Blood cis-eQTL Analysis Fails to Identify Novel Association Signals among Sub-Threshold Candidates from Genome-Wide Association Studies in Restless Legs Syndrome

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Abstract

Restless legs syndrome (RLS) is a common neurologic disorder characterized by nightly dysesthesias affecting the legs primarily during periods of rest and relieved by movement. RLS is a complex genetic disease and susceptibility factors in six genomic regions have been identified by means of genome-wide association studies (GWAS). For some complex genetic traits, expression quantitative trait loci (eQTLs) are enriched among trait-associated single nucleotide polymorphisms (SNPs). With the aim of identifying new genetic susceptibility factors for RLS, we assessed the 332 best-associated SNPs from the genome-wide phase of the to date largest RLS GWAS for cis-eQTL effects in peripheral blood from individuals of European descent. In 740 individuals belonging to the KORA general population cohort, 52 cis-eQTLs with \(p_{\text{nominal}}<10^{-3}\) were identified, while in 976 individuals belonging to the SHIP-TREND general population study 53 cis-eQTLs with \(p_{\text{nominal}}<10^{-3}\) were present. 23 of these cis-eQTLs overlapped between the two cohorts. Subsequently, the twelve of the 23 cis-eQTL SNPs, which were not located at an already published RLS-associated locus, were tested for association in 2449 RLS cases and 1462 controls. The top SNP, located in the DET1 gene, was nominally significant (\(p<0.05\)) but did not withstand correction for multiple testing (\(p=0.42\)). Although a similar approach has been used successfully with regard to other complex diseases, we were unable to identify new genetic susceptibility factor for RLS by adding this novel level of functional assessment to RLS GWAS data.

Citation: Schulte EC, Schramm K, Schurmanna C, Lichtner P, Herder C, et al. (2014) Blood cis-eQTL Analysis Fails to Identify Novel Association Signals among Sub-Threshold Candidates from Genome-Wide Association Studies in Restless Legs Syndrome. PLoS ONE 9(5): e98092. doi:10.1371/journal.pone.0098092

Editor: Mark R. Cookson, National Institutes of Health, United States of America

Received February 10, 2013; Accepted April 28, 2014; Published May 29, 2014

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Funding: Recruitment of the KORA cohort was supported by institutional (Helmholtz Zentrum München, Munich, Germany) and government funding from the German Bundesministerium für Bildung und Forschung (03.2007-02.2011 FKZ 01ET0713). SHIP is part of the Community Medicine Research net of the University of Greifswald, Germany, which is funded by the Federal Ministry of Education and Research (grants no. 01ZZ9603, 01ZZ0103, and 01ZZ0403), the German Center for Diabetes Research (DZD e.V.), the Ministry of Cultural Affairs as well as the Social Ministry of the Federal State of Mecklenburg-West Pomerania. This work is also part of the research project Greifswald Approach to Individualized Medicine (GANI_MED), which is funded by the Federal Ministry of Education and Research and the Ministry of Cultural Affairs of the Federal State of Mecklenburg-West Pomerania (03IS2061A). Genome-wide data have been supported by the Federal Ministry of Education and Research (grant no. 03SF00012) and the Federal State of Mecklenburg, West Pomerania. Whole-body MR imaging was supported by a joint grant from Siemens Healthcare, Erlangen, Germany and the Federal State of Mecklenburg West Pomerania. The University of Greifswald is a member of the ‘Center of Knowledge Interchange’ program of the Siemens AG and the CACHE.
Introduction

Restless legs syndrome (RLS) is a common sensory-motor disorder characterized by dysesthesias affecting the legs, triggered by periods of rest, relieved by movement and occurring mostly during the evening and at night. [1] Consequences are severe sleep disturbances, depression, anxiety and possibly also increased cardiovascular risk. [2,3] RLS is a complex polygenic phenotype and genome-wide association studies (GWAS) have identified a total of six genomic loci associated with the disease. [4–7] Still, the susceptibility alleles known to date only explain about 6.8% of the total heritability [6]. It is likely that additional risk loci of weaker effect sizes exist that have not yet been ascertained in the GWAS.

It has been shown that single nucleotide polymorphisms (SNPs) associated with complex genetic traits are more likely to have an effect on gene expression and, thus, represent expression quantitative trait loci (eQTLs). [8,9] The use of cis-eQTL analyses in prioritizing sub-threshold association signals for GWAS follow-up, has already been successfully employed with regard to several complex diseases such as Crohn’s disease [10], schizophrenia [12]. Accordingly, we sought to prioritize sub-threshold RLS association signals from an RLS GWAS [6] via cis-eQTLs in the human blood for follow-up association study seeking to highlight additional genetic factors involved in RLS.

