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Systemic Inflammation in Association with Periodic Limb Movements of Sleep

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Systemic Inflammation in Association with Periodic Limb Movements of Sleep
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An abstract of

A thesis submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Master of Science

in Clinical Research

2011

Abstract

Systemic Inflammation in Association with Periodic Limb Movements of Sleep

By Lynn Marie Trotti

Systemic inflammation is associated with cardiovascular disease. While restless legs syndrome (RLS) is also known to be associated with cardiovascular disease, the mechanism for this relationship is unknown. We evaluated the association between periodic limb movements (PLMs) and inflammatory markers as a possible mediator underlying this relationship.

A cross-sectional study of inflammation and PLMs was performed using a retrospective sample of 350 subjects evaluated for RLS/PLMs. PLMs were measured by actigraphy (n = 167) or polysomnography (n = 237). Subjects' demographic and clinical features were collected from existing databases. Banked plasma was assayed for CRP by nephelometry and for IL-6 and TNF-alpha by fluorokine multianalyte profiling. CRP was categorized as low-normal (<3 mg/L) or high (3-10 mg/L). IL-6 and TNF-alpha were divided into quartiles and lowest versus highest quartiles compared.

In subjects with actigraphically-measured PLMs, mean PLM/hour was significantly higher in the high CRP group (40.2/hr vs 26.1/hr, p = 0.04), but did not differ based on IL-6 or TNF-alpha quartile. No association was seen between polysomnographically-measured PLMs and the inflammatory markers. In an unadjusted logistic regression model using actigraphy subjects, the OR for each PLM/hr was 1.015 (95% CI 1.003, 1.03). Adding age, gender, and race did not substantially alter the estimate of the effect of PLMs (OR = 1.016), but improved predictive value (likelihood ratio test 9.23, p = 0.03). Further adding clinical conditions known to affect inflammation or cardiovascular disease risk did not substantially change the estimate for PLMs (OR = 1.015) and did not improve the predictive value (LRT = 8.76, p = 0.27), but resulted in the estimate of PLMs' effect becoming marginally non-significant (95% CI 0.999, 1.03). Body mass index, sleep length, RLS severity, current smoking, and ferritin could not be fully evaluated due to missing data, but did not appear to strongly confound the PLM-CRP association.

PLMs are associated with increased CRP, with each single PLM per hour corresponding to a 1.5% increase in odds of elevated CRP. After controlling for relevant confounders, the relationship between PLMs and CRP remains apparent. Further investigation into the relationship between PLMs and inflammation is warranted.

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Introduction

Periodic limb movements of sleep (PLMs) are a common motor phenomenon. Movements can vary but typically consist of dorsiflexion of the great toe and ankle. These occur with a periodicity of 5-90 seconds overlain on a circadian tendency to occur early in the sleep period (1). Most commonly associated with restless legs syndrome (RLS, in which they are present in over 90% of subjects), PLMs can also occur in isolation (2-3). The significance of isolated PLMs is debated, but recent literature has drawn attention to the possible role of PLMs and/or RLS symptoms in the generation of cardiovascular disease risk. PLMs occurring in the context of RLS are associated with prevalent hypertension, and each leg movement, whether occurring in patients with or without RLS, is associated with transient increases in heart rate and blood pressure (4-7). More broadly, the syndrome of RLS (without respect to the presence of absence of PLMs) has been shown to be associated with prevalent cardiovascular disease in three separate, population based studies (8-10). The mechanisms for this association are unknown, but the role of PLMs in mediating this risk is suggested by the physiologic changes that accompany these movements.

Cardiovascular risk can be generated through multiple mechanisms, including systemic inflammation, endothelial dysfunction, and increased sympathetic tone. Multiple markers of systemic inflammation are available, of which C-reactive protein (CRP) is most well-studied with relation to cardiovascular disease (11-12). Additional markers include tumor necrosis factor alpha (TNF-alpha) and interleukin-6 (IL-6). All three markers have been shown to be associated with cardiovascular risk and can be

elevated in conditions of sleep deprivation or sleep disorders (13-26). These markers have not been evaluated in association with PLMs. Therefore, we measured CRP, TNF-alpha, and IL-6 in patients with RLS, PLMs, or both to determine whether there is an association between increased numbers of PLMs and increased inflammation. We enrolled 350 patients, in whom PLMs were measured either by leg accelerometry (n = 167), surface electromyography during polysomnography (n = 237), or both. For those markers shown to vary with PLM counts, a logistic regression model was used to determine whether PLM counts predicted elevated levels of the inflammatory marker, after controlling for relevant confounders. This is the first study to evaluate the relationship between PLMs and systemic inflammation. If positive, it will provide preliminary evidence for a potential mechanism by which PLMs increase risk for cardiovascular disease, partially explaining the increased cardiovascular disease risk in people with RLS.

Background

Periodic Limb Movements and Restless Legs Syndrome

Periodic limb movements (PLMs) are recurrent leg movements occurring with a periodicity between 5 and 90 seconds (1). Although there is substantial variability of the movement quality, movements most typically involve flexion of the great toe and dorsiflexion of the knee and hip. Prevalence estimates for PLMs range from 5-11% for rates $\geq$5/hr (27). PLMs are most commonly seen during sleep, but may be present during wake in subjects who have co-morbid restless legs syndrome (RLS). RLS is a sensory phenomenon occurring during wake that consists of urges to move the legs that remit with movement, occur in the late evening and night, and worsen with rest (28). PLMs are considered supportive criteria for the diagnosis of RLS, and are seen in over 90% of RLS subjects (2). However, PLMs frequently occur in the absence of RLS, being seen in narcolepsy, rapid eye movement behavior disorder, and in otherwise asymptomatic individuals (3).

