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A Genetic Risk Factor for Periodic Limb Movements in Sleep

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ABSTRACT

BACKGROUND

The restless legs syndrome (RLS) is a common neurologic disorder characterized by an irresistible urge to move the legs. It is a major cause of sleep disruption. Periodic limb movements in sleep are detectable in most patients with RLS and represent an objective physiological metric.

METHODS

To search for sequence variants contributing to RLS, we performed a genomewide association study and two replication studies. To minimize phenotypic heterogeneity, we focused on patients with RLS who had objectively documented periodic limb movements in sleep. We measured serum ferritin levels, since iron depletion has been associated with the pathogenesis of RLS.

RESULTS

In an Icelandic discovery sample of patients with RLS and periodic limb movements in sleep, we observed a genomewide significant association with a common variant in an intron of *BTBD9* on chromosome 6p21.2 (odds ratio, 1.8; $P=2\times 10^{-9}$). This association was replicated in a second Icelandic sample (odds ratio, 1.8; $P=4\times 10^{-4}$) and a U.S. sample (odds ratio, 1.5; $P=4\times 10^{-3}$). With this variant, the population attributable risk of RLS with periodic limb movements was approximately 50%. An association between the variant and periodic limb movements in sleep without RLS (and the absence of such an association for RLS without periodic limb movements) suggests that we have identified a genetic determinant of periodic limb movements in sleep (odds ratio, 1.9; $P=1\times 10^{-17}$). Serum ferritin levels were decreased by 13% per allele of the at-risk variant (95% confidence interval, 5 to 20; $P=0.002$).

CONCLUSIONS

We have discovered a variant associated with susceptibility to periodic limb movements in sleep. The inverse correlation of the variant with iron stores is consistent with the suspected involvement of iron depletion in the pathogenesis of the disease.

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THE RESTLESS LEGS SYNDROME (RLS) IS A common neurologic disorder involving both sensory and motor elements. The combination of symptoms is manifested clinically as an uncomfortable and distressing sensory urge during rest or inactivity in the evening and at night that delays the onset of sleep. Sleep is often interrupted by involuntary, highly stereotypical, and regularly occurring limb movements, called periodic limb movements in sleep. Despite a high prevalence of RLS in North America and Europe (5 to 15%), its pathogenesis remains unclear. RLS has a negative impact on quality of life and chronic medical conditions.¹⁻¹¹

Iron deficiency and iron excess have been associated with the pathophysiology of various brain disorders. A deficiency of iron affects brain development, and iron accumulation has been related to neurodegeneration. Forty-seven years ago, Ekbom reported that 25% of people with RLS have low serum iron levels and that 24% of those with iron-deficiency anemia have RLS.¹²

A genetic contribution to RLS has been well documented and is substantial.^{13,14} There is striking ethnic disparity in reported prevalence of the condition: 5 to 15% in the populations of western European ancestry, as compared with 0.1% of people in Singapore,¹⁵ 2% in native Ecuadorians,¹⁵ 3.2% of people in Turkey,¹⁵ and 4.6% in elderly Japanese.¹⁶ The prevalence in African populations is unknown.¹⁷

Identification of the genetic underpinnings of RLS has been challenging because of age-dependent expressivity; the influences of anemia, uremia, diabetes, and peripheral neuropathy; and possibly the modulation of phenotype by body iron stores.¹⁸ At the onset of disease, sensory symptoms are mild and punctuated by asymptomatic periods of variable duration. Although one third of patients are affected before the age of 20 years, clinical presentation typically occurs between the fourth and sixth decades of life, when sensory symptoms occur nearly nightly.¹⁹⁻²² Periodic limb movements in sleep, the motor component of RLS, are increasingly recognized as integral to the phenotypic spectrum. Limb movements, which are present in most patients with RLS,^{2,23} can predate the RLS sensory disturbance^{13,21,24,25} and are more common in otherwise asymptomatic family members of patients with RLS than in the general population.^{19,21}