Materials and Methods

Ethics Statement and Data Availability

The KORA and SHIP-TREND studies as well as the recruitment of the RLS case/control sample was carried out in accordance with the recommendations of the Declaration of Helsinki and was approved by ethics committees of the “Bayerische Landesärztekammer” and the Technische Universität München (for KORA and the RLS case/control sample) and the University of Greifswald (for SHIP-TREND). Written informed consent was obtained from each of the study participants. Due to ethics constraints, full expression and genotyping data sets cannot be made available to the general public. However, interested researchers can apply for access to all data (KORA: http://www.helmholtz-muenchen.de/en/kora-en/information-for-scientists/; SHIP: http://www.medizin.uni-greifswald.de/cm/fv/english/ship_en.html).

Study Design and SNP Selection

The objective of the study was to use blood-based cis-eQTL analysis as a filter in the identification of new RLS susceptibility factors from sub-threshold association signals from a previously published GWAS. We selected all SNPs with an association signal of \( \text{p}_{\text{nominal}} \leq 1 \times 10^{-3} \) (\( \text{q} \)-corrected, \( n = 332 \)) from a recently published RLS GWAS [7] for cis-eQTL analysis to identify SNPs linked to differential mRNA expression (cis-eSNPs). These 332 SNPs represented 197 loci containing a single SNP and 101 loci with two or more SNPs in very high linkage disequilibrium (LD; \( r^2 \geq 0.8 \)). cis-eQTLs based on all 332 SNPs were identified in 740 individuals belonging to the KORA general population-based study and, in parallel, in 976 individuals belonging to the SHIP-TREND general population-based study. The cis-eSNPs with \( \text{p}_{\text{nominal}} \leq 1 \times 10^{-3} \) present in both cohorts and not located at loci of published association with RLS [4–7] were replicated in an independent case/control sample (Figure 1) with the objective of identifying new RLS-associated SNPs.

Cohorts and Case/Control Samples

KORA F4 cohort (discovery sample 1). Based in southwestern Germany, KORA (Cooperative Health Research in the Region of Augsburg) is a regional research platform for population-based surveys and follow-up studies. Whole-blood samples used for expression analysis in this study were collected from 740 subjects aged 62 to 81 years as part of the KORA F4 survey between 2006 and 2008 [13,14].

SHIP-TREND cohort (discovery sample 2). SHIP (Study of Health in Pomerania in northeastern Germany) is a population-based project consisting of two independent cohorts, SHIP and SHIP-TREND [15,16]. For eQTL analysis, the SHIP-TREND cohort was used where a total of 976 samples, from individuals aged 20 to 81 years, with both imputed genotypes and whole-blood gene expression data were available [16].

Case/control sample for eSNP association study. The sample comprised 2449 German and Austrian individuals with RLS (average age 48.0±34.7 years, 70.7% female) and 1462 individuals belonging to the S4 survey of the KORA general population cohort [17] (average age 49.9±13.4 years, 51.7% female), who were not genotyped in the genome-wide phase of the GWAS [6]. Both case and control populations were entirely of European descent. In all patients, diagnosis of primary RLS was based on the diagnostic criteria of the International RLS Study Group [1] as assessed in a personal interview conducted by an RLS expert.

Genome-wide Genotyping (Discovery Samples 1 and 2)

As described previously [16,19], genome-wide genotyping of the KORA sample was performed on Affymetrix Genome-Wide Human SNP Arrays 6.0. SNPs with minor allele frequency (MAF) <5%, a call rate <98% or a significant deviation from Hardy-Weinberg Equilibrium (HWE) (\( p <1 \times 10^{-5} \)) were excluded. Genotyping of the SHIP-TREND cohort has been described in detail [16]. In short, participants of the SHIP-TREND cohorts...
were genotyped using Illumina HumanOmni2.5-Quad arrays. Arrays with a call rate below 94% as well as individuals with a mismatch between the reported and genotyped sex were excluded. Imputation of autosomal genotypes in the SHIP-TREND cohort was performed using IMPUTE v2.1.2.3 [18] against the 1000 genomes phase I (interim) reference panel released June 2011 (all ancestries panel, build 37).