PLMs can be measured in one of two ways. Use of an actigraphy monitor (a tria-axial accelerometer) placed on the ankle allows recording of multiple consecutive nights of data in the home setting but cannot distinguish whether events arise from sleep or wake. Use of surface electromyography (EMG) over the anterior tibialis muscle during routine polysomnography (PSG) allows conclusive determination of sleep-wake state. However, given the expense of PSG, typically only a single night of monitoring is used for diagnosis. When actigraphy and PSG measurements of PLMs are collected on the same night, PLM counts are well correlated ($r = 0.87$, $p < 0.0001$) (29). However, the nightly variability of PLMs is substantial. When studied for up to 15 nights, subjects

exhibit a mean difference in hourly PLM counts of 25. Capture of 4-5 nights of data is required to approximate the results obtained with 10 nights of monitoring, and one night of PLMs monitoring will incorrectly classify a substantial proportion of RLS subjects as negative for PLMs who truly have PLMs on another night (2).

RLS and PLMs as risk factors for cardiovascular disease

In addition to the role of RLS in decreasing quality of life, recent work has implicated RLS and PLMs in increasing cardiovascular disease risk (30). RLS has been shown in three large, population-based studies to be associated with prevalent cardiovascular disease (CVD). In the 3433 subjects of the Sleep Heart Health Study, the odds ratio for CVD was 2.05 (95% CI 1.38, 3.04) for those with RLS versus those without (9). In the 2821 subject Wisconsin Sleep Cohort, the odds ratio was 2.58 (1.38, 4.84) for CVD in those with daily RLS symptoms compared to those without RLS (8). In a study of 2608 men in Sweden, a similar odds ratio of 2.5 (1.4-4.3) was found for CVD in those with versus without RLS (10).

These cardiovascular risks are of the same order of magnitude as those attributable to obstructive sleep apnea (OSA), a known vascular disease risk factor, yet the underlying biological substrate for the increased risk in RLS is not known (31). However, PLMs are a plausible candidate mechanism for several reasons. First, individual PLMs during the night are associated with physiologic changes likely to contribute to cardiovascular disease. They are associated with transient, robust increases in blood pressure (on average 11-22 mm Hg systolic) and changes in heart rate and heart rate variability related to autonomic activation (4-7). Furthermore, the presence of at

least 30 PLMs/hour during at least one night of ambulatory monitoring by actigraphy is associated with self-reported, physician-diagnosed hypertension with an odds ratio of 2.27 (95% confidence interval 1.12, 4.58), and hypertension itself is a known risk factor for cardiovascular disease (Rye, unpublished data).

Inflammation as a mechanism for development of cardiovascular risk

There are numerous mechanisms by which cardiovascular disease may develop, including systemic inflammation, increases in oxidative stress, and increased sympathetic drive. Several lines of evidence implicate inflammation in the genesis of cardiovascular disease. First, systemic inflammation plays multiple key roles in the development and progression of the atherosclerotic plaque, the central pathogenic finding in coronary artery disease (18, 32-34). Initial development of an atheromatous plaque requires attachment of blood leukocytes to the endothelial cells. Inflammatory cytokines induce expression of endothelial-leukocyte adhesion molecules that facilitate this process (35). Pro-inflammatory cytokines then allow leukocytes to migrate into the intimal layer of the blood vessel wall, where they are converted into macrophages which engulf lipid particles to form the "foam cells" seen microscopically within atherosclerotic plaque (35). Further, after an atherosclerotic plaque has formed, systemic inflammation can lead to plaque disruption and acute progression of vascular disease (i.e., myocardial infarction or stroke from acute thrombus formation) (34-35). In addition to the general role of inflammation in the formation of atherosclerotic plaque, specific inflammatory mediators may promote cardiovascular disease in other ways. C-reactive protein (CRP) may induce coagulation, a common step in the development of acute vascular events (36). Tumor

necrosis factor alpha (TNF-alpha) impairs relaxation of vascular smooth muscle (increasing risk of vascular events) by decreasing bioavailability of nitric oxide, a potent vasodilator (37). TNF-alpha also increases production of reactive oxygen species, causing oxidative stress and further endothelial damage (37).

In addition to evidence implicating systemic inflammation in the development of cardiovascular disease pathology, certain signaling components of the immune system may be useful for the prediction of future cardiovascular disease risk. CRP is the most widely used for such prediction and is supported by the strongest evidence. A recent meta-analysis of 22 studies showed a risk ratio for CVD of 1.58 (1.48, 1.68) for those with the highest tertile of CRP levels versus those in the lowest tertile (11). Despite the overall significant predictive value of CRP, not all studies have shown CRP to predict CVD, with those studies enrolling more women being less likely to be statistically significant (38). Current guidelines recommend measurement of CRP in those patients classified as intermediate risk based on other cardiovascular risk factors, to allow stratification into higher or lower risk groups (12). Although there are some data to support the use of TNF-alpha and IL-6 in the prediction of cardiovascular disease, these markers are subject to limitations based on half-life and circadian factors and are not as widely used as CRP (13-17). Finally, the link between cardiovascular disease and inflammation is supported by a recent placebo-controlled clinical trial that demonstrated that lowering CRP levels through the use of rosuvastatin decreased risk of vascular events, including in those subjects with elevated CRP levels but no other vascular risk factors (39). Thus inflammation, typically quantified with surrogate measures such as CRP, IL-6, and TNF-alpha, is intimately linked to cardiovascular disease risk.

Why might PLMs be associated with inflammation?

Systemic inflammation has not been evaluated with respect to PLMs. However, systemic inflammation is known to be associated with another sleep disorder and with abnormal sleep durations, suggesting that sleep disruptions may themselves result in inflammation. Sleep apnea is a condition in which breathing ceases or decreases repeatedly during sleep, either due to mechanical collapse of the airway (as in obstructive sleep apnea, OSA) or impaired central nervous system drive to breathe (as in central sleep apnea, CSA). OSA is associated with increased markers of inflammation (CRP, IL-6, TNF-alpha) (18-24). TNF-alpha and CRP levels may be lowered with continuous positive airway pressure therapy, the gold-standard treatment for OSA, although this finding has not been seen in all studies (40-42). In patients with disorders of excessive sleepiness, including sleep apnea, elevated TNF-alpha levels are seen in association with nocturnal sleep disruption (22). Sleep deprivation, self reports of poor sleep quality, and increased wakefulness during the sleep period all correlate with increased IL-6 levels (25-26). Thus we hypothesize that PLMs, as another cause of sleep disruption, may also be associated with increased markers of inflammation. In summary, RLS and PLMs are associated with increased cardiovascular risk, although the responsible mechanisms have yet to be clarified. Increased inflammation is associated with increased cardiovascular risk and has been shown to be increased in other causes of sleep disruption. Therefore, we hypothesize that some of the increased cardiovascular risk observed in subjects with RLS is caused by associated PLMs in these subjects, which in turn increase systemic inflammation and cardiovascular disease risk.

