

# Restless Legs Syndrome

## Confirmation of Linkage to Chromosome 12q, Genetic Heterogeneity, and Evidence of Complexity

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**Background:** Genes are involved in the etiology of restless legs syndrome, a common sensorimotor disorder.

**Objectives:** To replicate and to further characterize our previously reported chromosome 12q linkage results.

**Design:** Family linkage study.

**Setting and Participants:** A total of 276 individuals from 19 families have been examined using a selection of markers spanning the identified candidate interval on chromosome 12q.

**Results:** Two-point analyses of individual pedigrees indicated that 5 kindreds were consistent with linkage to chromosome 12q. When considering these 5 pedigrees along with the family in which linkage was originally reported, we observed a maximum 2-point logarithm-of-

odds score of 5.67 (at  $\theta=0.10$ ; for marker *D12S1636*; autosomal recessive) and a maximum multipoint logarithm-of-odds score of 8.84 between the interval defined by markers *D12S326* and *D12S304*. Furthermore, our results also suggest the presence of heterogeneity in restless legs syndrome as linkage was formally excluded across the region in 6 pedigrees. Interestingly, significantly higher periodic leg movements during sleep indices were observed for all probands with restless legs syndrome from linked families.

**Conclusions:** These results support the presence of a major restless legs syndrome–susceptibility locus on chromosome 12q, which has been designated as *RLS1*, and also suggest that at least one additional locus may be involved in the origin of this prevalent condition.

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**R**ESTLESS LEGS SYNDROME (RLS) is one of the leading causes of insomnia, affecting more than 5% to 10% of the white population.<sup>1-4</sup>

Clinically, this sensorimotor condition is characterized by motor restlessness as well as paresthesias-dysesthesias felt deep in the limbs, mostly in the legs, occurring preponderantly at rest and relieved by motor activity. The hallmark of the disease is the significant worsening of the symptoms in the evening or during the night, which often interferes with nocturnal sleep. Because of the incessant bedtime paresthesia, most patients are seen with delayed sleep onset and reduced sleep efficiency, which result in daytime somnolence, chronic sleep deprivation, and emotional disturbances in severe cases.

Although numerous causative factors have been proposed, the precise biological correlates underlying RLS remain unknown. Genetic contributions to RLS have been consistently recognized from popu-

lation, family, and twin studies.<sup>2,5-7</sup> Familial aggregation has been particularly well documented with more than 50% of the idiopathic cases reporting a positive family history for RLS.<sup>8-11</sup> In some pedigrees, it segregates in an autosomal dominant fashion,<sup>5,6,10</sup> with possible anticipation,<sup>5,6,10</sup> and a high penetrance rate (approximately 90%-100%).<sup>5,6,10</sup> However, remarkable interfamilial and intrafamilial phenotypic variation have been documented, ranging from a few episodic attacks per year to severe insomnia caused by incessant leg movements and paresthesias-dysesthesias.<sup>5,12</sup> This is also confirmed in our own studies, based on the investigation of a sample of more than 50 families with over 200 affected individuals (A.D., G.T., J.M., and G.A.R., unpublished data, 2005). Although 50% to 60% of RLS cases are idiopathic, it can be caused by anemia, renal failure, and peripheral neuropathy. Therefore, from many aspects of its clinical features, RLS can be considered as a complex trait. A recent study by Hening et al<sup>11</sup> showed that pa-

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tients with RLS who have a younger age of onset have an increased frequency of affected relatives, as high as 60.3% in first-degree relatives and 50.0% in second-degree relatives. This nonmendelian ratio, namely, a higher than expected ratio of affected-unaaffected relatives within families, could be owing to ascertainment bias; however, it may also indicate the complicated underlying genetic architecture of RLS.