Gene Expression Data and Quality Control (Discovery Samples 1 and 2)

Sample selection and preparation of whole-blood gene expression analyses in KORA F4 and SHIP-TREND have been described [16,19]. Briefly, in both studies blood was taken and stored in PAXgene blood RNA tubes, RNA was isolated using the PAXgene Blood miRNA Kit (Qiagen, Hilden, Germany) and reverse transcribed using the Illumina TotalPrep-96 RNA Amp Kit (Ambion, Darmstadt, Germany). The labelled cRNA was hybridized to Illumina HumanHT-12 v3 Expression BeadChip arrays and scanned using the Illumina Bead array reader. The GenomeStudio v2010.1 Gene Expression Module was used for quality control and to impute missing values. Subsequently, raw gene expression intensities were exported from Illumina’s GenomeStudio software to the R environment, where log2-transformation and quantile normalization where carried out.

After quality control (exclusion of individuals with less than 6000 significantly detected probes (p<0.01) and individuals with a reported vs. calculated gender mismatch), 740 KORA F4 and 976 SHIP-TREND samples with gene expression and genotype data where available for analysis.

Determination of cis- and Trans-eQTLs

The eQTL analysis was carried out for all 332 selected sub-threshold SNPs. For cis-eQTL analyses, all probes less than 500 kilobases (kb) away from the 332 selected SNP were used. Trans-eQTLs were determined for all probes more than 5 megabases (Mb) away from the SNP of interest.

Associations between the SNP and the respective mRNA probes were analyzed using a linear model with adjustment for age and sex. P-values were corrected using the Benjamini and Hochberg procedure.

Genotyping of Replicated cis-eQTL SNPs in Case/Control Replication Sample

Genotyping was performed on the MassARRAY system using MALDI-TOF mass spectrometry with iPLEX Gold chemistry (Sequenom Inc, San Diego, CA, USA). Genotypes were called with SpectroTyper 3.4. Genotype clustering was visually checked by an experienced evaluator. SNPs with a call rate <95%, MAF <5%, and Hardy-Weinberg p-value <1×10^-5 in controls were excluded. Known RLS-associated SNPs were not followed up in the replication. Associations were tested using the allelic test as implemented in PLINK [20].

Analysis of Brain-derived cis-eQTLs

The brain cis-eQTL status of all 23 replicated cis-eSNPs was analyzed using the NCBI GTEX cQTL browser (http://www.ncbi.nlm.nih.gov/gtex/GTEX2/gtex.cgi, accessed August 5, 2012; expression data from cerebral, frontal and temporal cortex and pons, n = 142 to 144) [22] and the seeQTL browser (http://gbrowse.csbio.unc.edu/cgi-bin/gle2/gbrowse/seeqtl/, accessed August 5, 2012; expression data from whole brain, n = 193) [23].

Results

RLS-associated SNPs are more likely to be cis-eQTLs

To test whether RLS-associated SNPs are more commonly cis-eQTLs than those not associated, we compared the number of cis-eQTLs among the 332 most significantly associated SNPs (all with \(p_{\text{nominal}} < 1 \times 10^{-5}\) from the latest RLS GWAS [6] to the 332 with the worst association p-values. MAF distribution was similar in both groups. Among the associated 332 SNPs, 52 cis-eQTLs (\(p_{\text{nominal}} < 1 \times 10^{-5}\)) were found while 37 cis-eQTLs were present among the 332 not-associated SNPs. After very stringent LD pruning (setting a threshold of \(r^2 < 0.05\)), which was necessary as there was significantly higher LD among the potentially associated SNPs compared to the not associated SNPs, we found evidence for an enrichment of cis-eQTLs \(p_{\text{nominal}} < 1 \times 10^{-6}\) in the associated vs. the not-associated SNPs (34 cis-eQTLs among 246 SNPs harboring the most significant association signals vs. 28 cis-eQTLs among 313 SNPs showing the least significant association signals; Fisher’s exact test, one-sided, p<0.05, OR = 1.63).

Analysis of Specific cis-eQTLs

Among the 332 best-associated SNPs, 52 cis-eSNPs with \(p_{\text{nominal}} < 1 \times 10^{-3}\) resulted in 45 independent cis-eQTLs were found when assessed in whole-blood samples from 740 KORA F4 controls. These 45 cis-eQTLs represent 33 loci with LD<0.4. Four SNPs (intronic rs17487227 in BARD1 as well as intronic rs6714954, rs7592599 and rs13307580 in SLCA15) represented cis-eQTLs of transcriptome-wide significance \(p_{\text{nominal}} < 1 \times 10^{-6}\) (Table 1). In the 976 SHIP-TREND samples, 59 cis-eSNPs with \(p_{\text{nominal}} < 1 \times 10^{-3}\) resulting in 46 independent cis-eQTLs and 29 independent loci were identified (Table 1).