METHODS

SUBJECTS

We obtained approval for the study protocol from the National Bioethics Committee and the Data Protection Authority (DPA) of Iceland. We contacted 451 persons who had answered a newspaper advertisement describing the clinical signs and symptoms of RLS. We then contacted 514 of their first-degree relatives. We surveyed 943 of the 965 subjects, using RLS questionnaires, and measured periodic limb movements; we obtained serum ferritin measurements for all 965 subjects. Written informed consent was obtained from all subjects. A nurse practitioner administered the questionnaire, which asked about the presence and severity of RLS symptoms²⁶ (according to the International Restless Legs Syndrome Study Group [IRLSSG] rating scale²⁷), clinical features, and coexisting conditions and collected venous blood for serum iron measurements and genotyping. The DPA encrypted all personal identifiers associated with information or blood samples with the use of the third-party encryption system developed by deCODE Genetics in collaboration with the DPA.²⁸ The Icelandic controls were chosen from persons who have participated in other genetic studies at deCODE Genetics (see the Supplementary Appendix, available with the full text of this article at www.nejm.org).

Subjects for the U.S. replication sample were recruited through the Program in Sleep at Emory University in Atlanta. An institutional review board at Emory approved the protocol, and all subjects provided written informed consent for inclusion in the study and for the collection of blood samples. Patients presenting to other general and subspecialty neurology clinics and their spouses were chosen as control subjects (as described in the Supplementary Appendix).

ASCERTAINMENT

The Icelandic portion of the study, which began in 2002, was designed to ask subjects questions regarding the four diagnostic criteria for RLS that were adopted in 1995.²⁶ These criteria were a desire to move the extremities, often associated with paresthesias or dysesthesias; motor restlessness; worsening of symptoms at rest with at least temporary relief during activity; and worsening of symptoms in the evening or at bedtime. In line

with a 2003 revision of the criteria, we removed the criterion of motor restlessness from the survey (Fig. 1 in the Supplementary Appendix). Thus, we considered subjects to be affected by RLS if they reported that at least two to four times per month, while at rest, they had an uncomfortable desire to move their legs that was relieved by movement and that predominated in the evening or at bedtime.

Under the best of circumstances, the sensitivities and specificities of subjective assessments regarding RLS do not exceed 0.80 to 0.90.¹ We therefore sought to enhance our discriminative abilities by measuring periodic limb movements in sleep and assessed the limitations intrinsic to our ascertainment strategy in Iceland by direct clinical examination of a subgroup of 123 Icelanders whose questionnaire responses conformed to the RLS consensus criteria (see data regarding phenotypic considerations in the Supplementary Appendix).

For detection of periodic limb movements, subjects wore a small (65-g), wristwatch-size triaxial accelerometer with 10-Hz sampling (PAM-RL detector, IM Systems) that was affixed by a hook-and-loop strap to their more affected ankle (or, as a default position, the nondominant ankle) for five consecutive nights. The PAM-RL provides an accurate assessment of polysomnographically derived frequencies (Pearson's correlation coefficient, 0.87; $P < 0.001$),²⁹ discriminates between periodic limb movements and normal nocturnal motor activity,³⁰ and is sensitive to treatment effects in subjects with RLS.^{7,31} The PAM-RL cannot discriminate between periodic limb movements that occur while subjects are awake and those that occur during sleep, but it can discriminate between movements that occur while the subject is recumbent and those that occur while the subject is upright. Thus, subjects who had more than five movements per hour for at least one night while recumbent during their major rest period (including sleep) were classified as having periodic limb movements in sleep (Fig. 2 of the Supplementary Appendix). Quantification of the frequency of movement was performed with the use of a software algorithm that accompanied PAM-RL version 7.5.70³² (see the Demographics section of the Supplementary Appendix). A diagram showing the overlap between the group with RLS and the group with periodic limb

movements in sleep is shown in Figure 3 of the Supplementary Appendix.

Ascertainment of RLS in the U.S. sample was based on the clinical judgment of an RLS specialist. All 188 genotyped subjects had periodic limb movements in sleep, according to ambulatory assessment with PAM-RL or polysomnographic measurements.

