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Association of low ferritin with PLM in the Wisconsin Sleep Cohort

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Abstract

Objective: The origins of periodic limb movements (PLMs), a strong correlate of Restless Legs Syndrome (RLS), are uncertain. This study was performed to assess the relationship between PLMs, and peripheral iron deficiency, as measured with ferritin levels corrected for inflammation.

Methods: We drew a cross-sectional sample of a cohort study of 899 men and women (n=1,245 assays, mean age 59.6±8.0) randomly selected from Wisconsin state employee agencies. A previously validated automatic detector was used to measure PLMs during sleep. Subjects were categorized into RLS symptoms-positive or RLS symptoms-negative based on a mailed survey response and prior analysis. Analyses were performed using a linear model with PLM category above and below 15 PLM/hour as the dependent variable, and adjusting for known covariates, including previously associated single nucleotide polymorphisms (SNPs) within BTBD9, TOX3/BC034767, MEIS1, MAP2K5/SKOR1 and PTPRD. Ferritin and C-reactive protein (CRP) were measured in serum, and ferritin levels corrected for inflammation using CRP levels.

Results—After controlling for cofactors, PLMI ≥ 15 was associated with low (≤50 ng/ml) ferritin levels (OR=1.53, p=0.009). The best model was found using quasi-least squares regression of ferritin as a function of PLMI, with an increase of 1.1 PLM/hr predicted by a decrease of 1 ng/ml ferritin (p=0.0026).

Conclusions—An association was found between low ferritin and greater periodic leg movements in a general population of older adults, independent of genetic polymorphisms, suggesting a role of low iron stores in the expression of these phenotypes. Subjects with high PLM index may need to be checked for iron deficiency.

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Disclosure Statement

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Keywords

Periodic limb movements; iron; restless legs syndrome; ferritin; Wisconsin Sleep Cohort; C-reactive protein

1. Introduction

Periodic limb movements (PLMs) are repetitive, involuntary limb movements that occur during sleep, most often in the lower extremities. PLMs are highly associated with restless legs syndrome (RLS), occurring 80–90% of the time in RLS patients(1, 2). Much research has been done to understand physiology of PLMs in RLS. In many cases, however, PLMs can be observed in isolation of RLS symptoms, notably in older adults, and there is current debate in the community on whether these can have health or behavioral consequences, such as cardiovascular problems(3) and daytime sleepiness(4, 5). For example, in the Wisconsin Sleep Cohort, a sample of older adults of mean age 59.6±8.0 year old, a prior study found that one-third had a PLM index above 15/hr (6), and similar figures are found in Osteoporotic Fractures in Men (MrOs), a sample of older males (7). PLM are also known to be associated with many other disorders and pathologies such as depression, rapid eye movement behavior disorder (RBD), narcolepsy, and Parkinson's disease(8–10).

Restless legs syndrome is a sensorimotor disorder affecting up to 10% of the population(5, 11) 12). It is characterized by an urge to move the limbs accompanied by uncomfortable sensations in the limbs, worsening at night and times of rest(11). RLS is classically differentiated in primary (idiopathic) and secondary cases. Secondary RLS is typically associated with low iron stores, anemia or kidney disease (12,13). Low levels of peripheral iron stores likely to play a role in causing RLS in these pathologies, with ferritin levels below 50 ng/ml (in the absence of associated inflammation that increases ferritin levels) used as a common cutoff to recommend iron supplementation. Iron supplementation may reduce RLS symptoms in patients with ferritin levels below 45 ng/ml (13, 14).

Reduced brain iron is believed to play a role in idiopathic RLS, as low levels in the substantia nigra are reported in these subjects(15). Further, reduced ferritin levels in cerebrospinal fluid (CSF) have been found in idiopathic RLS(16, 17). In these subjects, serum ferritin and CSF ferritin are correlated in both RLS and non-RLS subjects, though correlation is stronger in non-RLS patients(17).