Recently, we reported linkage in 1 large French Canadian (FC) family, positioning an *RLS*-susceptibility gene in a 15-centimorgan (cM) region on chromosome 12q (Online Mendelian Inheritance in Man \*102300).<sup>13</sup> Studying RLS in the FC population, where there is a well-characterized founder effect<sup>14,15</sup> and where the prevalence of RLS seems to be higher than in other populations,<sup>2</sup> may be advantageous to identify genetic risk factors for RLS. In the current study, we present further data supporting our previous findings in additional FC pedigrees with RLS. Our results also indicate that at least a second *RLS* locus may exist as linkage was formally excluded in 6 large pedigrees. In addition, we report results suggesting that some clinical features such as periodic leg movements during sleep (PLMS) indexes may explain part of the genetic heterogeneity.

## METHODS

### PATIENTS AND FAMILIES

The sample consisted of 276 genotyped individuals, including 146 affected individuals, 39 possibly affected individuals, and 91 unaffected family members from 19 clinically and genetically characterized multigenerational families with 4 to 19 affected or possibly affected individuals per family. In addition to the original kindred (sime001) that has been analyzed in a previous genomewide scan,<sup>13</sup> the investigated panel included 13 FC families ascertained through the Centre d'étude du sommeil, Montréal, Quebec, as well as 5 families unselected according to ethnic origin, who have been described previously.<sup>6</sup> French Canadian ancestry has been defined as having 4 grandparents of FC origin by history. Subjects were considered to be affected if they unequivocally exhibited the diagnostic criteria developed by the International Restless Leg Syndrome Study Group,<sup>16</sup> that is, imperative need to move the limbs associated with paresthesias-dysesthesias, motor restlessness, worsening of symptoms at rest with relief by movement, and worsening of symptoms during the evening or night. Many conditions known to cause RLS, such as anemia, renal failure, and peripheral neuropathy; or other sleep disorders such as narcolepsy, sleep apnea syndrome, and rapid eye movement sleep behavior disorder, were specifically investigated in both probands and family members and none of them were affected with these conditions.

### POLYSOMNOGRAPHIC MEASURES

Several measures have been used to support the clinical diagnosis of RLS. These clinical features were systematically investigated in all FC probands as described elsewhere.<sup>9</sup> Two indices of PLM were examined: the PLMS index, representing the number of PLMs per hour of sleep and the PLMW index representing the number of PLMs per hour of nocturnal wakefulness. Prior to polysomnographic (PSG) recordings, all FC subjects were investigated by means of the Suggested Im-

mobilization Test (SIT), a validated method used to quantify motor manifestations of RLS.<sup>17</sup> A movement index (SIT PLM index) was derived from the number of PLMs per hour of immobility. Polysomnographic procedures and methods for the administration of the SIT have been described in detail elsewhere.<sup>17</sup> The study protocol was approved by all of the institutional review board and each participant gave written informed consent.

## GENOTYPING

Genomic DNA was isolated from peripheral lymphocytes or from transformed lymphoblastoid cell lines.<sup>18</sup> All families have been investigated by use of a selection of markers spanning the critical region on chromosome 12q.<sup>13</sup> Microsatellite markers were amplified by polymerase chain reaction following standard protocols. Most markers were selected and ordered based on both the Marshfield genetic map and University of California, Santa Cruz, physical map (April 2003 freeze; Human Genome Working Draft [Available at: <http://genome.ucsc.edu>]). Primer sequences were obtained from the genome database. Amplification products were resolved by electrophoresis on a 6% denatured polyacrylamide gel and were visualized by autoradiography. The remaining amplimers were part of the ABI Linkage Mapping Set version 2.5 (Applied-Biosystems Inc, Foster City, Calif). The fluorescently labeled polymerase chain reaction products were pooled, separated by electrophoresis, and detected on an automated sequencing system (ABI model 377; Applied-Biosystems Inc). Alleles were scored using Genescan 3.7 and Genotyper 3.7 softwares (Applied Biosystems Inc).

