogenic RLS in multigenerational families, linkage studies and next-generation sequencing of exome or genome are approaches to search for rare variants with strong effects [2–4]. In terms of identifying common risk variants with small effects for the multifactorial or complex form of RLS, case–control association studies are the method of choice [5]. These can be broadly categorized into hypothesis-driven candidate gene studies and hypothesis-free genome-wide association studies (GWAS). In general, studies focused on single candidate genes or variants are increasingly superseded by GWAS due to the existence of large phenotype-specific consortia and comparably cheap and reliable microarrays for genotyping [6, 7].

For RLS, recent meta-analyses of GWAS have identified a total of 22 risk loci containing 23 independent genome-wide significant risk variants [8, 9]. These associations have been replicated, ie, confirmed in an independent sample using similar phenotype definitions, which is considered the gold standard for identifying bona-fide significant signals. The most recent GWAS meta-analysis identified two further risk loci still awaiting replication, one located on chromosome 2q32.2 (lead variant rs10188680) and one on chromosome 18q21.32 (lead variant rs58127855) [9]. In addition, hypothesis-based candidate gene studies have been performed for RLS before and also after GWAS became feasible [2]. In general, their sample sizes were below 1000 individuals per study and only a few selected single nucleotide polymorphisms (SNPs) and variable number of tandem repeats (VNTRs) located in individual candidate genes were analyzed. To date, the results of these studies have not been validated in independent cohorts.

Therefore, we assessed them in a large dataset well powered for association testing. We reviewed the literature for published candidate gene studies for RLS and compiled a list of all genetic variants tested for association to idiopathic RLS in populations of European ancestry. The individual variants as well as the respective genes were then screened for confirmation using the International EU-RLS-GENE Consortium GWAS dataset.

Methods

The general study workflow is depicted in Figure 1.

Selection of published candidate gene studies for replication

We compiled a list of candidate gene variants by searching PubMed for genetic association studies published between 1996 and January 4, 2022. The search term "genetics AND restless legs syndrome" returned 383 publications. These were screened manually to identify case–control association studies in which single candidate genes or single candidate variants were tested for association to idiopathic RLS. We excluded candidate genes studies conducted in populations of non-European ancestry as well as studies conducted originally in samples included in the International EU-RLS-GWAS cohort [8].

Statistical analysis

For single-variant association testing, we extracted nominal *p* values from an existing dataset, the International EU-RLS-GENE Consortium GWAS, which had been included in our meta-analysis published in 2017 [8]. In brief, this GWAS had included 6228 RLS cases and 10 992 population-matched controls recruited in eight European countries, Canada, and the United States. In the case group, face-to-face interviews had been used for diagnosing RLS based on the International Restless Legs Syndrome Study Group diagnostic criteria. Details of genotyping, imputation, quality control procedures, and statistical analyses yielding these *p* values have been described previously [8].

The published GWAS had included both sexes, but one candidate gene study had reported associations for MAOA in females only. Therefore, we ran an additional sex-specific association analysis in the current study for this gene. We extracted imputed dosages of female cases and controls from the quality-controlled International EU-RLS-GENE Consortium dataset and ran a logistic regression analysis in SNPTEST (v2.5.4) under an additive model, including age and the first 10 principal components from an MDS analysis in PLINK as covariates.

For gene-based association testing, we ran MAGMA (v1.08, SNP-wise mean model) on the single-variant association p values of the EU-RLS-GENE Consortium GWAS dataset [10]. Input files of gene location definitions (genome build 37) and linkage disequilibrium reference data (European population of the 1000 Genomes phase 3) were downloaded from the MAGMA homepage (https://ctg.cncr.nl/software/magma). For this analysis, variants were mapped to a gene if they were either located within the gene body or within 5 kb upstream or downstream of the gene's end or start point (buffer regions). Since the test results may vary depending on the variants included or the model used, we performed two additional gene-based tests: (1) same gene analysis model in MAGMA, but using 10 kb buffer regions, and (2) analysis with 5 kb buffer regions, with and without including regulatory variants outside of the buffer regions, and applying a different gene-based test, the aggregated Cauchy association test (ACAT) using the GeneScan3DKnock (v0.3) R package [11]. The ACAT has been shown to perform better in settings where only a small number of the variants included in a gene-based test are causal variants [12].