Methods

Null Hypothesis

In people with RLS or PLMs, plasma levels of CRP, IL-6, and TNF-alpha will not vary based on the number of PLMs present, after controlling for potential confounders such as gender, age, race, BMI, and medical conditions known to increase cardiovascular disease risk.

Specific Aims:

The primary aim of this study is to determine whether increasing numbers of PLMs are associated with increasing levels of inflammation and, by extension, increased cardiovascular risk.

Study Design

Cross-sectional study analyzed as a case control study.

Participants:

Participants for this study were drawn from the larger Clinical Research in Neurology Study, a repository of biospecimens and clinical/demographic information on subjects with neurologic diseases (including RLS/PLMs).

Inclusion Criteria

1) Patient seen at the Emory Sleep Center between November 2001 and December 2009 for diagnoses of RLS, PLMs, or both

2) Available measurement of PLMs by actigraphy, PSG, or both

3) Banked plasma available for testing of inflammatory markers

Exclusion Criteria

1) Treatment with dopaminergic medications (e.g., pramipexole, ropinirole, levodopa), opiate medications (e.g., hydrocodone, oxycodone), or gabapentin/gabapentin-derivatives at time of evaluation

2) Failure of serum assays for inflammatory markers

Clinical procedures and data collection:

Measurement of PLMs

PLMs were measured in one of two ways, at the discretion of the treating physician. Actigraphic measurements of PLMs were obtained using the PAM-RL tri-axial accelerometer (Philips Respironics). For subjects with asymmetric RLS symptoms, the monitor was affixed to the ankle in which RLS symptoms were more severe. For those with symmetric or no RLS symptoms, the monitor was worn on the non-dominant ankle. Actigraphs were worn for five consecutive nights, then returned and downloaded. Software accompanying the monitor was used to calculate PLM index (PLMI, number of PLMs per hour of monitoring) during the major rest period, following standard criteria. As is typically done in PAM-RL studies, the major rest period (a surrogate measure of sleep period) was defined beginning after five minutes in the horizontal position without movement following a major vertical body movement (presumed to represent the subject getting into bed). The rest period end was defined as a major vertical body movement

followed by a total lack of movement (presumed to represent the subject sitting up and removing the monitor).

PLMs measured during PSG were recorded via surface EMG placed over the bilateral anterior tibialis muscles. PLMs were scored by an experienced polysomnographic technician and confirmed by a sleep specialist. Standard scoring criteria were used (43). Typically, PLMs were measured only on a single night of polysomnography. However, in subjects diagnosed with obstructive sleep apnea who underwent a CPAP titration study, a second study night was available with PLMs measurement.

Measurement of Inflammatory Markers:

High sensitivity CRP was measured on banked, frozen plasma by nephelometry (Dade-Behring). IL-6 and TNF-alpha were measured by flurokine multianalyte profiling (R&D systems). The investigators performing these assays were blinded to all clinical and demographic details of the subjects.

Data collection:

Demographic and clinical information about individual subjects was compiled from two sources. When available, information recorded for the parent study (Clinical Research in Neurology) was used. For those variables not included in the parent study, medical records were reviewed and data abstracted.

<u>Study Definitions:</u>

PLMI (Periodic Limb Movement Index): Average number of PLMs per hour of PSG-recorded sleep time (for subjects studied by PSG) or per hour of time spent horizontal during the major rest period (for subjects studied by actigraphy) during a single night. When multiple nights of data were available (as with all subjects measured by actigraphy and some subjects measured by PSG), the highest single night PLMI was used for analysis.

Body mass index (BMI): BMI was calculated based on measured or patient-reported height and weight using the standard formula: (weight in pounds)(703)/(height in inches)^2

Cardiovascular disease: Cardiovascular disease was defined as physician-diagnosed coronary artery disease, heart failure, or cardiomyopathy.

Current use of CRP-lowering medication: Use of a medication known or suspected to lower CPR levels at the time of blood draw. These medications included: HMGCoA reductase inhibitors, ezetimibe, fenofibrate, niacin, beta-antagonists, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, vitamin E, rosiglitazone, pioglitazone, omega-3 fatty acids, and cod liver oil (44-46).

Family history of RLS: Defined as a first-degree relative (parent, sibling, or child) with a clinical diagnosis of RLS

Ferritin: Serum ferritin levels (ng/mL) drawn at the clinic visit closest to the PLM measurement were collected from the medical record

Hyperlipidemia: Hyperlipidemia was defined as physician-diagnosed hyperlipidemia or dyslipidemia.

Hypertension: Hypertension was defined as physician-diagnosed hypertension or a blood pressure greater than 140 systolic or 90 diastolic at the time of study enrollment.

Inflammation: Inflammatory conditions were considered present if the patient had any of the following conditions: any type of arthritis other than osteoarthritis, asthma, chronic sinusitis, chronic bronchitis/chronic obstructive pulmonary disease, any type of malignancy, chronic inflammatory demyelinating polyneuropathy, eczema, Crohn's disease, dermatomyositis, hepatitis C, recurrent bladder infections, human immunodeficiency virus (HIV), celiac sprue, ulcerative colitis, or endometriosis.

Insomnia: Because difficulty initiating and maintaining sleep is commonly seen in subjects with RLS, insomnia was only diagnosed in those subjects in whom the treating sleep physician felt insomnia was a problem separate from the RLS symptoms.

Isolated PLMs: Subjects were considered to have isolated PLMs if they did not experience RLS symptoms. Those subjects with PLMs having rare RLS symptoms or RLS symptoms induced by medications were not considered to have isolated PLMs. Those subjects with a history of PLMs who subsequently developed RLS were considered to have RLS rather than isolated PLMs.