## STATISTICAL AND LINKAGE ANALYSIS

Two-point linkage analysis was computed using the MLINK routine of the LINKAGE package, FASTLINK version 5.1.<sup>19-21</sup> The empirical significance level was assessed by simulations under the hypothesis of no linkage and genotypes were generated using SIMULATE.<sup>22</sup> The replicates were subsequently analyzed with the MSIM program of the SLINK software.<sup>22,23</sup>

The logarithm-of-odds (lod) score calculations were performed assuming the model that provided the most significant results in our previous study,<sup>13</sup> namely, the inheritance pattern was considered to be more likely as autosomal recessive with a common disease allele and a reduced penetrance of 0.8. An estimated phenocopy frequency of 0.005 was used throughout the analysis and the disease-allele frequency in the population was set at 0.25 ( $p=0.75$ ,  $q=0.25$ ,  $f_1=0.005$ ,  $f_2=0.005$ , and  $f_3=0.80$ —note that in our original article,<sup>13</sup> there was an error in the annotation used to report the model, but not in the actual parameters used, which are those reported above. These parameters have remained constant and have been used in all the analyses carried out to date with the recessive model). Recombination fractions were equal in both sexes. Multipoint analysis was performed by the SIMWALK2 program<sup>24</sup> using the computer facility of the United Kingdom Human Genome Mapping Project Resource Centre, Wellcome Trust Sanger Institute, Cambridge, England. Marker allele frequencies were based on data derived from all founder individuals for ethnic background.

To investigate the presence of clinical characteristics that could segregate as endophenotypes, selected clinical and PSG features were considered as dependent variables and their distribution was examined across families, using the appropriate test according to the nature of the variables. These parameters included age at onset, PLMW index, PLMS index, and movement index during the SIT.

**Table. Maximum lod Scores for Individual Linked Families Observed Across the Candidate Region on Chromosome 12q**

Marker	Genetic Distance, cM	Physical Position, Mb	Sime001		Sime002		Sime003		Sime025		Sime026		Sime044	
			$z_{max}$	$\theta_{max}$										
<i>D12S1064</i>	95.03	90.66	1.99	0.10	NA	NA	0.47	0.20	0.07	0.30	0.04	0.30	0	0.40
<i>D12S1044</i>	97.00*	94.01	3.5	0.05	0	0.40	0.14	0.30	0.14	0.10	0.15	0.10	0.80	0
<i>D21S1716</i>	101.45	96.78	0.04	0.40	0.05	0.20	1.06	0.10	0.15	0.10	1.19	0	-0.03	0.40
<i>D12S1300</i>	NA	98.33	3.17	0.05	0.28	0.20	0.58	0	0.39	0	0.43	0	0.80	0
<i>D12S346</i>	104.65	99.46	1.86	0.10	0.12	0.30	0.11	0.30	1.60	0	0.17	0.10	0.11	0.30
<i>D12S78</i>	111.87	104.10	2.11	0.10	NA	NA	NA	NA	0.51	0	1.36	0	1.09	0
<i>D12S1636</i>	114.28	105.66	2.05	0.05	1.25	0	0.59	0.20	0.77	0	0.92	0	0.81	0.10
<i>D12S1613</i>	116.08	107.47	2.27	0.10	1.68	0	-0.02	0.40	0.02	0.01	0.38	0	-0.01	0.40
<i>D12S1583</i>	119.55	109.72	3.08	0.05	-0.01	0.40	0.37	0.20	0.15	0.10	0.06	0.01	0.85	0.10
<i>D12S1646</i>	121.84	114.49	NA	NA	0.77	0	-0.03	0.40	1.30	0	0.09	0.10	2.05	0
<i>D12S86</i>	134.54	118.85	1.11	0.20	-0.03	0.40	0.41	0.20	0.02	0.40	0.18	0.10	2.29	0
<i>D12S1349</i>	139.61	122.00	0.43	0.30	NA	NA	NA	NA	-0.06	0.40	0.14	0.10	1.39	0
<i>D12S304</i>	144.83	125.50	0.18	0.30	-0.02	0.40	0.21	0.20	-0.02	0.40	0.14	0.10	1.09	0
<i>D12S1659</i>	155.94	129.05	0.65	0.20	0.13	0.10	-0.03	0.40	0.84	0	0.14	0.10	0.79	0