In spite of these findings, it has been difficult to reconcile known genetic association results with the iron deficiency hypothesis, which involve mostly developmental genes(18). Polymorphisms associated with abnormal ferritin levels in genome wide association studies do not correlate with RLS(19). It is therefore possible that low iron is universally involved in the pathology of both primary and secondary RLS, although this is debated, and may involve effects in the CNS.

The gold standard for determining iron deficiency is examining the bone marrow for absent iron stores. Because of the invasiveness of the procedure and the unavailability of the subjects, blood tests are used instead. Serum ferritin has been shown to be the best screening

test for iron deficiency in older people(20). Iron deficiency, in the context of RLS, is usually defined as serum ferritin less than 50 ng/ml, though in a broader clinical context it is defined as below 15–30 ng/ml. Serum ferritin is an acute phase reactant, and in patients with acute or chronic inflammation, it may be artificially elevated(21), which obscures iron deficiency. For example, a patient with inflammation or liver disease can be iron deficient when ferritin is 70 ng/ml, as opposed to 45 ng/ml in the general population(22). As a result, many studies account for elevated ferritin levels by assaying transferrin saturation, which stays relatively constant throughout the inflammatory response, or CRP, another acute phase reactant.

Whereas much is known in the area of clinical RLS and iron regulation, virtually nothing is known regarding the relationship of iron status with PLMs, and even less so in a population based sample. There has been a study showing a negative correlation between iron and PLMs, but only in a small sample of children (23). In this study, we used the Wisconsin Sleep Cohort, a cohort of older adults, to explore the possible relationship of low ferritin levels with PLM events and RLS symptoms.

2. Methods

2.1. Demographics

The Wisconsin Sleep Cohort (WSC) study is an ongoing longitudinal community-based study designed to investigate sleep patterns and problems. The University of Wisconsin Health Sciences Institutional Review Board approved WSC protocols and informed consent documents. A random sample of men and women aged 30–60 was drawn in 1988 from a sampling frame of Wisconsin state employees. Participants were mailed questionnaires every five years that asked about their sleep and medical history. A sub-sample was studied every 4 years by in-laboratory polysomnography(24). Current mean age is 59.6±8.0.

2.2. PLMS

Periodic limb movements (PLMs) occur most frequently in the lower limbs. Typically they involve extension of the big toe in combination with partial flexion of the ankle, the knee and hip. To be counted as “periodic”, there need to be at least four movements in a 90 sec period. Contractions should last no less than 0.5 sec and no more than 5 sec. When they are recorded from both anterior tibialis muscles, they need to be separated by at least 5 sec to be scored as two separate movements(25). These movements may be associated with an autonomic arousal, a cortical arousal, or an awakening. PLMs are highly associated with RLS. Five or more PLMs occur per hour in 80% to 90% of RLS patients. Periodic limb movement index (PLMI) is calculated by dividing total number of PLMs by sleep time in hours.

In this study, we used the Stanford PLM Automatic Detector (S-PLMAD), an automatic detector of PLMs that was validated in the Wisconsin Sleep Cohort (26) and a clinical sample in relation to a gold standard. PLM indices derived from the detector, which do not include arousal-associated PLM events, correlated with self-reports of RLS symptoms in the cohort, as well as with most single nucleotide polymorphisms (SNPs) previously shown to

be associated with RLS (6). The detector was optimized to remove false signals from leg channels, such as ECG contamination, or fragmentary myoclonus-like patterns (26).