Race: Race was extracted from the medical record. Due to small numbers of non-Caucasian, non-African-American subjects, only Caucasian and African-American subjects were included in the regression analyses.

RLS severity: Although results of a validated RLS severity questionnaire were not available on the majority of subjects, an RLS severity score was generated from a series of three routine questions. For the first two questions ("when you try to relax in the evening or sleep at night, do you ever have unpleasant, restless feelings in your legs that

can be relieved by walking or movement" and "do you experience a strong urge to move your legs usually accompanied or caused by unpleasant sensations in your legs – for example, restlessness, creepy-crawly, or tingly feelings?"), up to five points were assigned based on the frequency of symptoms (with more points corresponding to more frequent symptoms). For the third question ("At what time(s) have you experienced this urge to move or unpleasant sensation"), subjects were given one point for each of four time periods they selected. Scores could range from 2 to 14, with higher scores indicating more severe RLS symptoms.

Sleep apnea: For subjects who did not undergo a PSG, sleep apnea was considered to be present if the subject had a history of witnessed apneas during sleep. Snoring in the absence of witnessed apneas was not diagnosed as sleep apnea. For subjects who underwent a PSG, a respiratory disturbance index (RDI, number of disordered breathing events per hour of sleep) greater or equal to 5 was considered diagnostic of sleep apnea.

Sleep length: Typical sleep length as reported by the subject

Smoking status: Smoking status was classified as current (any current use of tobacco products), prior (any history of tobacco use), or never (no history of tobacco use)

Stroke: Stroke was defined as physician-diagnosed ischemic stroke, hemorrhagic stroke, or transient ischemic attack.

<u>Sample Size:</u>

Because we did not have a priori knowledge of the number of patients who would be likely to meet inclusion/exclusion criteria to use to power our study, we screened all

patients who were enrolled into the parent study who were seen since we began routinely using actigraphy to measure PLMs at our center (i.e., late 2001).

Statistical Analysis:

Because PLM counts measured by actigraphy and by PSG are correlated but not identical, the study sample was divided into two samples for analysis – one sample containing subjects whose PLMs were measured by actigraphy, and the other containing those subjects whose PLMs were measured by PSG. Subjects who had both types of PLM measurement available were included in both samples for analysis.

Descriptive analyses of demographic and clinical characteristic were performed on the overall sample and the two sub-samples (i.e., actigraphy and PSG groups). Based on published standards, CRP was treated as a categorical variable such that values up to 3 mg/L were considered low-normal, greater than 3 mg/L were considered elevated, and values greater than 10 were excluded as likely representing acute rather than chronic inflammation (12). As no analogous standards exist for TNF-alpha and IL-6, they were considered first as continuous variables, then categorized into quartiles to allow for comparisons between highest and lowest quartile and highest quartile versus all other quartiles. For dichotomous inflammatory markers, the difference in PLMs between groups was compared by t-test. For continuous markers, the association between each marker and PLMs was tested using a Spearman correlation.

For those inflammatory markers shown to be significantly correlated with PLMI, further analyses were performed. Comparisons between high and low-normal CRP subjects were performed using t-tests for continuous variables (PLMI, age, BMI, ferrritin,

RLS severity, and sleep length) and chi-square tests for categorical variables (cardiovascular disease, diabetes, first degree relative with RLS, gender, hyperlipidemia, hypertension, inflammatory conditions, insomnia, medications known to lower CRP, obstructive sleep apnea, race, RLS symptoms versus isolated PLMs, smoking, and stroke). When expected cell counts were too small to allow for chi-square analyses, Fisher's exact test was performed. Although variables were not always normally distributed, parametric tests were performed because sample sizes were large.

All potential predictor variables were then dichotomized to allow for stratified analyses of the relationship between CRP and PLMs by strata of the predictor variable. For each stratified analysis, a Breslow-Day test was performed to assess stratum heterogeneity. Unless significant heterogeneity was observed, a Mantel-Haenszel odds ratio was calculated for the association of PLMs and CRP by level of each stratified variable. These adjusted odds ratios were compared to the crude PLMs-CRP odds ratio to further evaluate for potential confounders. For the purpose of these analyses, PLMs were dichotomized at the mean PLMI.

Logistic regression was then used to model the relationship between CRP and PLMs. Prior to including continuous variables in a model, they were confirmed to be log-linear with respect to CRP. PLMs were modeled in several ways. First, PLMs were treated as a continuous variable to determine the effect of a single PLM. Second, they were treated as a categorical variable using 15 PLM/hour increments (with the final 7.9% of subjects, all those with PLMI greater than or equal to 75, being considered as a single group), based on current consensus recommendations that < 15 PLMs/hour be considered

normal (1). Third, they were grouped into four categories (< 15 = “normal”, 15 to <30 = “mild”, 30 to <60 “moderate”, ≥ 60 = severe), modeled using dummy variables.

Three models were tested. The first model contained only a single predictor variable, PLMs. The second model contained PLMs and demographic variables (age, gender, and race). The third model contained PLMs, demographic variables, and clinical conditions known to influence CRP and/or cardiovascular risk (cardiovascular disease, diabetes, hyperlipidemia, hypertension, inflammatory conditions, OSA, stroke, and use of CRP lowering medications). Stroke and cardiovascular disease were combined into a single composite variable due to small numbers of events. All three models were tested with a Hosmer-Lemeshow goodness of fit test and evaluated for collinearity using the Rosen-Kleinbaum collinearity macro (47). Each model was compared to the preceding model via likelihood ratio test.

For those potential confounders for which there was substantial missing data, such that they could not all be included in the full model, each potential confounder was considered separately. The demographic model was run on the subset of subjects for whom a value of the confounder was known (to isolate the effect of loss of power compared to running the demographic model on the entire sample) and then run controlling for the confounder. The OR for the effect of PLMs on CRP was compared in the two models to determine whether controlling for the potential confounder substantially altered the estimate of the effect of PLMs (i.e., to determine whether the variable was confounding the PLMs-CRP association).

Statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC).

Human Subjects Protection:

All subjects provided informed consent for use of their clinical and research information and blood samples through a research protocol approved by the Emory University Institutional Review Board.