Abbreviations: cM, centimorgan; NA, not applicable;  $z_{max}$ , lod (logarithm-of-odds) score maximum;  $\theta_{max}$ , maximum recombination frequency. \*Inferred.

## RESULTS

### LINKAGE ANALYSIS

Although a brief inspection of most pedigrees suggests a mode of transmission compatible with an autosomal dominant mode of inheritance, in agreement with our original findings,<sup>13</sup> a recessive model with common disease alleles consistently yielded higher lod scores compared with dominant models with either common or rare disease alleles or a recessive model with a rare disease allele (data not shown). Therefore, only linkage results with a recessive model are presented here. To determine which families were linked to the chromosome 12q RLS locus, a panel of polymorphic microsatellite markers spanning the critical region was genotyped over the interval defined by markers *D12S398* and *D12S1638* (sime001).<sup>13</sup> Two-point linkage analysis of individual pedigrees indicated that 5 of the 18 additional families analyzed were consistent with linkage to chromosome 12q. These families are all of FC ancestry. For each of these kindreds, 2-point lod score values individually provided significant results for confirmation of our previous linkage findings, that is, a lod score of 1.0 or more, which has traditionally been the accepted threshold to replicate a previously identified locus<sup>25,26</sup>: sime002 ( $z_{max}$  = 1.68 at  $\theta$  = 0;  $P$  = .002 for *D12S1613*), sime003 ( $z_{max}$  = 1.06 at  $\theta$  = 0.10;  $P$  = .02 for *D12S1716*), sime025 ( $z_{max}$  = 1.60 at  $\theta$  = 0;  $P$  = .008 for *D12S346*), sime026 ( $z_{max}$  = 1.36 at  $\theta$  = 0;  $P$  = .002 for *D12S78*), and sime044 ( $z_{max}$  = 2.29 at  $\theta$  = 0;  $P$  = .001 for *D12S79* and *D12S86*). Results of pairwise lod scores for individual linked families estimated over the chromosome 12q-interval are given in the **Table**. When considering these 5 families together with the first family in which linkage was originally reported, we observed an accumulated  $z_{max}$  2-point lod score of 5.67 (at  $\theta$  = 0.10 for *D12S1636*).

Six other families, including 1 non-FC family<sup>6</sup> and 5 FC large multiplex pedigrees with RLS, yielded lod scores