2.3. RLS symptoms

Subjects in the WSC were identified as having RLS symptoms using a previously published questionnaire response(24). However, the questionnaire did not address all needed diagnostic criteria(11); notably, it did not ask if the symptoms were worse at night. For this reason, subjects were designated as positive for these questions as having “RLS symptoms” and data used as such in prior published studies The questionnaire asked subjects to provide the frequency with which they felt (a) Repeated urge to move legs, (b) Strange and uncomfortable feelings in the legs, (c) periods of several leg jumps or jerks. The choice of frequency included never, less than once a month, monthly, weekly, and nightly. If the subject answered “never” for these questions they skipped ahead, otherwise two more questions were presented: (d) Do these feelings just mentioned get better when you get and start walking? (e) Do these feelings just mentioned disrupt your sleep? Based on extensive prior analysis, we defined four categories based on these responses. Category A (n=184), definite RLS symptoms, were defined as response to (a) weekly or more often, (d) yes, and (e) yes. Category B (n=185), possible RLS symptoms, was defined by responses (a) monthly or more frequent, (d) yes. Category B could not include members already in Category A. Category C (n=515), no RLS symptoms, was defined by the responses (a) less than monthly and (b) missing or less than monthly. Category D (n=171), unknown or uncertain, included the remaining response possibilities and cases with missing responses. Because all correlations were similar in subjects with RLS group A or B, subjects in these groups were considered with “possible RLS symptoms”. Group C, which did not endorse any symptom, was considered “negative RLS symptoms.” Subjects from group D were excluded from any analysis where RLS symptoms were used as covariates. Because we believe RLS symptoms are poorly characterized in this cohort, our work with the cohort focuses on PLMs.

2.4. Other Parameters

Additional parameters of interests were included in the analysis when they were known correlates of RLS, PLMs, or ferritin based on prior studies, including those from this cohort. Drugs that exacerbated RLS symptoms were grouped together: antidepressants, antipsychotics, tricyclic antidepressants (TCA), selective serotonin reuptake inhibitors (SSRI), SSRI antagonists, and antihistamines. Drugs that inhibited RLS symptoms were benzodiazepines, opioids, Parkinson’s medication, and anticonvulsives/gabapentin. Blood pressure medication was its own category.

2.5. RLS-associated polymorphisms

Fourteen SNPs from six loci, BTBD9 (chromosome 6), TOX3/BC034767 (chromosome 16), MEIS 1 (chromosome 2, two loci), MAP2K5/SKOR1 (chromosome 15) and PTPRD (chromosome 9) were used. Genotyping for 13 of these SNPs was performed on the MassARRAY system using MALDI-TOF mass spectrometry with the iPLEX Gold chemistry (Sequenom Inc, San Diego, CA, USA), as reported by Winkelmann *et al.* (18). The 14th SNP, rs11693221 (T) is a MEIS1 polymorphism recently shown to be more strongly associated with RLS than previously reported rs6710341 (G) or rs2300478(G) (27).

Genotypes from this SNP were derived from imputing Genome Wide Data from the cohort using Affymetrix 6.0 arrays.

2.6. Biochemical assays

Blood sampling was performed the morning after their corresponding sleep study. A ferritin ELISA kit (Eagle Biosciences, Nashua, NH, USA) was used for quantitative determination of ferritin in serum. A human C-reactive protein enzyme-linked immunosorbent assay (ELISA) kit (Alpha Diagnostic International, San Antonio, TX, USA) was used to quantitatively measure C-reactive protein (CRP) in the serum. Both assays were performed in duplicate. Ferritin and CRP are both acute phase reactants, and elevated levels of CRP are an indicator of inflated levels of ferritin. To remove the influence of inflammation, ferritin values were transformed (28): when CRP values were greater than 5000 μg/L, the corresponding ferritin value was converted by a corrective multiplier of 0.67.

2.7. Statistical Analysis

All statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, NC) and MATLAB (Mathworks, Natick, MA). Generalized estimating equations (GEE) were used to optimize power in the presence of multiple observations for most subjects, and mixed models were used to study association between ferritin, RLS and PLMI, with a robust covariance matrix used to estimate significance and effect sizes. We used the GENMOD procedure in SAS and the GEEQBOX toolbox (29) in MATLAB. GEE with a Markov correlation structure assumes that the correlation of repeated measures depends on the timing of their measurement, which was subject age in this study.