In order to map regulatory variants to genes, we extracted enhancers linked to the 27 candidate genes from two publicly available enhancer–target–gene datasets, the GeneHancer database 2017 release (http://www.genecards.org, GeneHancer_Version_4-4) and the activity-by-contact (ABC)-model

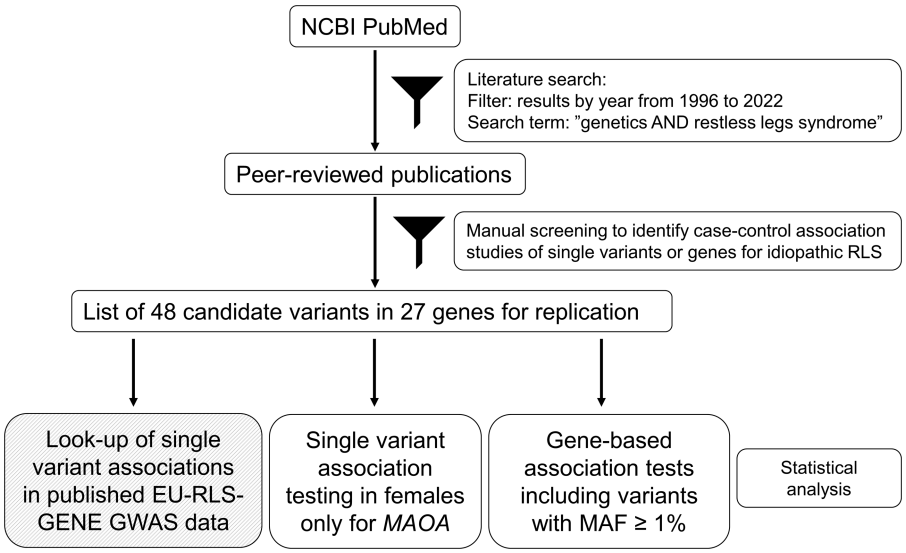

Figure 1. Study workflow. Text boxes on the right-hand side of the filter symbols describe the filtering criteria used to obtain the list of candidate variants for replication. Gray shading of boxes indicates that previously published data are used without conducting any new analyses.

predictions for neuronal cell types from the Engreitz lab (https://www.engreitzlab.org/resources/; cell types: H1-derived neuronal progenitor cultured cells, bipolar neurons from induced pluripotent stem cells, and fetal spinal cord) [13, 14]. We limited the regulatory elements to high-confidence elements (ABC score ≥ 0.015 or GeneHancer score ≥ 0.5) and performed mapping of variants to these elements in GeneScan3DKnock based on their genomic position.

Study power analysis

We ran power calculations with the R package genpwr (v1.0.4) [15] for single variants using an additive model and the sample size of the International EU-RLS-GENE GWAS (17 220 individuals with a case ratio of 0.3617). This was done for a range of odds ratios (1.05–2) and minor allele frequencies (0.01, 0.05, 0.1–0.5) in order to cover the estimates and frequencies given in the published candidate gene studies. Moreover, we performed individual power calculations for the variants with reported positive associations with RLS in the candidate gene studies. For the proxy SNP used for the MAOA uVNTR, we performed these power calculations using the sample size of the female-only GWAS and the published odds ratio estimate of the uVNTR. For evaluation of the potential effect of winner's curse on study power, we calculated adjusted odds ratios based on the published data of the discovery studies using the method implemented in the program WINNER (v1.1) [16]. WINNER analytically calculates the bias on the odds ratios as a function of sample size, allele frequencies, and statistical significance level and uses an ascertainment-corrected maximum likelihood method to estimate the adjusted risk allele frequency differences and odds ratios.

Results

Our search in PubMed yielded a total of 14 candidate gene association studies in populations of European ancestry published for idiopathic RLS between 1996 and 2022, which were independent of the International EU-RLS-GENE Consortium GWAS [17–30]. Their sample sizes were rather small, with a maximum of 285 for cases and 505 for controls. Overall, 45 SNPs and three VNTR (variable number of tandem repeats) variants located in 27 different genes had been analyzed in these studies (Table 1). Five variants in five genes had shown per-study significant association to idiopathic RLS. These included a VNTR polymorphism in the monoamine oxidase a (*MAOA*) gene and four SNPs in the genes encoding heme oxygenase 1 (*HMOX1*), vitamin D receptor (*VDR*), alcohol dehydrogenase 1B (*ADH1B*), and gamma-aminobutyric acid type A receptor subunit rho3 (*GABRR3*) [17, 23, 25, 26, 28]. Interestingly, the association in *MAOA* had been significant in females only.