SERUM IRON LEVELS

Serum levels of soluble transferrin receptor and ferritin were assayed in 362 men and 603 women (subjects with RLS or their relatives) with the use of a clinical chemical analyzer and a ferritin reagents kit (Hitachi 912 Chemical Analyzer and Tina-quant kit, Roche Diagnostics). These measurements were used to derive the ferritin index (the ratio of soluble transferrin receptor to \log_{10} ferritin), which is inversely related to total-body iron stores.³³

ASSOCIATION ANALYSIS

For the discovery phase, samples were genotyped with the use of genotyping systems and software (Human Hap300 and Human Hap300-duo+ Bead Arrays, Illumina).³⁴ In total, 311,388 single-nucleotide-polymorphism (SNP) markers, distributed across the human genome, were common to both platforms. For the association analysis, we used 306,937 of the SNP markers; the other 4451 were deemed unusable because of low yield, deviations from Hardy-Weinberg expectations, or discrepancies in genotype frequencies between the two arrays. A total of 306 case subjects and 15,664 control subjects from Iceland were tested for an association with the 306,937 SNP markers. The genomewide significance threshold, after the Bonferroni correction for the number of SNPs tested, was set at 2×10^{-7} (approximately 0.05 of 306,937). Samples with a yield below 98% were excluded from the analysis.

In subsequent replication studies, the SNP rs3923809 was genotyped in an additional 123 case subjects and 1233 control subjects in Iceland and in 188 case subjects and 662 controls in the United States.

STATISTICAL ANALYSIS

For the association analyses, we used a likelihood procedure described previously³⁵ (see the Supplementary Appendix). Evaluation of statistical sig-

nificance took the relatedness of the subjects into account by applying a correction factor to the distribution of the chi-square test statistic. On the basis of the method of genomic control³⁶ and a simulation procedure using the known genealogy, which we had previous used (see the Supplementary Appendix), this correction factor was 1.117 for subjects in Iceland and 1.216 for subjects in the United States. We used the Mantel-Haenszel model³⁷ to combine results from the Icelandic and U.S. samples.

RESULTS

GENOMIC MARKERS

To minimize phenotypic heterogeneity, we focused our initial genomewide association analysis on 306 subjects with RLS who also had periodic limb movements in sleep. Two markers, rs3923809 and rs6923737, in an intron of the BTB (POZ) domain-containing 9 (*BTBD9*) gene on chromosome 6p21.2 (Fig. 1) showed genomewide associations that were significant (for rs3923809: odds