PLMI was used as a continuous variable and as a dichotomous one (with categories of PLMI≤15 and PLMI>15). A 15 PLM/hr cutoff was selected because it is commonly used in clinical studies, especially considering the older age of our population. Prior studies in the cohort have explored other cutoffs for RLS and SNP association with very similar results(6). Data on sex, age, body mass index (BMI), drugs exacerbating and inhibiting RLS symptoms, depression, RLS category, and genetic factors significant for RLS in this cohort were examined as potential confounders. Based on prior studies, RLS symptoms were split into RLS A+B (definitely RLS and RLS possible) versus RLS C (no RLS) (6). BMI was calculated as kilograms per meters squared. Quasi least squares (QLS) regression was used to assess statistical significance for continuous variables. Ferritin as a categorical variable was split into ferritin levels greater than or equal to 50 ng/ml and less than 50 ng/ml. GEE models and χ^2 tests were used to analyze categorical data. Associations for categorical variables were expressed as odds ratios (OR) and 95% confidence intervals (CI), while for linear effects changes in PLMI as a function of changes in ferritin were reported. A two-tailed p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Demographic and Clinical Data

Ferritin values were obtained for 1,245 samples, representing 899 subjects. Ferritin levels were only measured within 4 years of each other and of the response to the RLS

questionnaire. To determine factors associated with low ferritin levels, we dichotomized CRP-transformed ferritin less than 50 ng/ml versus CRP-transformed ferritin greater than 50 ng/ml, as this value is often used clinically. Results are presented in Table 1. Not all measures were usable, hence why number of observations may not sum to the same total across the tables.

Subjects with low ferritin were older, more frequently male in this cohort, and had slightly less sleep disordered breathing (independently of BMI). They also scored higher on the Zung depression scale, and took more medication known to exacerbate RLS (including antidepressants). In unadjusted comparisons, PLMI and RLS were not significantly different in subjects with low ferritin versus high ferritin (Table 1). Subjects with low ferritin also took less blood pressure medication, although they did not differ in percentage of subjects with high blood pressure.

RLS polymorphisms known to affect RLS and PLMI in this cohort were also examined by Ferritin category (Table 1), after correction for sex, age, blood pressure medication, Zung depression scale and AHI. Subjects with low ferritin had higher frequency of MEIS1 rs11693221 (T) and TOX3 rs3104767(G) (these associations are also found with RLS and high PLM in prior studies). The association of low ferritin with rs3104767 disappeared after controlling for PLMI, a factor previously known to be strongly associated with these polymorphisms, while the rs11693221 association remained significant (data not shown).

We next grouped our subsample into those with PLMI below 15 (n=650, 1022 observations) and greater than or equal to 15 (n=249, 465 observations) (supplementary table 1). Regression modeling showed that people in the high PLMI category were older and less male as found in the entire cohort(24). Looking at cardiovascular health factors, there was higher usage of blood pressure medication with high PLMI. All other factors were found to not differ significantly, including BMI or CRP (Table 1). Other analysis confirmed that PLMI was not associated with high CRP, including for subjects with PLMI≥ 45, unlike in a smaller prior study(3). As previously reported, in this sample with a larger number, many RLS SNPs were associated with high PLMI in the cohort (6). The most significant SNPs at each loci were TOX3/BC034767, rs3104788 (T); MAPS2k5/SKOR1, rs6494696(G); BTBD9, rs3923809(A); and MEIS1, rs12469063(G). These covariates were used in subsequent models exploring the effects of ferritin on PLMI. Factors that differed significantly in cases with and without low ferritin levels, such as the Apnea Hyponea Index, were also considered as covariates, but did not affect results (data not shown).

We also grouped patients into RLS-likely (n=546) and RLS-negative (n=709) and excluded patients that had “unknown” RLS status (supplementary Table 1). Medication that aggravates and inhibits RLS factors was more common in the RLS-possible group. Regarding SNPs, only TOX3/BC034767 rs3104774 (G) on chromosome 15 was increased in subjects with RLS but this was borderline (p=0.04). In the whole cohort, there were no SNPs that were significantly increased in subjects with RLS (6). These covariates were used in subsequent models exploring the effects of ferritin on RLS symptoms.