The International EU-RLS-GENE GWAS provided association results for 42 out of the 45 SNPs. The three remaining SNPs had not been genotyped directly in the dataset and there were no appropriate tagging variants (defined as strongly correlated with a linkage disequilibrium [LD] of $r^2 \geq 0.5$ with the target SNP in 1000Genomes European dataset) which could have served as proxies. The VNTR variants were not directly measured in the GWAS due to the use of SNP microarrays. However, we identified a proxy SNP (rs909525) for the uVNTR in *MAOA* based on a published haplotype analysis of the *MAOA* gene locus in individuals of European ancestry [31]. Therefore, a total of 43 variants out of the 48 reported could be tested in our single-variant association analyses.

The four SNPs located in *HMOX1*, *VDR*, *ADH1B*, and *GABRR3*, which had been reported as significantly associated with RLS in the candidate gene studies, were present in the EU-RLS-GENE GWAS dataset. They did not show significant association, neither using a genome-wide significance threshold ($p \leq 5 \times 10^{-8}$), a study-wise significance threshold ($p \leq 0.0012$, adjusting for 43 variants tested), or nominal significance without accounting for multiple testing (Table 2). For all remaining SNPs, we confirmed the negative results of the candidate genes studies (Supplementary Table 1).

In order to assess the reported significant association of the uVNTR in *MAOA*, we tested the proxy SNP rs909525 in the EU-RLS-GENE GWAS data using females only (4141 RLS cases and 4957 controls). It was not significantly associated with RLS in our study (Table 2).

Table 1. Overview of published single-variant candidate gene case–control association studies for idiopathic RLS in populations of European ancestries

Gene	SNP	OR (95% CI)	$P_{nominal}$	Study population ancestry (country)	N cases	N controls	PMID
DBH	rs1108580	NR	0.977	European (Quebec, Canada)	82	192	11591853
DRD1	rs4532	NR	0.721				
DRD2	rs1801028	NR	0.249				
DRD3	rs6280	NR	0.966				
DRD4	rs1800955	NR	0.969				
DRD5	rs6283	NR	0.134				
TH	rs6356	NR	0.693				
SLC6A3 (DAT)	3'UTR 40bp VNTR	NR	0.514				
MAOA all	uVNTR	NR	> 0.08	European (Quebec, Canada)	96	200	12136060
MAOA female	**uVNTR**	**2 (1.06–3.77)**	**< 0.05**				
MAOB	Intron 2 (GT) VNTR	NR	0.29				
SLC11A2 (DMT1)	rs1005559	NR	0.4413	European (Quebec, Canada)	179	180	17510944
	rs12424509	NR	0.4494				
	rs12830073	NR	0.7055				
	rs149411	NR	0.5625				
	rs224575	NR	0.458				
	rs224589	NR	0.8519				
	rs370462	NR	0.0829				
	rs407135	NR	0.8211				
	rs427020	NR	0.0935				
	rs829022	NR	0.8243				
GABRA4	rs2229940	1.05 (0.79–1.40)	0.725	European (Spain)	205	230	29720720
GABRE	rs1139916	1.13 (0.85–1.50)	0.376				
GABRQ	rs3810651	1.1 (0.82–1.47)	0.506				
GABRR1	rs1186902	1.53 (0.73–3.27)	0.227				
GABRR1	rs12200969	1.11 (0.82–1.51)	0.467				
GABRR2	rs282129	1.22 (0.87–1.72)	0.231				
GABRR3	**rs832032**	**1.66 (1.16–2.37)**	**0.004**				
MAPT1	rs1052553	1.02 (0.76–1.36)	0.906	European (Spain)	205	324	23001634
DRD3	rs6280	1.05 (0.80–1.38)	0.713	European (Spain)	206	324	23312624
SLC1A2	rs3794087	1 (0.75–1.35)	0.991	European (Spain)	205	328	24424098
NOS1	rs693534	1.16 (0.89–1.51)	0.256	European (Spain)	205	328	25300364
NOS1	rs7977109	1.01 (0.78–1.30)	0.946				
HNMT	rs11558538	1.33 (0.88–2.011)	0.157	European (Spain)	205	410	27837280
HMOX1	**rs2071746**	**1.37 (1.07–1.74)**	**0.01**	European (Spain)	205	445	26313808
HMOX1	rs2071747	1.44 (0.74–2.87)	0.259				
HMOX2	rs1051308	1.15 (0.89–1.49)	0.265				
HMOX2	rs2270363	1.11 (0.85–1.45)	0.425				
VDR	**rs731236**	**1.37 (1.07–1.76)**	**0.01**	European (Spain)	205	445	26632733
VDR	rs2228570	1.13 (0.88–1.46)	0.314				
ADH1B	**rs1229984**	**1.88 (1.26–2.79)**	**0.001**	European (Spain)	205	505	29045753
ADH1B	rs6413413	1.63 (0.51–5.81)	0.378				
NOS3	rs1799983	1.03 (0.82–1.31)	0.788	European (Spain)	273	325	33732155
NOS3	rs2070744	1.13 (0.90–1.42)	0.304				
NOS3	rs79467411	1.04 (0.79–1.36)	0.8				
VDR	rs2228750	1 (0.79–1.26)	0.995	European (Spain)	285	325	33219423
	rs731236	1.17 (0.93–1.47)	0.185				
	rs739837	1.24 (0.99–1.55)	0.063				
	rs7975232	1.11 (0.89–1.40)	0.347				
	rs78783628	0.82 (0.66–1.03)	0.088				
GC	rs4588	0.99 (0.78–1.26)	0.936				
GC	rs7041	0.86 (0.68–1.07)	0.173				