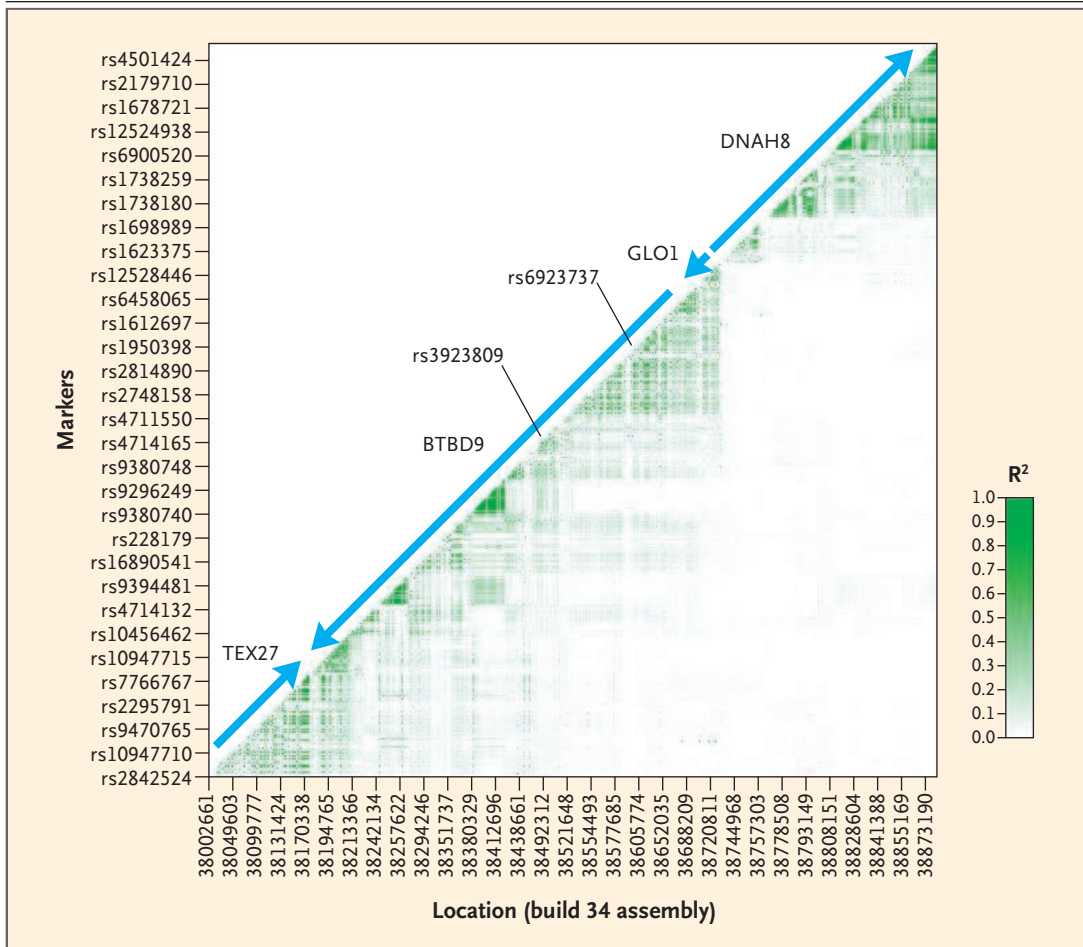


Figure 1. Linkage Disequilibrium at the *BTBD9* Locus.

The genomewide significant markers, rs3923809 and rs6923737, are located in a diffuse linkage-disequilibrium block overlapping portions of the BTB (POZ) domain-containing 9 (*BTBD9*) gene, the glyoxalase I (*GLO1*) gene, and markers in the promoter region of the dynein, axonemal, heavy polypeptide 8 (*DNAH8*) gene. The block is adjacent to the testis expressed sequence 27 (*TEX27*) gene. Data are from the single-nucleotide-polymorphism (SNP) International HapMap Project (release 19) of the Centre d'Etude du Polymorphisme Humain samples from Utah.

ratio, 1.8; $P=2\times 10^{-9}$; for rs6923737: odds ratio, 1.7; $P=1\times 10^{-7}$) (Table 1, and Fig. 1 and Table 2 of the Supplementary Appendix). After adjustment for rs3923809, the association with rs6923737 was no longer significant ($P=0.16$), whereas the association with rs3923809 remained significant after adjustment for rs6923737 ($P=0.001$). None of the other 70 SNPs in a 600-kb region around rs3923809 remained significant after adjustments for rs3923809 and for multiple testing. The association with rs3923809 remained significant after adjustment for each of the SNPs individually (Table 2 of the Supplementary Appendix).

To validate these results, we analyzed a second Icelandic sample of 123 subjects with RLS and periodic limb movements in sleep and 1233 controls. The results with the second sample significantly replicated the original results for rs3923809 (odds ratio, 1.8; $P=4\times 10^{-4}$) (Table 1, and Table 2 of the Supplementary Appendix). Extending the replication effort to a third sample of 188 subjects with RLS and periodic limb movements in sleep and 662 controls from the United States further confirmed the initial result for rs3923809 (odds ratio, 1.5; $P=0.004$) (Table 1). With all three samples combined, the association between the A allele of rs3923809 and RLS and periodic limb movements in sleep was highly significant (odds ratio, 1.7; $P=3\times 10^{-14}$). There was no significant deviation from the multiplicative model, which

assumed that the ratio of risk for homozygous carriers (AA) to heterozygous carriers (AG) was the same as the ratio of risk for heterozygous carriers to homozygous noncarriers (GG) in both the Icelandic subjects and the U.S. subjects (Table 3 of the Supplementary Appendix). The odds ratio for homozygous carriers was estimated at 3.2 for the Icelandic subjects and 2.3 for the U.S. subjects under the multiplicative model and at 4.3 for the Icelandic subjects and 2.0 for the U.S. subjects under the full model.

Among the 229 subjects who reported having RLS symptoms in the absence of periodic limb movements (35%), there was no association with the A allele of rs3923809 (odds ratio, 1.0; $P=0.81$) (Table 2). Conversely, among the 105 subjects who had periodic limb movements in sleep but who did not meet the RLS consensus criteria, there was an association with the A allele of marker rs3923809 (odds ratio, 2.3; $P=2\times 10^{-6}$) (Table 2). The odds ratio for this group did not differ significantly from that for the group that had RLS plus periodic limb movements in sleep ($P=0.19$). With the combined data from all the Icelandic subjects who had periodic limb movements in sleep (i.e., those with and those without RLS), the strength of the association (odds ratio, 1.9; $P=1\times 10^{-17}$) was greater than that for the group with RLS plus periodic limb movements in sleep alone.