3.2. Influence of low ferritin on RLS symptoms and PLMI

Supplementary Table 2 reports on the effect of ferritin levels as a dichotomous variable (with cutoff of 50 ng/ml), and as a continuous variable on the presence of possible RLS symptoms (dependent variable), after adjustment for RLS covariates. Average ferritin levels did not differ significantly between the RLS-likely and RLS-negative categories, even after correction for CRP and covariates. Table 2 reports on the influence of ferritin on PLMI as the dependent variable as a dichotomous (PLMI cutoff of 15 movements/hr), after controlling for covariates identified in supplementary Table 1. As a dichotomous variable, PLMI≥15 was associated with low ferritin (≤ 50 ng/ml) both with adjustment for RLS symptoms (OR=1.53, p=0.009) and without (OR=1.35, p=0.04). Table 3 shows the relationship of ferritin and PLMI as continuous variables. Quasi Least Squares (QLS) regression indicates that there is a significant association between high PLMI and low ferritin ($\beta = -1.12$, $p=1.8 \times 10^{-3}$), with an increase of 1.12 PLM/hr in PLMI predicted by a decrease in ferritin of 1 ng/ml when adjusted with RLS symptoms. The association was also significant when the model was non-adjusted for RLS symptoms ($\beta = -1.14$, $p=2.6 \times 10^{-3}$).

4. Discussion

This study is the first evidence for a significant association between iron deficiency as measured by low ferritin, and PLMI in the general population, independent of genetic polymorphisms. In agreement with this finding, a prior study in children found a significant association of PLMS with low serum iron, and non-significantly lower ferritin levels (23). In our study, the effect was weakly statistically significant but effect size was notable, predicting an increase of 1.1 movements per hour per decrease of 1 ng/ml of ferritin. This effect was seen in the GEE model using ferritin as a dichotomous variable. It is likely that separating ferritin into ≤50 ng/ml and >50 ng/ml categories is more meaningful than using the entire spectrum of ferritin values with the linear model. This cutoff is used clinically so it has heuristic value.

We could not find a significant association between iron deficiency and RLS symptoms, with or without control of covariates. Iron deficiency has long been established as a secondary cause of RLS, although evidence for population based association of iron deficiency with RLS is lacking. The likely explanation for this unanticipated result is measurement error for RLS symptoms, as our questionnaire did not address all RLS symptoms. In contrast, measuring PLMI (a partial correlate of RLS), an objective PSG parameter is likely more accurate than a subjective, non-clinically-based assessment of RLS, explaining why we found a clear association of PLMI with low ferritin.

As reported in the GWAS study of ferritin levels, we did not find strong genetic associations between low ferritin and any RLS-associated SNPs, suggesting these genes do not impact ferritin directly (30). Of the nine candidate single nucleotide polymorphisms we examined in this study, only SNP rs11693221 in MEIS1 has a weak association with low ferritin which remained significant after adjusting for sex, age, and other confounding factors. It has been hypothesized that MEIS1 has a role in hematopoietic cell development(31). Although Stefansson *et al.* found lower ferritin in RLS subjects with BTBD9 rs3923809 (A) (32), the association was only within subjects with RLS, suggesting that maybe the low ferritin

interacts with the genetic polymorphism to exacerbate RLS symptoms, raising them to the level of severity consistent with a clinical diagnosis. Overall, RLS associated polymorphisms in this study were most strongly associated with PLMI, not ferritin or RLS symptoms.

In summary, we found an association between low ferritin and PLMI in the Wisconsin Sleep Cohort. In contrast, we were still unable to find a relationship between iron deficiency and RLS symptoms, probably because this phenotype was poorly ascertained. Overall, our results support the concept that RLS-PLMI is associated with low ferritin level independent of genetic factors. Subjects with high PLMI may need ferritin screening to exclude iron deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.