Of the six known RLS loci [5,6,7,8], only SNPs located on chromosome 15q were cis-eSNPs with \(p_{\text{nominal}} < 1 \times 10^{-3}\) in both cohorts (Table 1). The expression change seen, however, did not affect the primary candidate genes at these loci but rather another gene in the vicinity.

Of the identified cis-eSNPs, 23 overlapped between the KORA and SHIP-TREND samples and eleven of these reached transcriptome-wide significance in either cohort but only rs17487227 in BARD1 reached transcriptome-wide significance independently in both cohorts. The 25 replicated cis-eSNPs contained nine that were dependent upon SNPs at the known RLS-associated locus on chromosome 15q (MAP2K3/SLCA15). None of these were associated with altered gene expression levels of 5OR1 and only two cis-eSNPs (rs4409954 and rs20670272) affected the gene expression levels of MAP2K3, the two candidate genes underlying the GWAS association signal at this locus. Instead, seven cis-eSNPs coincided with differential expression of CAEMLA, located approximately 400 kb upstream of the known locus.

The remaining 14 cis-eSNPs represented twelve individual loci as three SNPs (rs7592599, rs6714954 and rs13307580) located in SLCA15 all associated with decreased expression levels of two neighboring genes, ACU1 and MRPL53. Directions of differential expression concurred in 42.9% (6 out of 14) of cis-eSNPs in the two cohorts (Table 1).

Trans-eQTLs Linked to RLS-associated SNPs

We also assessed transcriptome-wide trans-eQTLs in the whole-blood samples for 13 SNPs known to be associated with RLS [4–7]. However, none of the trans-eQTLs identified in KORA F4 or SHIP-TREND were also found at \(p_{\text{nominal}} < 1 \times 10^{-3}\) in the respective other cohort (data not shown).
### Table 1. RLS-associated SNPs representing cis-eQTLs in peripheral blood.