Results

Study participants

A total of 350 subjects met inclusion & exclusion criteria. Of these, 167 had available PLMI from actigraphy and 237 from PSG. Fifty-four subjects had PLMI measurements available using both techniques. In these subjects, mean PLMI were similar using the two techniques (27.3 +/- 28.8 for actigraphy, 28.1 +/- 44.3 for PSG, p = 0.91). PLMI measured by the two techniques were significantly but not strongly correlated (Spearman's correlation coefficient 0.37, p = 0.006).

Demographic and clinical features of the entire group of subjects, as well as the actigraphy and PSG sub-groups, are reported in Table 1. Subjects in the actigraphy group were more likely to be female (58.1% versus 45.6% in the PSG group), were younger (42.4 years old versus 51.2 years old), had lower BMIs (27.2 versus 29.1), were more likely to have RLS (83.8% versus 65.0%), were more likely to have a first degree relative with RLS (57.4% versus 31.4%), and were less likely to have hypertension (28.7% versus 42.8%), hyperlipidemia (12.7% versus 25.3%), or sleep apnea (27.9% versus 70.9%). Median PLMI were higher in the PSG group than the actigraphy group (30.0/hour versus 19.8/hour). No other clinical or demographic features differed between the two groups. There were no differences in average CRP, IL-6, or TNF-alpha measurements between the two groups.

Unadjusted relationships between inflammatory markers and PLMI

In the actigraphy sample, PLMI were significantly higher in the high CRP group compared to the low-normal CRP group (40.2 versus 26.1, t = -2.1, p = 0.04). Among

the PSG group, there was no significant difference in PLMI between the high and low-normal CRP groups (37.1 versus 45.1, t = 1.39, p = 0.17). Considering IL-6 as a continuous variable, there were no significant correlations between IL-6 levels and PLMI in the actigraphy (rho = 0.11, p = 0.17) or PSG (rho = 0.008, p = 0.90) groups. Categorizing IL-6 into quartiles and comparing highest versus lowest quartiles of IL-6 did not reveal any differences between average PLMI between the two groups for the actigraphy (30.0 versus 27.4, p = 0.71) or PSG (44.1 versus 45.5, p = 0.84) samples. Similarly, there were no correlations between TNF-alpha measurements and PLMI in either the actigraphy or PSG groups (rho = 0.09, p = 0.24 for the actigraphy group; rho = 0.03, p = 0.65 for the PSG group). Categorizing TNF-alpha into quartiles and comparing highest versus lowest quartiles of TNF-alpha revealed no significant differences in average PLMI in the actigraphy (31.0 versus 24.4, p = 0.24) or PSG (45.5 versus 41.1, p = 0.52) groups.

Multivariate analyses of CRP and PLMI

Because CRP and actigraphically-measured PLMs were found to be significantly related in the univariate analyses, multivariate analyses were performed to further evaluate this relationship. Multivariate analyses were not performed on TNF-alpha, IL-6, or in the PSG subgroup based on non-significant results in the univariate analyses.

Individual predictor variables were compared in the high and low-normal CRP groups (see Tables 2 and 3). Of these, BMI was significantly higher in the high CRP group (31.5 versus 25.4, t = -4.03, p = 0.0003). The high CRP group had a higher percentage of women than did the low-normal CRP group (76.5% versus 52%, t = 6.57, p

= 0.01). The presence of OSA symptoms approached statistical significance (40.0% in the high CRP group versus 24.3% in the low-normal CRP group, t = 3.05, p = 0.08). No other demographic or clinical features were different by CRP level. Predictor variables were dichotomized and the Mantel-Haenszel odds ratio for the effect of PLMs on CRP calculated (see Table 4). Comparison of adjusted ORs to the crude OR for CRP and PLMs suggested additional possible confounders of sleep length, RLS severity, current smoking, serum ferritin, and BMI*gender. Breslow-Day tests of these stratified analyses revealed no significant interactions.

In the logistic regression model uncontrolled for any possible confounders, the odds ratio for each additional PLM was 1.015 (1.003, 1.03; see Table 5). In the unadjusted model using PLMs as a categorical variable by groups of 15 PLMs/hour, the odds ratio for an increase of 15 PLMs/hour was 1.35 (1.08, 1.69). Using PLMs modeled with indicator variables (normal, mild, moderate, or severe), the ORs were 1.0 for normal (reference group), 0.91 for mild, 1.16 for moderate, and 4.02 for severe, suggesting that the relationship between CRP and PLMs is largely driven by those subjects with at least 60 PLMs per hour.

Multivariate logistic regression was then performed in a model including PLMs and demographic information (gender, race, age; see Table 5). Treating PLMs as a continuous variable, the OR for each additional PLM was 1.016 (1.002, 1.03). As a categorical variable, the OR for each additional 15 PLMs was 1.40 (1.07, 1.84). Comparing this model to the unadjusted model, the OR for the effect of PLMs on CRP did not markedly change. However, the likelihood ratio test for this comparison was significant (LRT = 9.23, p = 0.03) due to the additional predictive value of gender on

CRP. Next, a model was run including PLMs, these demographic features, and clinical conditions. In this model, the OR for an individual PLM was 1.015 (0.999, 1.03) and for each additional 15 PLMs was 1.31 (0.97, 1.78). The likelihood ratio test comparing this model to the demographic model was not significant (LRT = 8.76, p = 0.27).

Several potential confounders (BMI, BMI*gender, sleep length, RLS severity, current smoking, and ferritin) could not be combined with this model due to missing data on a substantial number of subjects. For each of these potential confounders, the demographic model was run on the reduced sample (the subgroup of subjects for whom a value of the potential confounder was known) and then run again with the potential confounder included in the model. Whether considering PLMs as continuous or categorical by 15, inclusion of BMI, BMI*gender, sleep length, RLS severity, current smoking, and ferritin individually did not substantially alter the relationship between CRP and PLMs (see Table 6), suggesting that these were not major confounders.

Sensitivity Analysis

To determine whether use of maximum PLMI of the five nights of monitoring was equivalent to use of mean PLMI of the five nights, the three models were run using mean PLMI instead of maximum PLMI. ORs for the effect of PLMs on CRP increased a small amount (OR in unadjusted model = 1.018, OR in demographic model = 1.020, OR in full model = 1.020) using mean PLMs.