Table 1. Association between Allele A of SNP rs3923809 and RLS with Periodic Leg Movements in Sleep among Subjects in Iceland and the United States.*

Population	No. of Case Subjects/ No. of Controls	Odds Ratio (95% CI)	Population Attributable Risk	Allele Frequency		P Value
				Case Subjects	Controls	
Iceland						
Discovery group	306/15,633	1.8 (1.5–2.2)	0.57	0.775	0.656	2×10^{-9}
Replication group 1	123/1233	1.8 (1.3–2.4)	0.56	0.772	0.657	0.0004
Combined group	429/16,866	1.8 (1.5–2.1)	0.57	0.774	0.656	2×10^{-12}
United States						
Replication group 2	188/662	1.5 (1.2–2.0)	0.46	0.766	0.681	0.004
Iceland and United States						
Combined group	NA	1.7 (1.5–2.0)	0.54	NA	NA	3×10^{-14}

* NA denotes not applicable.

Table 2. Association between Allele A of SNP Rs3923809 and RLS with or without Periodic Leg Movements in Sleep among Subjects in Iceland.*

Phenotype	No. of Case Subjects/ No. of Controls	Odds Ratio (95% CI)	Allele Frequency		P Value
			Case Subjects	Controls	
RLS with PLMs	429/16,866	1.8 (1.5– 2.1)	0.774	0.656	2×10 ⁻¹²
RLS without PLMs	229/16,866	1.0 (0.8– 1.2)	0.651	0.656	0.81
PLMs without RLS	105/16,866	2.3 (1.6– 3.2)	0.814	0.656	2×10 ⁻⁶
RLS	658/16,866	1.4 (1.2–1.6)	0.731	0.656	6×10 ⁻⁸
PLMs	546/16,866	1.9 (1.5–2.2)	0.783	0.656	1×10 ⁻¹⁷

* For the group of subjects reporting RLS symptoms without periodic limb movements in sleep (PLMs), no association with rs3923809 was detected. A total of 12 subjects with PLMs supplied incomplete answers on the RLS questionnaire and therefore were excluded from the categories of RLS with PLMs and PLMs without RLS.

We found that the frequency of periodic limb movements in sleep correlated with the presence of allele A of marker rs3923809 (Fig. 2A) and that AA homozygotes had almost twice as many limb movements per hour of sleep as did non-carriers ($P<0.001$) (Fig. 2B). The odds ratio for the group of subjects with the most severe symptoms (>20 movements per hour of sleep) was 2.0, whereas it was 1.0 for the group with the least severe symptoms (≤ 5 movements per hour of sleep) (Fig. 2A). No significant correlation was observed between allele A and the severity of RLS symptoms, as assessed on the basis of the IRLSSG rating scale ($P=0.35$) or the self-reported age at the onset of RLS symptoms ($P=0.73$).

OTHER RISK FACTORS

Female sex, advanced age, depletion of body iron stores, and western European ancestry were risk factors for RLS.^{38,39} To determine whether these factors interact with the at-risk variant, we analyzed them as covariates in conferring a risk of RLS. The risk of RLS and periodic limb movements in sleep conferred by allele A of rs3923809 in men was greater than that for women (odds ratio, 2.0 vs. 1.7), although the difference was not significant ($P=0.28$). A similarly insignificant trend was observed for the combined groups with periodic limb movements in sleep (odds ratio for men, 2.3; odds ratio for women, 1.7; $P=0.09$). The number of periodic limb movements in sleep was significantly higher after the age of 50 years than at a younger age ($P<0.001$ for both sexes), a finding that is consistent with a

previous study.²³ The difference was significantly less in women ($P=0.04$).

The principal clinical measures of iron availability are serum iron, transferrin iron-binding capacity, and ferritin. Serum soluble transferrin receptor, ferritin, total iron-binding capacity, and iron were assayed in 965 Icelandic subjects (subjects with RLS and their relatives). The ferritin index, a measure inversely related to body iron stores, was increased by 5.5% per A allele of marker rs3923809 (95% confidence interval [CI], 1 to 10; $P=0.02$). In line with this observation, serum ferritin levels were decreased by 13% per A allele (95% CI, 5 to 20; $P=0.002$) (Fig. 3).