95% CI, 95% confidence interval; Gene, official gene symbol (for *SLC6A3* and *SLC11A2*, the aliases used in the candidate genes studies are listed in brackets); N, number; NR, not reported in publication; OR, odds ratio; $P_{nominal}$, nominal *p* value; PMID, PubMed identifier; SNP, single nucleotide polymorphism. Bold text indicates variants for which candidate gene studies reported significant association to RLS. For *MAOA*, "all" refers to association results from the combined analysis of both sexes while "female" refers to the results from females only.

Our GWAS dataset was well powered to replicate the positive associations reported for the four SNPs with close to 100% power for the odds ratio estimates of the original studies (Figure 2). The female-only dataset also had a power of 100% for rs909525 based on the odds ratio estimate for the *MAOA* uVNTR (odds ratio: 2.0; 95% confidence interval: 1.07–3.77). In general, our study had

Table 2. Single-variant association results in the International EU-RLS-GENE GWAS dataset

Gene	Variant	Effect allele	EAF	Beta (SE)	$P_{nominal}$
ADH1B	rs1229984	T	0.044	−0.065 (0.062)	0.29
HMOX1	rs2071746	A	0.58	−0.026 (0.025)	0.31
VDR	rs731236	G	0.39	−0.031 (0.024)	0.20
GABRR3	rs832032	T	0.21	0.044 (0.030)	0.14
$MAOA_{females}$	rs909525	A	0.67	0.018 (0.033)	0.86

Beta, effect size estimate from logistic regression analysis; EAF, effect allele frequency in the GWAS dataset; $P_{nominal}$, nominal p value; SE, standard error. In *MAOA*, the uVNTR had not been assayed in the genome-wide association study (GWAS) and single nucleotide polymorphism (SNP) rs909525 serves as a tagging SNP with allele A correlated with the long 4 and 5 repeat alleles, and allele G with the short 3 repeat allele of the uVNTR. *Females*, for *MAOA* results of the association analysis in females only is reported; for all other genes, results were extracted from the published International EU-RLS-GENE GWAS.