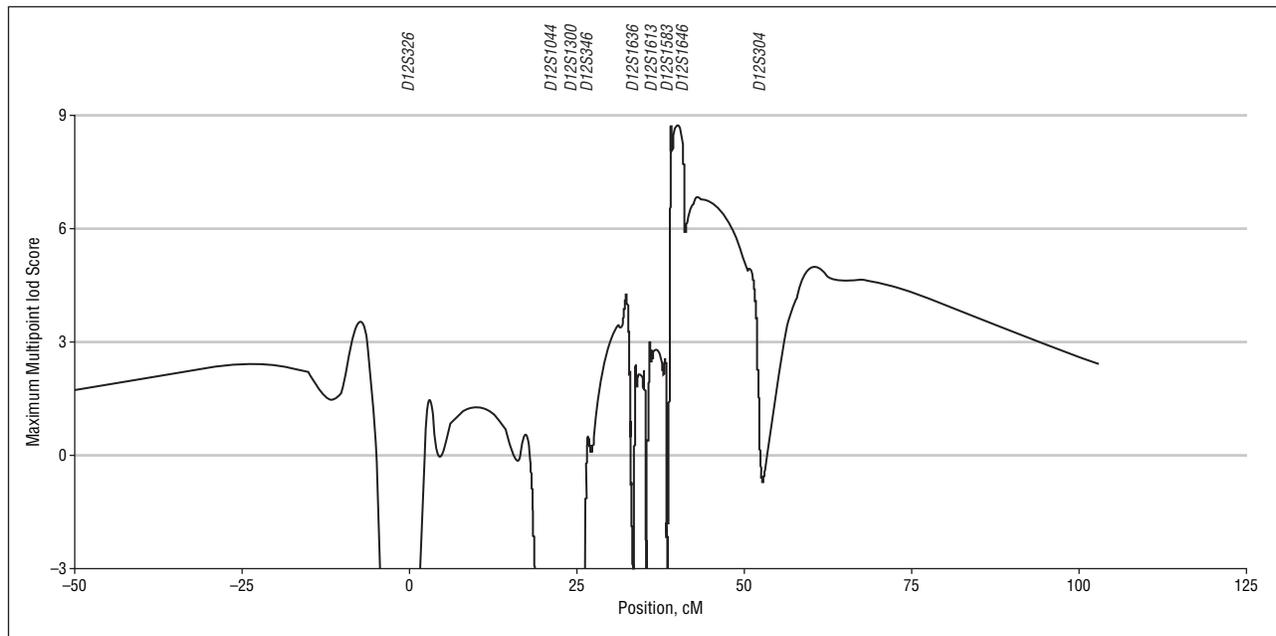
of less than -2.0 across the region, excluding linkage to the chromosome 12q RLS locus and suggesting evidence of additional RLS loci, whereas 7 other families (ie, 4 non-FC and 3 FC families) gave negative lod scores at  $\theta$  = 0 for nearly all markers analyzed within the interval but could not formally exclude linkage in this region (lod scores > -2.00, but < 0) (data not shown). Multipoint linkage analysis was further performed against a fixed map of 9 markers on chromosome 12q21-24 in the set of 6 linked families and provided a  $z_{max}$  lod score of 8.84 between the interval defined by markers *D12S326* and *D12S304* (**Figure 1**).

### DEFINITION OF A GENETICALLY HOMOGENOUS PHENOTYPE

To assess whether genetic heterogeneity found in our sample could be explained by phenotypic differences in families, we compared RLS clinical and PSG features between probands from linked and unlinked families. While there were no obvious between-group differences for criteria such as age at onset ( $t$  test = -0.33; 11 denominator  $df$  [ddf];  $P$  = .75), PLMW index ( $t$  test = -0.45; 7 ddf;  $P$  = .66), and SIT PLM index ( $t$  test = 0.82; 9 ddf;  $P$  = .44), we found a significant locus-specific effect on the PLMS index. Probands from linked families exhibited significantly higher PLMS indices than those from unlinked families (Mann-Whitney test = 34;  $P$  = .01) enabling us to differentiate between kindreds linked to chromosome 12q and other kindreds (**Figure 2**).

### COMMENT

The current study corroborates our previous findings supporting the presence of a major RLS locus on chromosome 12q, which we designated as *RLS1*. Linkage to *RLS1* has been observed in a subgroup of 6 families (31.6% of the total number of families investigated) in which the disease was found to be associated with high PLMS in-



**Figure 1.** Multipoint linkage analysis for the candidate locus relative to selected markers on chromosome 12q. The highest probability for location of the restless leg syndrome gene is between markers *D12S1583* and *D12S1646*. cM indicates centimorgan.