DISCUSSION

The association between a sequence variant and subjects who have periodic limb movements without RLS — and the absence of such an association in subjects with RLS without periodic limb movements — suggest that we have identified a genetic determinant of periodic limb movements in sleep. Among the minority of subjects with periodic limb movements who did not fulfill the subjective criteria for RLS, sensory discomfort might have been absent — or the subjective RLS questionnaire for assessing the sensory component might have lacked sensitivity (see the Supplementary Appendix). Further study is required to determine whether the A allele of marker rs3923809 is associated with periodic limb movements outside the context of RLS. It also remains to be determined whether a sequence

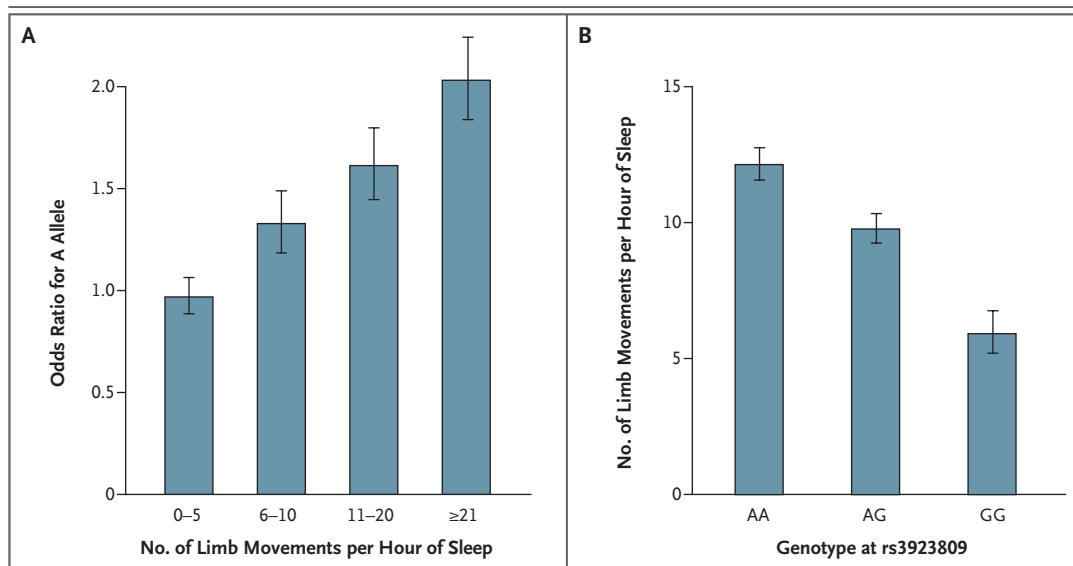


Figure 2. Correlation between the Frequency of Periodic Limb Movements in Sleep with the Presence of Allele A of Marker rs3923809 and of Homozygosity for the AA Genotype.

A total of 943 Icelandic subjects with RLS and their relatives were genotyped for marker rs3923809 and were evaluated for the frequency of periodic limb movements per hour of sleep. The subjects were then grouped into four categories on the basis of the frequency of limb movements, as follows: 271 subjects with 0 to 5 movements, 182 subjects with 6 to 10 movements, 212 subjects with 11 to 20 movements, and 278 subjects with 21 or more movements (Panel A, and data in the Supplementary Appendix). The odds ratio for having allele A of marker rs3923809 increased with the number of periodic limb movements, from 1.0 in the group with 5 or fewer movements to 2.0 in the group with 21 or more movements. Subjects were also grouped according to genotype into AA homozygotes (502 subjects), AG heterozygotes (371 subjects), and GG homozygotes (70 subjects) (Panel B). For marker rs3923809, the frequency of movements was greater in AA homozygotes than in AG heterozygotes ($P=0.003$) and greater in AG heterozygotes than in GG homozygotes ($P<0.001$). AA homozygotes moved nearly twice as often during each hour of sleep as did noncarriers ($P<0.001$). I bars indicate standard errors.