Discussion

The results of this study show that PLMs are a predictor of increased levels of CRP, after controlling for relevant demographic features (i.e., gender, age, and race). Additionally controlling for clinical conditions associated with cardiovascular disease and inflammation did not improve the predictive ability of the model and did not substantially change the estimate of the effect of PLMs on CRP, although this larger model became marginally non-significant. Although they could not be fully evaluated in this sample due to incomplete data, BMI, sleep length, RLS severity, smoking and ferritin did not appear to markedly confound the PLM-CRP association. The increased odds of high CRP conferred by each additional PLM per hour was 1.5%, although this appears to be largely driven by those with at least 60 PLMs per hour.

There are several potential explanations for the finding of a significant association between PLMs and CPR when PLMs are measured by actigraphy but not by polysomnography. First, because the decision to measure PLMs by actigraphy versus polysomnography was made clinically, with patients suspected to have another comorbid sleep disorder (e.g., sleep apnea, narcolepsy) more likely to be studied by polysomnography, the two groups ended up with significantly different characteristics. Subjects in the actigraphy group were younger, more likely to be female, more likely to have RLS symptoms and a family history of RLS, and less likely to have hypertension, hyperlipidemia, and clinically-suspected OSA. It may be that the relationship between PLMs and inflammation is stronger in this healthier group, although we did not determine any evidence of interaction between PLMs and these clinical features with respect to CRP levels in our study. A second possible explanation for the significant association

between CRP and PLMs in our study is that we may have inadvertently been measuring OSA and PLMs rather than PLMs alone, and the relationship between OSA and CRP could be driving the association we observed. More specifically, leg movements may occur at the termination of an apnea, but in this case are not considered to be PLMs. This distinction between such respiratory-related leg movements (RRLMs) and PLMs can easily be made during polysomnography, as respiratory events are monitored in concert with leg movements. However, this distinction cannot be made during actigraphy, such that in a patient with both RRLMs and PLMs, both types of events would be counted as PLMs. To control for this possibility, we included clinically-suspected OSA as a covariate in the model, and it was not a significant predictor of high CRP in the multivariate model. This suggests that a confounding relationship between OSA and CRP is not the cause of our results. Finally, our observation of a relationship between PLMs and CRP only in the actigraphy group may be a reflection of the superior ability of actigraphy to capture the nightly variability of PLMs. PSG provides a single (or at most two-night) estimate of PLMs severity, but actigraphy provides a five-night estimate. Prior research has shown that a single night of study does not adequately reflect the true PLMs severity when measured over 10-15 nights, yet 5 nights of study does capture this variability (2). Thus the improved accuracy of PLMs estimates in the actigraphy group may have allowed us to see the true association between PLMs and CRP in this group.

Another interesting finding in our study was a significant relationship between CRP and PLMs but not the other tested inflammatory markers (i.e., IL-6, TNF-alpha). Similar to PLMs, this may be an effect of our ability to adequately capture the variability of the inflammatory markers tested. CRP has a long half-life of 19 hours and does not

have substantial circadian variability, so a single measurement is considered an accurate reflection of true CRP levels (11, 38, 48). In contrast, both IL-6 and TNF-alpha have been shown to have circadian variability, with IL-6 peaking at 1 am and nadiring at 10 am and TNF-alpha peaking at 9 pm and nadiring at 9 am (13). IL-6 additionally has substantial intra-subject variability with repeated measures (14). Plasma samples for all inflammatory markers were collected at a single point without respect to circadian phase, which may have introduced noise into the IL-6 and TNF-alpha signals, reducing our ability to see the true association, if any, with PLMs. Additionally, while IL-6 and TNF-alpha are important pieces of the inflammatory response, CRP may be consider the "final common pathway" of the inflammatory response (49); that is, regardless of which inflammatory pathway is activated (some of which would involve IL-6 or TNF-alpha), CRP will be involved. Thus CRP may be a better marker of general systemic inflammation than IL-6 or TNF-alpha.

Strengths and Weaknesses

The major strengths of this study were objective measurement of PLMs and face-to-face diagnosis of RLS. Prior studies of cardiovascular risk in RLS have not included measurements of PLMs, despite physiologic evidence to suggest that they may be the most plausible mediator of this increased risk. In contrast, in our study we considered PLMs as our predictor variable. Additionally, although RLS can be diagnosed for research purposes based on standard questionnaires, face-to-face interview is considered the gold standard for diagnosis. All our subjects were classified as having or not having RLS based on face-to-face interview.

There were several limitations to our study. First, because it required retrospective collection of information, data were missing on several potentially important variables, including BMI, sleep length, serum ferritin levels, smoking status, and RLS severity (for which we also did not have a validated rating scale). This limited our ability to control for these variables, although available analyses did not suggest them to be important confounders. Second, we only collected blood at a single time point for each subject, without respect to clock time, which did not allow us to account for the variability and circadian fluctuations of IL-6 and TNF-alpha. Third, the cross-sectional design of this study is by nature not able to assess temporality. Thus, while we hypothesize that increasing PLMs cause increased inflammation, from these data we can only conclude that these two factors are associated, without drawing conclusions about the direction of the relationship. Finally, our population consisted only of subjects with either RLS or PLMs (or both). Had we had a group of controls with neither RLS nor PLMs, we would have been able to better tease apart the contribution of RLS itself to increased inflammation.

Future study should confirm these results in another population with complete data on the covariates identified in the literature and in this study as potentially important for modifying the PLMs-CRP association. Additionally, evaluation for the underlying mechanisms linking PLMs with CRP is necessary to add weight to this finding. Also, this study used CRP as a surrogate for cardiovascular disease risk, as is commonly done, but a study showing a direct association between PLMs and cardiovascular risk would be beneficial to confirm the health-related significance of this finding. Finally, both to help determine causality of the relationship between PLMs and CRP levels (or cardiovascular

disease) and to determine the relevance to clinical practice, a trial evaluating treatment of PLMs for the effect on CRP or cardiovascular risk is needed. These data provide support for the pursuit of these additional projects by demonstrating the association between PLMs and CRP.