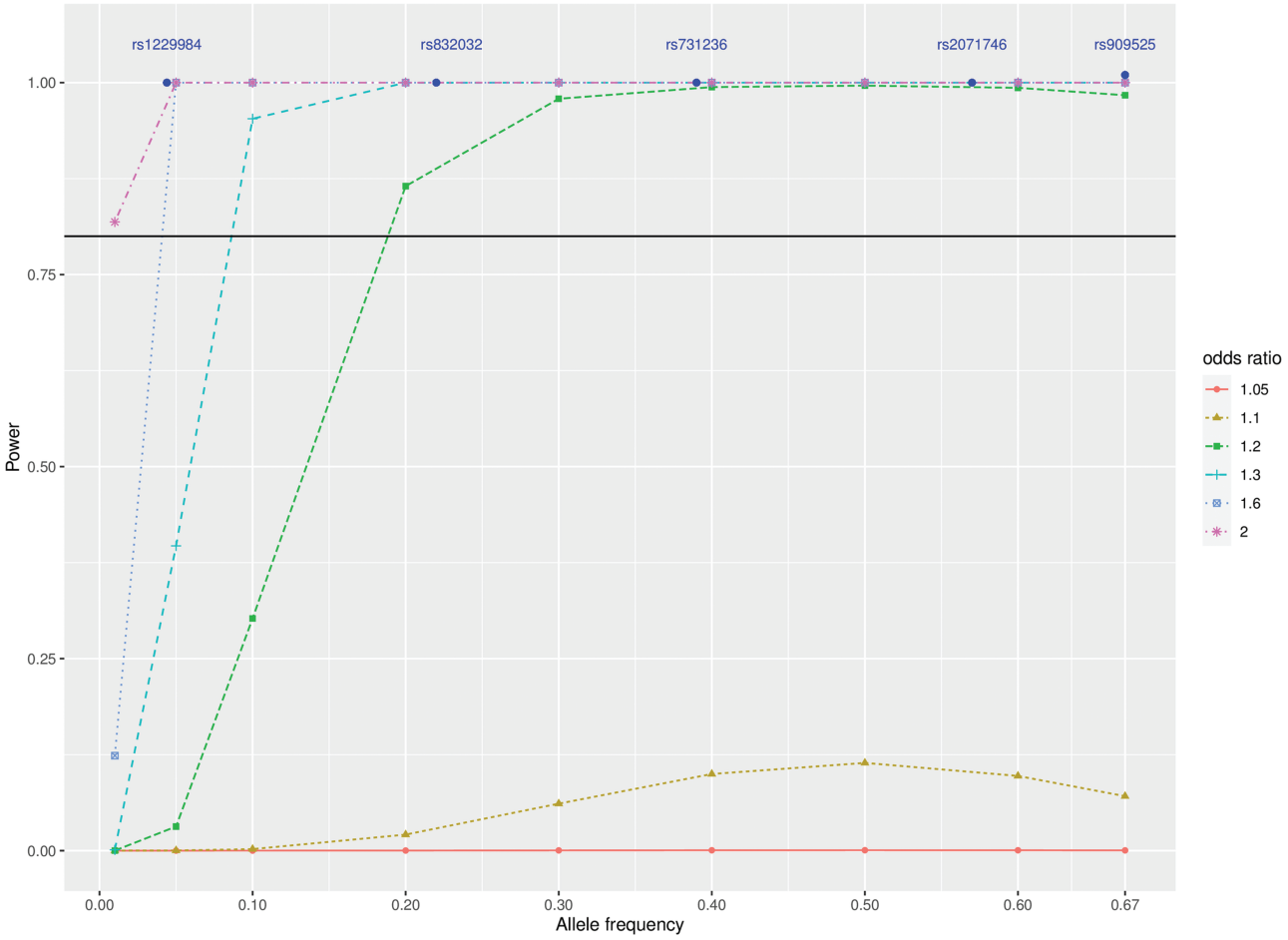

Figure 2. Study power analysis. Study power was calculated across a range of risk allele frequencies and odds ratios using a total sample size of 17 220 and a significance level of 5×10^{-8}. Allele frequency for the risk allele is depicted on the x-axis, and the power estimate is given on the y-axis. The solid horizontal line without any data points indicates a study power of 80%. The different line types and point shapes refer to the different odds ratios used in the power calculation. The labeled single dots refer to the five variants with nominally significant association results in candidate gene studies and show the study power for both adjusted and unadjusted odds ratios. For the proxy SNP rs909525 tagging the *MAOA* uVNTR, power calculations were done using the female-only sample size of 9098.

a power of at least 80% for common variants (minor allele frequency [MAF] > 0.2) with odds ratios of 1.2 or larger. For variants with lower MAFs, odds ratios of 1.3 or higher were needed for 80% study power (Figure 1). Published effect size estimates can be biased upwards due to winner's curse in discovery studies. Winner's curse refers to the fact that because genetic association studies usually report effect size estimates for variants with significant associations only, these estimates tend to be larger than the true effects [16]. Therefore, we ran additional power analyses using adjusted odds ratios, which confirmed the excellent power of our study for all SNPs (Figure 2 and Supplementary Table 2).