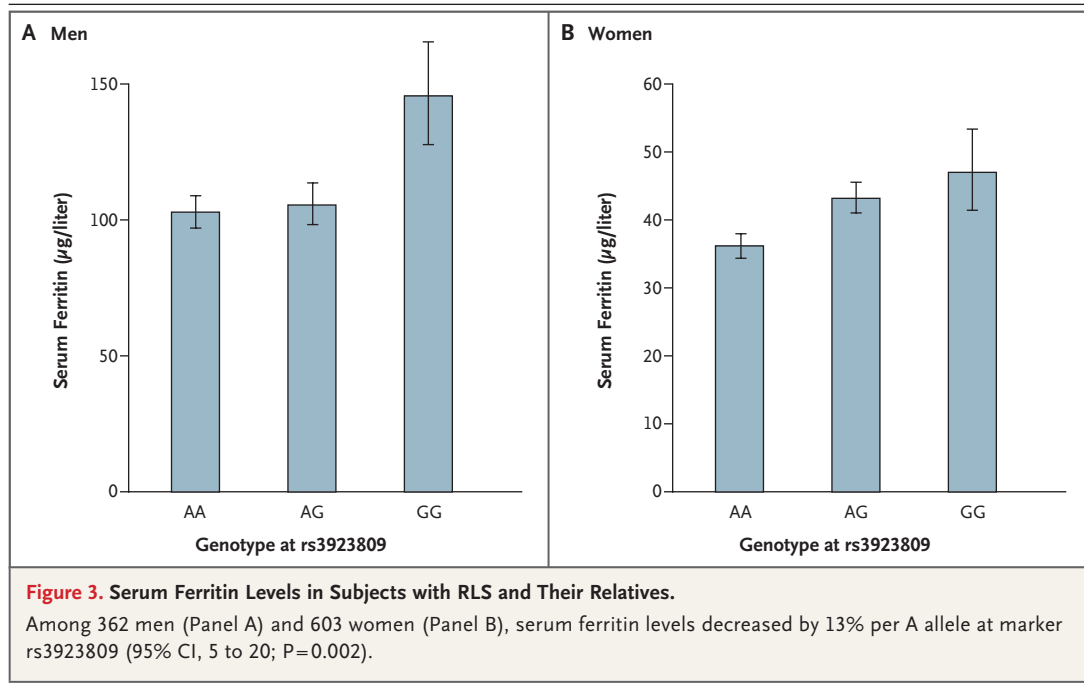
variant for RLS without periodic limb movements can be found.

The association between allele A of rs3923809 and RLS with periodic limb movements is evident in different populations of subjects with RLS: two population-based groups of mostly untreated Icelandic subjects, whose disease status was determined by self-report and ambulatory assessment, and U.S. subjects drawn from a sleep center, where the diagnosis rested on clinical judgment and ambulatory and laboratory assessment.

Although the authenticity of RLS has recently been questioned,⁴⁰ our study provides evidence that periodic limb movements in sleep is a genuine syndrome with an ascertainable phenotype and a genetic basis. In fact, our study provides insight into the manner in which common medical conditions are, and might come to be, defined in the genomics era. Given the prevalence of the “risk” SNP, the population attributable risk —

the fraction of cases that would be eliminated from the population if the risk among carriers were to be reduced to that among noncarriers — is approximately 50% for RLS with periodic limb movements on the basis of combined data from Iceland and the United States. However, the high population attributable risk does not eliminate the possibility that additional major susceptibility variants for the syndrome exist.

The results of the genomewide association analysis point to three genes: *BTBD9*, *GLO1* (encoding glyoxalase I), and *DNAH8* (encoding axonemal dynein heavy chain). *BTBD9* is widely expressed in parts of the brain, such as the amygdala, cerebellum, hippocampus, and caudate and subthalamic nuclei, and in other organs, such as the heart, kidneys, pancreas, and liver. The *BTBD9* protein is not well characterized, and its function has not been determined. It contains a BTB domain, also called POZ, which is known to be



a protein–protein interaction motif. GLO1 is a glutathione-binding protein involved in the detoxification of methylglyoxal, a by-product of glycolysis. Dyneins, on the other hand, are microtubule-associated motor protein complexes whose heavy chains are responsible for force production and ATPase activity. All three genes — *BTBD9*, *GLO1* and *DNAH8* — are candidates for affecting the risk of periodic limb movements in sleep. The SNPs that are most strongly associated with periodic limb movements and that also confer a risk of RLS are not in exons or known regulatory elements.

The discovery of sequence variants that are strongly associated with susceptibility to periodic limb movements in sleep, such as the one identified in our study, may lead to new approaches

for preventing or alleviating the symptoms associated with this condition.

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