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Table 1: Demographic and clinical characteristics of subjects

	Entire Sample	Actigraphy	PSG	p-value*
Sample size	350	167	237	
Age	48.3 (19.5)	42.4 (20.9)	51.2 (17.8)	**< 0.0001**
BMI	28.6 (7.0)	27.2 (7.0)	29.1 (6.8)	**0.01**
Cardiovascular disease	7.6%	4.9%	9.0%	0.13
Current use of CRP-lowering medication	36.9%	34.7%	37.6%	0.56
Diabetes	10.2%	7.4%	11.6%	0.16
Family history of RLS	42.6%	57.4%	31.4%	**< 0.0001**
Ferritin	49.0 (9-988)	41.5 (10-988)	51.0 (9-589)	0.80
Hyperlipidemia	20.8%	12.7%	25.3%	**0.002**
Hypertension	38.4%	28.7%	42.8%	**0.004**
Inflammatory disease	26.0%	21.7%	28.0%	0.16
Insomnia	34.3%	33.6%	36.4%	0.58
Isolated PLMs	27.7%	16.2%	35.0%	**< 0.0001**
Male gender	49.4%	41.9%	54.4%	**0.01**
Measured RDI	----	----	11.0 (0-110)	
Race	84.4% Cauc, 13.0% Af-Am	85.4% Cauc, 12.8% Af-Am	83.7% Cauc, 13.7% Af-Am	0.77
RLS severity	10.1 (2.8)	10.1 (2.6)	9.9 (2.9)	0.61
Sleep apnea	56.4%	27.9%	70.9%	**< 0.0001**
Sleep length	7.5 (1.8)	7.5 (1.7)	7.6 (1.9)	0.87
Smoking	7.0% current; 17.5% prior	3.6% current; 13.4% prior	7.9% current; 18.5% prior	0.14
Stroke or TIA	4.7%	3.1%	5.6%	0.24
PLMs (per hour)	------	19.8 (0.7-145.8)	30.0 (0-222.8)	**0.0001**
CRP	1.20 (0.15-49.2)	1.03 (0.15-47.1)	1.42 (0.15-49.2)	0.35
IL-6	0.87 (0.01-13.3)	0.75 (0.01-6.4)	0.92 (0.01-13.3)	0.10
TNFa	3.89 (0.16-33.2)	3.81 (0.21-7.88)	3.98 (0.16-33.2)	0.10

Values represent mean (SD) for normally distributed continuous variables, median (range) for non-normally distributed continuous variables, and percentages for categorical variables.

* p-value compares actigraphy and PSG groups; performed as t-tests for continuous variables and chi-square or Fisher's exact tests for categorical variables

BMI = body mass index
RLS = restless leg syndrome
PLMs = periodic limb movements
TIA = transient ischemic attack
Cauc = Caucasian
Af-Am = African-American
RDI = respiratory distress index (number of respiratory events per hour of sleep)
Sleep apnea = known or suspected diagnosis of sleep apnea

Table 2: Potential risk factors (categorical variables) in subjects with low-normal versus high CRP among those subjects with PLMs measured by actigraphy

	Low-Normal CRP	High CRP	T-test	p-value
PLMs (n = 161)	26.1	40.2	-2.1	**0.04**
Age (n = 161)	40.9	46.2	-1.32	0.19
BMI (n = 126)	25.4	31.5	-4.03	**0.0003**
Ferritin (n = 122)	80.1	58.8	0.80	0.42
RLS severity (n = 72)	10.0	10.4	-0.62	0.54
Sleep length (n = 92)	7.7	7.2	1.01	0.32

PLMs = periodic limb movements
BMI = body mass index
RLS = restless leg syndrome

Table 3: Potential risk factors (categorical variables) in subjects with low-normal versus high CRP among those subjects with PLMs measured by actigraphy

	Low-Normal CRP	High CRP	Chi-square	p-value
CVD (n = 156)	5 (4.1%)	3 (8.8%)	**	0.37**
Caucasian race (vs Af-Am) (n = 155)	105 (86.8%)	30 (88.2%)	**	1.00
Current use of a CRP-lowering medication (n = 161)	44 (34.7%)	11 (32.4%)	0.06	0.80
DM (n = 157)	9 (7.3%)	3 (8.8%)	**	0.72**
Family History of RLS (n = 118)	55 (59.1%)	14 (56.0%)	0.08	0.78
HTN (n = 158)	33 (26.6%)	11 (32.4%)	0.44	0.51
Hyperlipidemia (n = 151)	15 (12.7%)	5 (15.2%)	**	0.77**
Inflammatory disease (n = 151)	23 (19.5%)	10 (30.3%)	1.77	0.18
Insomnia (n = 140)	38 (34.2%)	8 (27.6%)	0.46	0.50
Isolated PLMs (n = 161)	22 (17.3%)	5 (14.7%)	0.13	0.72
Male gender (n = 161)	61 (48.0%)	8 (23.5%)	6.57	**0.01**
Sleep apnea (n = 141)	27 (24.3%)	12 (40.0%)	3.05	0.08
Smoking (now) (n = 106)	2 (2.5%)	2 (7.7%)	**	0.25**
Stroke (n = 157)	3 (2.4%)	2 (5.9%)	**	0.30**

** Fisher's exact test

CVD = cardiovascular disease
DM = diabetes mellitus
HTN = hypertension
Af-Am = African American
RLS = restless leg syndrome
PLMs = periodic limb movements

Table 4: Summary of stratified analyses evaluating the relationship between PLMs and CRP by strata of potential confounders