We also performed gene-based association tests for all candidate genes using the EU-RLS-GENE GWAS dataset. No gene was significantly associated after correction for multiple testing (significance threshold of $p \leq 0.0019$, adjusting for 27 genes) in a standard analysis with MAGMA including only variants in the gene body and the most proximal potential regulatory regions (Table 3). Neither extending the proximal region size from

Table 3. Gene-based association results for 27 candidate genes obtained with MAGMA

Gene symbol	Chr	Start	Stop	N SNPs	Z score	$P_{nominal}$
ADH1B	4	100227527	100242572	32	1.42	0.078
DBH	9	136501485	136524466	9	0.005	0.498
DRD1	5	174867675	174871163	6	−0.42	0.664
DRD2	11	113280317	113346413	179	−1.80	0.964
DRD3	3	113847499	113918254	221	−1.61	0.947
DRD4	11	637305	640706	7	1.51	0.066
DRD5	4	9783258	9785633	4	−1.31	0.905
GABRA4	4	46920917	46996424	249	0.14	0.444
GABRE	X	151121596	151143156	26	1.05	0.146
GABRQ	X	151806637	151821825	22	−0.04	0.516
GABRR1	6	89887223	89941007	264	1.78	0.036
GABRR2	6	89966840	90025018	216	0.24	0.407
GABRR3	3	97705527	97754148	114	−0.01	0.505
GC	4	72607410	72671237	157	0.42	0.337
HMOX1	22	35777060	35790207	32	0.20	0.42
HMOX2	16	4524719	4560348	97	1.07	0.142
HNMT	2	138721808	138773934	96	1.23	0.109
*MAOA**	X	43514155	43606071	97	−0.73	0.768
MAOB	X	43625857	43741721	26	−1.75	0.96
MAPT	17	43971702	44105700	728	−0.09	0.535
NOS1	12	117645921	117799607	447	0.32	0.373
NOS3	7	150688144	150711687	36	1.40	0.08
SLC11A2 (DMT1)	12	51373566	51422118	125	0.06	0.476
SLC1A2	11	35272752	35441610	559	−0.07	0.53
SLC6A3 (DAT)	5	1392905	1445543	132	−0.29	0.613
TH	11	2185159	2193107	5	0.30	0.381
VDR	12	48235320	48298814	174	−1.47	0.929

Chr, chromosome; End, genomic position of end of gene body for gene; *N* SNPS, the number of single-nucleotide polymorphisms annotated to the gene in the data; $P_{nominal}$, nominal *p* value for the gene; Start, genomic position of start of gene body for gene; *Z* score, *Z*-value for the gene. Genomic positions are based on genome build 37. The significance threshold after study-wise correction for multiple testing for 27 genes was $P_{nominal} \leq 0.0019$.
*The genome-wide association study data of females only were used for the analysis in MAGMA.

5 to 10 kb nor including also more distal regulatory elements by annotating enhancers to the genes nor using a different method for association testing (ACAT) changed these results (Supplementary Table 3).

Discussion

We performed the first GWAS-based evaluation of published candidate gene studies for idiopathic RLS in populations of European ancestry. Four out of the five variants which had been reported as significantly associated in these studies could be directly tested for replication in existing GWAS data. For the fifth variant, the uVNTR in *MAOA*, we performed association tests of a highly correlated SNP as a proxy. None of these variants reached even nominal significance in our study. Additional gene-based tests of the respective genes *HMOX1*, *VDR*, *ADH1B*, *GABRR3*, and *MAOA* were not significant either. Taken together, none of the published significant associations in candidate gene studies were replicated in our dataset.