| SNP           | Loci   | Gene expression altered | Association (p<sub>nominal</sub> from past GWAS) [7] | KORA cis-eQTL (p<sub>nominal</sub>) | KORA major allele | KORA beta (p<sub>nominal</sub>) | SHIP-TREND cis-eQTL (p<sub>nominal</sub>) | SHIP-TREND major allele | SHIP-TREND beta (p<sub>corrected</sub>) | Association Replication (p<sub>nominal</sub>) | Association Replication (p<sub>corrected</sub>) |
|---------------|--------|-------------------------|---------------------------------------------|---------------------------------|------------------|-----------------|---------------------------------|---------------------|--------------------------|---------------------------------|---------------------------------|
| rs17487827    | BARD1  | BARD1                   | 0.00021                                      | 1.02E-13                         | C                | −0.171         | 9.20E-17                        | C                    | −0.110                    | 0.255                           | NS                              |
| rs6746899     | intergenic | intergenic             | 0.00089                                      | 1.53E-08                         | A                | −0.172         | 1.61E-08                        | A                    | −0.130                    | no assay                        | NS                              |
| rs6714954     | NBC4   | MRPL53/AUP1             | 0.00021                                      | 2.22E-08                         | G                | −0.145         | 8.50E-07                        | G                    | 0.104                     | 0.148                           | NS                              |
| rs9920066     | DET1   | DET1                    | 0.00053                                      | 2.26E-08                         | T                | −0.071         | 2.31E-08                        | T                    | −0.054                    | 0.038                           | NS (0.418)                     |
| rs9354792     | CRS95314 | CRS95314               | 0.00078                                      | 3.20E-08                         | A                | 0.086          | 3.36E-10                        | A                    | 0.048                     | 0.521                           | NS                              |
| rs17125761    | intergenic | ERO1L                   | 0.00054                                      | 9.40E-07                         | T                | −0.091         | 4.85E-05                        | T                    | 0.066                     | 0.965                           | NS                              |
| rs11024433    | SERGEF | SAAL1                   | 0.00088                                      | 5.58E-06                         | G                | −0.074         | 1.35E-06                        | G                    | 0.051                     | 0.557                           | NS                              |
| rs7670748     | intergenic | intergenic             | 0.00113                                      | 7.86E-06                         | C                | 0.134          | 8.78E-12                        | C                    | 0.125                     | 0.499                           | NS                              |
| rs2029361     | intergenic | SMC4                   | 0.00095                                      | 9.95E-06                         | C                | 0.099          | 0.0003                         | C                    | 0.063                     | 0.385                           | NS                              |
| rs738415      | intergenic | BCO333837             | 0.00062                                      | 1.04E-05                         | G                | 0.142          | 1.49E-07                        | G                    | −0.119                    | 0.937                           | NS                              |
| rs8488643     | ZNF364 | ANKRID3S                | 0.00047                                      | 1.51E-05                         | G                | 0.072          | 4.41E-06                        | G                    | −0.055                    | 0.533                           | NS                              |
| rs28670272    | MAP2K5 | CALML4                  | 0.00025                                      | 0.00019                          | A                | −0.064         | 4.62E-06                        | A                    | 0.053                     |                                 |                                 |
| rs28670272    | MAP2K5/LBXCOR1 | CALML4     | 0.00025                                      | 1.93E-04                         | A                | −0.064         | 4.62E-06                        | A                    | −0.053                    |                                 |                                 |
| rs12593813    | MAP2K5 | CALML4/MAP2K5           | 1.49E-06                                     | 0.00022                          | G                | −0.059         | 3.65E-06                        | G                    | 0.048                     |                                 |                                 |
| rs638356      | KCNC3  | NAPSB                   | 0.00018                                      | 0.00034                          | T                | −0.125         | 1.64E-07                        | T                    | 0.116                     | 0.618                           | NS                              |
| rs448954      | MAP2K5 | CALML4                  | 8.26E-06                                     | 0.00038                          | G                | −0.057         | 1.32E-06                        | G                    | −0.052                    |                                 |                                 |
| rs1026732     | MAP2K5 | CALML4                  | 4.01E-06                                     | 0.00051                          | G                | −0.055         | 3.56E-06                        | G                    | 0.048                     |                                 |                                 |
| rs694696      | MAP2K5/LBXCOR1 | CALML4     | 3.70E-06                                     | 0.00042                          | G                | −0.056         | 3.23E-06                        | G                    | 0.049                     |                                 |                                 |
| rs448954      | MAP2K5 | MAP2K5                  | 8.26E-06                                     | 0.00044                          | G                | −0.057         | 0.00083                        | G                    | −0.035                    |                                 |                                 |
| rs28670272    | MAP2K5/LBXCOR1 | MAP2K5     | 0.00098                                      | 7.13E-05                         | A                | −0.049         |                                 | A                    | −0.045                    |                                 |                                 |

A total of 23 cis-eQTLs were found in both KORA F4 and SHIP-TREND at p<sub>nominal</sub> < 1 x 10<sup>-5</sup>. SNPs which were carried into the replication phase are printed in bold. NS = not significant.
Replication of Sub-threshold SNPs Representing cis-eQTLs

Twelve cis-eSNPs with $p_{\text{nominal}} < 10^{-3}$ in both the KORA F4 and the SHIP-TREND study were selected for replication in an independent sample comprising 2449 German and Austrian RLS cases and 1462 KORA general population-based controls. Due to technical reasons, intergenic SNP rs6746899 could not be included in the replication. One SNP in DET1 (rs9920066) showed nominally significant association ($p_{\text{nominal}} < 0.05$) but did not withstand Bonferroni correction ($p_{\text{corrected}} = 0.42$) while the other ten SNPs were not associated with the RLS phenotype in the replication sample (Table 1).

Expression in Brain

The relevance of blood-based cis-eQTLs or cis-eSNPs to neurologic and psychiatric diseases has been shown. [9] However, differences between blood eQTLs or eSNPs and brain-based eQTLs or eSNPs have also been demonstrated. [23] Therefore, we analyzed the brain cis-eQTL status of all 23 cis-eSNPs seen in both general population cohorts using the NCBI GTEx eQTL and the seeQTL browsers. None of the 23 brain cis-eSNPs were also cis-eSNPs with $p_{\text{nominal}} < 1 \times 10^{-3}$ in the cerebellum, frontal and temporal cortex orpons (n = 142 to 144) [22] or in whole brain (n = 193) [23].