dices. However, our results also indicate that several other large pedigrees with sufficient power to detect linkage were not linked to chromosome 12q, suggesting that RLS is a genetically heterogeneous trait that is likely to be influenced by other genes in other chromosomal regions even among the FC population. This is also consistent with linkage studies from other groups, which failed to replicate our original findings in non-FC families.<sup>27,28</sup> Bonati et al<sup>28</sup> recently excluded *RLS1* locus on chromosome 12q but reported a new locus on chromosome 14q in 1 large Italian family with RLS. A more recent genome scan of 15 pedigrees with RLS reported another novel candidate region on chromosome 9p.<sup>29</sup> Genetic heterogeneity was somewhat predictable in the context of RLS given the high prevalence of the disease in some populations, including the FCs,<sup>2-4</sup> and the reported variability in clinical presentation.<sup>5,12</sup>

The fact that our most significant results were obtained under a recessive model may at first appear surprising since most pedigrees under study suggest segregation ratios that are consistent with a dominant mode of transmission with high penetrance. This paradox could be partly explained if one assumes, for instance, a pseudodominant mode of inheritance. This is a situation in which the real transmission is autosomal recessive, but given a high frequency of the disease allele(s), there is a higher proportion of affected individuals that approximates that seen in dominant conditions with affected subjects in all generations. For example, parkin gene mutations were first found in juvenile recessive Japanese families with parkinsonism<sup>30</sup>; further investigations discovered families with pseudodominant inheritance of parkin gene mutations.<sup>31-33</sup> A pseudodominant mode of transmission is conceivable in our sample considering that (1) the occurrence of RLS in the FC population is one of the highest in the world, (as high as 15%-20%)<sup>2</sup>; (2) only FC families were linked to this locus; and (3) a founder

effect has been well described and characterized in the FC population.<sup>14,15</sup> In addition, maximizing our results over different model parameters, such as changing estimates of penetrance and phenocopy rates, as well as decreasing the frequency of the disease gene did not substantially change our results, indicating that the lod score calculations were robust to parameter modifications in the autosomal recessive model.

One may speculate that the pseudodominant mode of inheritance may be also a consequence of an ascertainment bias that was introduced by recruiting exceptionally loaded families with RLS who have multiple affected members. In such kindreds the presence of intrafamilial allelic heterogeneity is highly plausible, as suggested, for instance, in the case of Parkinson disease discussed earlier. Following the original report of autosomal recessive juvenile parkinsonism,<sup>30</sup> further studies in large cohorts of patients with Parkinson disease have not only identified numerous additional mutations in the same gene but also uncovered a mixed mode of inheritance of different variants within and between families.<sup>34,35</sup> In addition, some mutations may act only as susceptibility alleles for late-onset forms of Parkinson disease.<sup>34</sup>

The 5 additional FC RLS pedigrees showed significant linkage results with the recessive mode of inheritance. However, the presence of likely phenocopies and nonpenetrants in all 6 linked families made it difficult to detect a common segregating disease haplotype between all the linked families, at least with our current resolution of markers (approximately 5.0-cM average between markers) within the region. Although it is possible that the difficulty in defining the candidate region be related to inaccuracies in recombination estimates consequent to the underlying genetic complexity, it is also possible that intrafamilial heterogeneity with mixed modes of transmission such as those observed in Parkinson dis-

ease may account for this difficulty. Further support for this hypothesis as well as for intrafamilial nonallelic heterogeneity comes from studies of other complex traits such as diabetes mellitus, hereditary hearing loss, and Alzheimer disease. All of these cases involve multiple loci and genes; each gene contains several disease-associated variants with different functional effects (Online Mendelian Inheritance in Man).