One of the most common reasons for nonreplication is insufficient power due to a small sample size [32]. However, power analyses for our sample revealed a power of 98%–100% to detect association at genome-wide significance for the five variants with published positive results. This was the case both when using the original discovery odds ratios and when using odds ratios adjusted for winner's curse. This type of correction tends to bias the estimates downward, so we consider them to reflect the worst case scenario, not necessarily the exact true odds ratio estimate [16]. A second common reason for nonreplication is a difference of phenotype definitions used in the discovery and the replication efforts [32]. We addressed this issue by selecting only the International EU-RLS-GENE GWAS subset from our published larger meta-analysis dataset for this study. It represents the largest RLS GWAS in which all cases have been diagnosed by face-to-face interviews of expert clinicians based on the IRLSSG diagnostic criteria. This matched with the case ascertainment used in the candidate gene studies. These had also been conducted in a general sample of idiopathic RLS patients without selection based on family history, severity, or age at onset. Furthermore, genetic differences due to differing ancestries may also affect replication power negatively. Our study included samples of European ancestry only, thereby matching the continental ancestry and reducing the risk of nonreplication due to a different ethnic background. We had no samples from Spain in our study, raising the concern that more subtle differences within Europe may have contributed to the nonreplication. However, our GWAS consisted of samples from the north-east (Czech Republic) to south-west (France) of Europe and the GWAS associations were consistent across these populations, suggesting a rather conserved genetic architecture of RLS in European ancestry populations. Similar results have been described for other complex diseases, for which GWAS conducted in Spanish samples reproduced the associations identified in previous GWAS of other populations of European ancestry [33, 34].

Taking all these factors into account, the nonreplication of the five variants is most likely explained by false-positive associations reported in the candidate gene studies. One of the negative results confirmed by our study further underlines the reproducibility challenge posed by small-scale genetic association studies: We had previously reported a significant association with RLS for SNPs rs7977109 and rs693534 in *NOS1* in a targeted follow-up study of the linkage region on chromosome 12 [35]. Here, we had applied a study-wise multiple testing correction, but did not use the genome-wide significance level of 5×10^{-8}. A later candidate gene association study did not replicate these associations, but this could have been due to the small sample size [20]. However, the EU-RLS-GENE GWAS dataset was sufficiently powered (power > 80% for both SNPs with reported as well as adjusted odds ratios) and did not replicate these associations either. Therefore, the previously reported associations of rs7977109 and rs693534 were also likely false-positive results. Our nonreplication results are in line with observations for other disorders such as schizophrenia or depression, where most candidate gene and variant associations failed to replicate in larger studies [36, 37].

One limitation of our study is the lack of large-scale genetic data for populations of non-European ancestry. Therefore, we could not assess the signals reported in two candidate gene studies in Asian populations. These had reported single-variant and haplotype associations at the gene loci of bone marrow stromal cell antigen 1 (*BST1*) in a Chinese sample and neuronal PAS domain protein 2 (*NPAS2*) in a sample from South Korea, respectively [38, 39]. The bias towards European ancestry is a general issue in genetic studies and more work towards a more balanced representation of different ancestries is needed in the future. A further limitation is the fact that the VNTR variants were not directly measured in our GWAS dataset. However, we could address the only VNTR with a positive report by using a highly correlated proxy SNP as well as a corresponding gene-based test. Even though we could not test the variant itself, both substitute tests indicated nonreplication of the *MAOA* association to RLS. Although the gene-based tests provided good coverage of common variants due to the use of GWAS summary statistics of variants with a MAF ≥ 1%, they were not comprehensive. We could not test the contribution of rare variants and some common and low-frequency variants may not have been present in our GWAS dataset. Therefore, we cannot ultimately rule out the possibility that variants in these genes contribute to RLS susceptibility.

Finally, the phenotype information of our GWAS was limited; therefore, we could not perform any analyses with regard to family history, age of onset, or iron levels, which had been performed in some of the candidate gene studies. This a minor limitation, though, since the main aim of our study was to reassess reported associations for idiopathic RLS in the general RLS population.

In conclusion, none of the reported significant single-variant associations in *HMOX1*, *VDR*, *ADH1B*, *GABRR3*, and *MAOA* from candidate gene studies were replicated in our large, well-powered GWAS dataset. Therefore, we consider them false-positive associations. This is also the case for the signals in *NOS1*, for which we confirmed the nonreplication of the previously described association [20, 35]. Our results emphasize the need for large sample sizes and stringent significance thresholds in association studies. Even when study-wise correction for multiple testing is performed, sequential testing of variants in the same study population would translate to testing these variants all at once in terms of the multiple testing burden. This would have to be accounted for when setting the significance thresholds for a candidate gene study. Compared to single-variant association studies, joining forces in large consortia for discovery as well as replication efforts on a genome-wide scale seems more promising for identifying common risk variants for RLS.

Supplementary material

Supplementary material is available at *SLEEP* online.

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None declared.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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