	Stratum 1 OR (variable = 1)	Stratum 2 OR (variable = 2)	Breslow-Day test p-value	OR-MH	95% CI
Absence of family history	5.78	1.54	0.17	2.64	1.08, 6.50
African American race	0.73	2.61	0.33	2.26	1.04, 4.91
Age	3.25	1.36	0.34	2.43	1.05, 5.60
BMI	1.80	1.89	0.95	1.85	0.75, 4.56
Current use of CRP-lowering medication	2.53	2.42	0.96	2.46	1.12, 5.41
CVD	3.0	2.29	0.86	2.33	1.07, 5.10
Diabetes	1.6	2.51	0.76	2.41	1.10, 5.28
Ferritin	4.61	1.89	0.33	2.93	1.20, 7.16
HTN	3.2	2.02	0.61	2.34	1.05, 5.23
Hyperlipidemia	4.57	2.21	0.57	2.44	1.09, 5.43
Inflammatory disease	3.43	2.09	0.76	2.39	1.08, 5.26
Insomnia	4.09	1.86	0.41	2.31	1.01, 5.30
Isolated PLMs	2.33	2.63	0.91	2.38	1.09, 5.16
Male gender	3.17	2.47	0.78	2.65	1.19, 5.93
RLS severity	1.77	0.63	0.65	1.18	0.40, 3.45
Sleep apnea	1.51	2.82	0.48	2.21	0.96, 5.09
Sleep length	0.83	1.59	0.54	1.11	0.39, 3.16
Smoking (ever)	2.25	1.87	0.89	1.92	0.77,4.77
Smoking (now)	(empty cell)	1.6	0.34	1.74*	0.71, 4.29
Stroke	(empty cell)	2.14	0.26	2.35*	1.08, 5.12

For these analyses, PLMI was treated as a dichotomous variable divided at the mean (29/hour). The crude OR for the relationship between dichotomous PLMs and CRP is 2.34 (1.08, 5.07).

For all categorical variables, variable 1 is presence of the listed risk factor, variable 2 is its absence.

Age: variable 1 is age $\geq$ 42, variable 2 is age < 42

BMI: variable 1 is BMI $\geq$ 30, variable 2 is BMI < 30

Ferritin: variable 1 is ferrtin < 41.5, variable 2 is ferritin $\geq$ 41.5

RLS severity: variable 1 is severity $\geq$ 11, variable 2 is severity < 11

Sleep length: variable 1 is sleep length < 7.5 hours, variable 2 is sleep length $\geq$ 7.5 hours

OR-MH = Mantel-Haenszel odds ratio

BMI = body mass index

CVD = cardiovascular disease

HTN = hypertension

PLMs = periodic limb movements

RLS = restless legs syndrome

*estimate correcting for empty cell

Table 5: Logistic regression models for odds of high CRP

	PLMs as a continuous variable			PLMs categorized into groups of 15/hour		
	LRT	Parameter Estimate for each PLM	OR (95% CI) for PLMs	LRT	Parameter Estimate for 15 PLMs/hour	OR (95% CI) for PLMs
Model 1: PLMs (n = 161)	5.64 (p=0.02)	0.015 (p=0.02)	1.015 (1.003, 1.03)	6.61 (p=0.01)	0.30 (p = 0.01)	1.35 (1.08, 1.69)
Model 2: PLMs and demographics (n = 155)	14.59 (p=0.01)	0.016 (p=0.02)	1.016 (1.002, 1.03)	15.35 (p= 0.004)	0.34 (p = 0.01)	1.40 (1.07, 1.84)
Model 3: PLMs, demographics, and diseases (n = 132)	23.38 (p=0.02)	0.015 (p=0.07)	1.015 (0.999, 1.03)	23.16 (p=0.02)	0.27 (p = 0.08)	1.31 (0.97, 1.78)

Model 1: controls for periodic limb movements (PLMs)
Model 2: controls for PLMs, age, gender, and race
Model 3: controls for PLMs, age, gender, race, cardiovascular disease or stroke, CRP-lowering medications, diabetes, hyperlipidemia, hypertension, inflammatory conditions, and obstructive sleep apnea

LRT = likelihood ratio test comparing the model to the model containing only the intercept

Table 6: Partially adjusted logistic regression models for those suspected confounders with missing data

	PLMs as a continuous variable		PLMs categorized into groups of 15/hour	
	Parameter Estimate for each PLM	OR (95% CI) for PLMs	Parameter Estimate for 15 PLMs/hour	OR (95% CI) for PLMs
PLMs/demographics model, with known BMI (n = 122)	0.012 (p = 0.13)	1.012 (0.997, 1.03)	0.249 (p = 0.10)	1.28 (0.95, 1.73)
Model with PLMs, demographics, and BMI	0.011 (p = 0.20)	1.011 (0.994, 1.03)	0.223 (p = 0.17)	1.25 (0.91, 1.72)
Model with PLMs, demographics, BMI, and BMI*gender	0.011 (p = 0.18)	1.011 (0.995, 1.03)	0.237 (p = 0.16)	1.27 (0.91, 1.76)
PLMs/demographics model, with known sleep length (n = 87)	0.009 (p = 0.35)	1.009 (0.990, 1.03)	0.175 (p = 0.33)	1.19 (0.84, 1.69)
Model with PLMs, demographics, and sleep length	0.008 (p = 0.39)	1.008 (0.989, 1.03)	0.165 (p = 0.36)	1.18 (0.83, 1.68)
PLMs/demographic model, with known RLS severity (n=72)	0.015 (p = 0.15)	1.015 (0.995, 1.04)	0.264 (p = 0.19)	1.30 (0.88, 1.93)
Model with PLMs, demographics, and RLS severity	0.016 (p = 0.13)	1.016 (0.995, 1.04)	0.286 (p = 0.17)	1.33 (0.88, 2.01)
PLMs/demographic model, with known smoking (n = 102)	0.011 (p = 0.15)	1.011 (0.996, 1.03)	0.227 (p = 0.14)	1.26 (0.93, 1.69)
Model with PLMs, demographics, and smoking	0.011 (p = 0.16)	1.011 (0.996, 1.03)	0.218 (p = 0.16)	1.24 (0.92, 1.69)
PLMs/demographic model, with known ferritin (n = 118)	0.016 (p = 0.03)	1.017 (1.002, 1.03)	0.369 (p = 0.01)	1.45 (1.08, 1.94)
Model with PLMs, demographics, and ferritin	0.016 (p = 0.03)	1.016 (1.001, 1.03)	0.363 (p = 0.02)	1.44 (1.07, 1.93)

PLMs = periodic limb movements
Demographics = age, gender, and race