Demographics by ferritin level

	transformed ferritin≤50	transformed ferritin>50	R	p
Number of observations	378	1050		
Age	58.12 +/− 8.34	60.33 +/− 7.84	0.047	0.075
BMI	32.26 +/− 8.19	31.45 +/− 6.70	0.021	0.437
AHI	**11.83 +/− 15.04**	**13.60 +/− 16.23**	**0.078**	**0.004**
PLMI	15.90 +/− 21.91	14.15 +/− 18.29	−0.008	0.777
	transformed ferritin≤50	transformed ferritin>50	OR	p
rs9357271(T)	154 (40.7%)	395 (37.6%)	1.140	0.285
rs9296249(T)	144 (38.1%)	363 (34.6%)	1.165	0.220
rs3923809(A)	198 (52.4%)	511 (48.7%)	1.160	0.216
rs6494696(G)	198 (52.4%)	550 (52.4%)	1.000	1.000
rs2300478(G)	144 (38.1%)	401 (38.2%)	0.996	0.974
rs12469063(G)	156 (41.3%)	414 (39.4%)	1.080	0.531
rs6710341(G)	83 (22.0%)	267 (25.4%)	0.825	0.179
rs11693221(T)	**45 (11.9%)**	**83 (7.9%)**	**1.574**	**0.020**
rs6747972(A)	240 (63.5%)	718 (68.4%)	0.804	0.083
rs1975197(A)	99 (26.2%)	271 (25.8%)	1.020	0.885
rs4626664(A)	109 (28.8%)	268 (25.5%)	1.182	0.210
rs3104788(T)	230 (60.8%)	686 (65.3%)	0.825	0.119
rs3104774(G)	221 (58.5%)	673 (64.1%)	0.789	0.052
rs3104767(G)	**229 (60.6%)**	**697 (66.4%)**	**0.778**	**0.043**
sex	**239 (63.2%)**	**427 (40.7%)**	**2.509**	**<1E-13**
High blood pressure	84 (22.2%)	228 (21.7%)	1.030	0.838
blood pressure drugs	**147 (38.9%)**	**474 (45.1%)**	**0.773**	**0.035**
RLS-aggravating drugs	**171 (45.2%)**	**350 (33.3%)**	**1.652**	**<1E-4**
RLS-inhibiting drugs	51 (13.5%)	113 (10.7%)	1.293	0.153
Zung>45 (depression)	**118 (31.2%)**	**235 (22.4%)**	**1.574**	**<1E-3**
RLS	128 (40.4%)	401 (44.9%)	0.833	0.167

Table 2:

PLMI GEE model

	PLMI≥15	PLMI<15	β	OR	95% CI		P
Number of observations	446	979					
ferritin	167.06 ± 15.26	172.08 ± 10.95					0.727
ferritin, CRP corrected	151.50 ± 14.03	158.86 ± 10.61					0.777
ferritin, CRP corrected and adjusted**	**113.36 ± 19.49**	**146.19 ± 14.44**	**0.302**	**1.353**	**1.013**	**1.807**	**0.041**
ferritin, CRP corrected and adjusted + RLS**	**114.10 ± 19.58**	**148.30 ± 15.75**	**0.422**	**1.526**	**1.111**	**2.093**	**0.009**

** sex, age, blood pressure drugs, Zung, rls_aggravators, rls_inhibitors, rs3104774

Sleep Med. Author manuscript; available in PMC 2021 March 23.

Table 3:

QLS regression with ferritin (CRP-transformed)

dependent variable	β	p
PLMI (covaried with factors*)	**−1.12**	**0.0018**
PLMI (covaried with factors and RLS)	**−1.14**	**0.0026**
RLS (covaried with factors**)	5.67E-08	0.9789
RLS (covaried with factors ** and PLMI)	9.99E-08	0.9703

* sex, age, blood pressure drugs, Zung, rls_aggravators, rs12469063, rs6494696, rs3923809, rs3104788

** sex, age, blood pressure drugs, Zung, rls_aggravators, rls_inhibitors, rs3104774