Discussion

Blood cis-eQTL analysis has been successfully used in enhancing output from GWAS. [10,11,12] Here, we evaluated cis-eSNPs and cis-eQTLs linked to (potential) RLS susceptibility genes identified in previous RLS GWAS in order to prioritize sub-threshold candidates for follow-up evaluation.

Apart from one SNP in the de-etiolated 1 encoding gene DET1 (rs9920066) that reached nominal significance in the replication phase but did not withstand correction for multiple testing, we did not identify any novel susceptibility factors for RLS.

Next to the possibility that eQTLs in general participate very little in bringing about the RLS phenotype, it is possible that our study lacks the power to establish an association between the RLS phenotype and the SNPs underlying relevant eQTLs. Statistical power calculation using the Purcell Power Calculator [24] revealed that in order to replicate an association for one SNP such as, for example, DET1 rs9920066 (OR = 1.11 (95% confidence interval: 1.00–1.22)), with MAF = 0.30 at $\alpha = 0.05$, one would need a minimum of 5,767 cases and 5,767 controls to achieve 80% power.

Another caveat has to be that cis-eQTLs employed in selecting SNPs for replication were evaluated in peripheral blood and not a more disease-specific tissue. Although the underlying pathophysiology is not entirely clear, an involvement of the central nervous system in RLS pathophysiology seems likely. Evaluation of the 23 common cis-eSNPs in two human brain expression data sets showed that none of the blood cis-eQTLs were also found in the brain. Whether this is due to the smaller number of samples (347 brain samples vs. 1,716 blood samples) or the fact that cis-eQTLs depend on (potentially) RLS-associated SNPs in the peripheral blood do not overlap with those in the brain and are not functionally relevant for disease pathogenesis, remains unclear. It is known that eQTLs can be specific to developmental time points [22,23] and brain regions [21] and that they were, therefore, not detected in the available data. In this context, an RLS-linked common variant was recently shown to alter gene expression in the murine ganglionic eminences, the primordial basal ganglia, during development [25]. Accordingly, it will be of great interest to evaluate eQTLs in specifically this neuroanatomic region and at this developmental time point in the future.

Although none of the RLS-associated SNPs selected for follow-up could be replicated, two additional interesting aspects emerged. Firstly, one of the known RLS susceptibility loci on chromosome 15q [4–6] comprising RLS candidate genes MAP2K5 and SKOR1 harbored nine cis-eSNPs with $p_{\text{nominal}} < 1 \times 10^{-3}$. Two of these showed altered MAP2K5 expression dependent on the RLS-risk allele though in different directions in KORA F4 and SHIP-TREND, while none were related to altered expression of SKOR1. Interestingly, seven RLS-linked SNPs in MAP2K5 were further related to altered expression of calmodulin-like 4 (CALML4), a gene located approximately 400 kb upstream of the RLS-associated MAP2K5/SKOR1 locus. However, here, too, the direction of differential expression was not the same in both cohorts. Despite the fact that several studies have been successful in using cis-eQTLs to fine-map or provide functional support for specific genes at a GWAS locus [11,26,27], in our study, the situation is not as clear. It is possible that potential RLS-associated expression changes in CALML4 are due to SNPs in CALML4, which are in high LD with RLS-associated SNPs at the MAP2K5/SKOR1 locus. Alternatively, it cannot be excluded that variation in CALML4 instead of, or in addition to, MAP2K5/SKOR1 could play a role in RLS pathogenesis, as has been postulated in other complex traits such as the body mass index [28] or that these expression changes are artificial as they do not concur in the two cohorts.

Overall, we were unable to establish a new genetic susceptibility factor for RLS, although, at least in the case of DET1, this may be due to the lack of power to replicate alleles conferring only a small risk increase. Our study is challenged by the fact that cis-eQTLs were evaluated in peripheral blood and not a tissue of more pathophysiologic relevance to RLS. In the future, as the neuroanatomic correlates of RLS become more defined and more expression profiles of different brain regions become available, it will be interesting to assess whether the blood cis-eQTLs also play a role in brain-region-specific, RLS-allele-dependent eQTLs and in disease development.

Author Contributions

Conceived and designed the experiments: ECS KS CS BMM JW. Performed the experiments: ECS KS CS BMM. Analyzed the data: ECS KS CS PL BMM. Contributed reagents/materials/analysis tools: CG AP CT BH BF KB IF NG KSK WO CGB WP AZ TM. Recruitments and management of RLS cases: CT BH BF KB IF NG KSK WO CGB WP AZ TM.

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