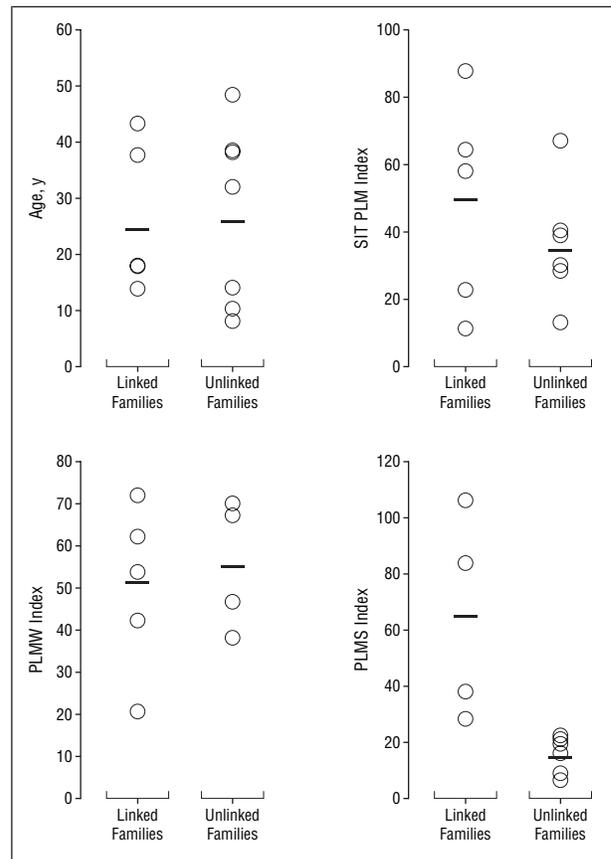
Certainly, a more accurate understanding of the contribution of *RLS1* to the genetic origin of RLS will have to await its isolation. Nevertheless, incomplete penetrance, phenocopy, between-family and likely intrafamilial locus heterogeneity, as well as potentially complicated allelic heterogeneity at *RLS1* locus all indicate that RLS is more likely a complex trait than a simple mendelian condition as previously suggested by different segregation analyses.<sup>5,6,10</sup>

The investigation of subclinical features that could be correlated with the chromosome 12 locus revealed significantly higher PLMS indices in probands with RLS from linked families. This is an interesting finding that reinforces the importance of careful phenotypic characterization in the study of complex traits and parallels the utility of clinical features such as age at onset in the successful identification of genes involved in conditions such as breast cancer and Alzheimer disease.<sup>36,37</sup> Whether this finding suggests that *RLS1* is more directly related to PLMS or that the presence of higher PLMS indices may indicate a more severe phenotype with increased familial loading will need to be further investigated by examining additional probands and family members.

A positional candidate approach has been previously undertaken by our group and allowed the exclusion of neurotensin as a potential candidate gene for RLS.<sup>38</sup> Other promising candidates located in the proximity of the mapped locus on 12q include (1) the nitrogen fixation clusterlike *N*-terminal-containing gene, which shows many functional features that are in agreement with the current pathophysiological models of RLS, in which iron disturbances have been repeatedly reported<sup>39-41</sup>; and (2) ataxin 2, which is a causative gene involved in spinocerebellar ataxia type 2 (SCA2). Ataxin 2 is relevant in the context of RLS since a recent study showed that 27% of 22 patients with SCA2 had RLS symptoms.<sup>42</sup>

## CONCLUSIONS

These results further support the involvement of an *RLS*-susceptibility locus on chromosome 12q in the FC population and also provide evidence that there must be other loci involved in this common sleep disorder. Furthermore, our findings illustrate that extensive characterization of subclinical differences represents a major tool in the identification of susceptibility loci for complex diseases. Given the findings of the current study, attempts at fine mapping the *RLS1* locus should probably focus, at least in the first instance, on individuals and families with high PLMS indices. Although the background of RLS is most likely complex, this finding may offer a new starting point for further dissecting the genetic cause of RLS.



**Figure 2.** Clinical and polysomnographic variables compared between probands from linked and unlinked families. A circle indicates each subject; a solid bar, the mean of each parameter; PLM, periodic leg movement; PLMS Index, the number of PLMs per hour of sleep; PLMW Index, the number of PLMs per hour of nocturnal wakefulness; SIT, Suggested Immobilization Test (the SIT PLM Index was derived from the number of PLMs per hour of immobility).

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