ORIGINAL ARTICLE

A potential role for zinc in restless legs syndrome

Pan Chen¹, Julia Bornhorst², Stephanie Patton³, Kanika Bagai⁴, Rachana Nitin⁵, Mahfuzur Miah¹, Dominic J. Hare⁶, Kai Kysenius⁷, Peter J. Crouch^{7,8}, Lan Xiong⁹, Guy A. Rouleau^{9,*}, Tanja Schwerdtle¹⁰, James Connor³, Michael Aschner¹, Aaron B. Bowman^{11,*} and Arthur S. Walters^{4,*}

¹Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY ²Food Chemistry, Faculty of Mathematics and Natural Sciences, University of Wuppertal, Wuppertal, Germany ³Department of Neurosurgery, Penn State Hershey Medical Center, Hershey, PA ⁴Department of Neurology, Sleep Division, Vanderbilt University Medical Center, Nashville, TN ⁵Vanderbilt University, Vanderbilt Brain Institute, Nashville, TN ⁶School of Biosciences, The University of Melbourne, Victoria, Australia ⁷Department of Pharmacology and Therapeutics, The University of Melbourne, Victoria, Australia ⁸Florey Institute of Neuroscience and Mental Health, the University of Melbourne, Victoria, Australia ⁹Montreal Neurological Institute, McGill University, Montréal, Québec, Canada ¹⁰Institute of Nutritional Science, Department of Food Chemistry, University of Potsdam, Nuthetal, Germany ¹¹School of Health Sciences, Purdue University, West Lafayette, IN

*Corresponding authors. Arthur S. Walters, Division of Sleep Medicine Vanderbilt University School of Medicine, Medical Center North A-0118, 1161 21st Ave. South, Nashville, TN 37232-2551. Email: Walters@Vanderbilt.edu; Art.Walters@VUMC.org.

Aaron B. Bowman, School of Health Sciences, Purdue University, 550 Stadium Mall Drive – HAMP 1173Am West Lafayette, IN 47907-2051. Email: Bowma117@Purdue.Edu.

Abstract

Study Objectives: Evaluate serum and brain noniron metals in the pathology and genetics of restless legs syndrome (RLS).

Methods: In two independent studies (cohorts 1 and 2), in which subjects either remained on medications or tapered off medications, we analyzed serum levels of iron, calcium, magnesium, manganese, copper, and zinc both in RLS patients and controls, and assessed the prevalence of the MEIS1 and BTBD9 risk alleles previously established through genome-wide association studies. Human brain sections and a nematode genetic model were also quantified for metal levels using mass spectrometry.

Results: We found a significant enrichment for the BTBD9 risk genotype in the RLS affected group compared to control ($p = 0.0252$), consistent with previous literature. Serum ($p = 0.0458$ and $p = 0.0139$ for study cohorts 1 and 2, respectively) and brain ($p = 0.0413$) zinc levels were significantly elevated in the RLS patients versus control subjects.

Conclusion: We show for the first time that serum and brain levels of zinc are elevated in RLS. Further, we confirm the BTBD9 genetic risk factor in a new population, although the zinc changes were not significantly associated with risk genotypes. Zinc and iron homeostasis are interrelated, and zinc biology impacts neurotransmitter systems previously linked to RLS. Given the modest albeit statistically significant increase in serum zinc of ~20%, and the lack of association with two known genetic risk factors, zinc may not represent a primary etiology for the syndrome. Further investigation into the pathogenetic role that zinc may play in restless legs syndrome is needed.

Statement of Significance

Zinc has not been previously been investigated in RLS. Here we show, for the first time, that zinc is increased in RLS patients compared to control subjects. We also verify the previously known increased prevalence of BTBD9 at risk gene alleles in our human RLS population. In addition, zinc levels are increased in *Caenorhabditis elegans* with a deletion mutant *hpo-9(tm3719)* serving as a model of the BTBD9 gene risk alleles. We present novel findings that are pertinent to RLS, since independent approaches both support a role for elevated zinc in RLS. The implications of these findings are discussed in terms of the possible role that zinc may play in the pathogenesis of RLS.

Key words: restless legs syndrome; essential metals; zinc

Submitted: 26 May, 2020; Revised: 13 October, 2020

© Sleep Research Society 2020. Published by Oxford University Press on behalf of the Sleep Research Society.

All rights reserved. For permissions, please e-mail journals.permissions@oup.com.

Introduction

Except for iron, trace metals have been little investigated in restless legs syndrome (RLS). Iron supplementation is a well-established therapy for RLS [1]. Iron parameters are altered in RLS compatible with the iron deficiency hypothesis of RLS as investigated by magnetic resonance imagery (MRI), cerebrospinal fluid (CSF), and autopsy material [2–4]. On the other hand, serum levels of the iron-binding protein ferritin are variably reduced in RLS, and normal serum iron levels have been reported in RLS [5, 6]. Both parathyroid hormone and Vitamin D are well known to play a key role in calcium metabolism. To our knowledge, calcium and parathyroid hormone levels have only been studied in cases of RLS secondary to hyperparathyroidism or uremia where some increases might be expected to be seen [7, 8]. However, two proteomic studies, one in CSF and one in serum, show that Vitamin D binding protein is elevated even in idiopathic RLS [9]. Preliminary evidence also exists for Vitamin D as a therapy for RLS [10]. Evidence for magnesium as a therapy for RLS is shown in open-label studies, but serum magnesium and CSF levels seem to not be altered in RLS [11–13]. Other metals, including copper, manganese, and zinc have not been investigated in RLS. The current study evaluates the previously investigated metals and these additional metals and, although our original hypothesis was that serum manganese would be elevated in RLS based upon the known reciprocal relationship between manganese and iron [14], this study shows increased levels of zinc both in serum and in the brain by mass spectrometry analysis.

Methods

The study was comprised of the following elements: (1) in two cohorts of patients and controls serum metals were analyzed. In the second cohort, genotyping was done for the BTBD9 and MEIS-1 genes, which have been found to be highly allelically associated with RLS in previous studies; (2) in the third cohort, brain autopsy material was analyzed for the presence of zinc by laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS); (3) in the fourth cohort, lymphoblastoid cell lines generated from patients and controls were analyzed for zinc; (4) mutations that are the counterpart of the BTBD9 and MEIS-1 genes were created in *Caenorhabditis elegans* worms and zinc levels were compared with the wild type.

Study design and participants

The study was conducted separately in two adult (above 21 years of age) patient groups referred to as study 1 and study 2. We obtained IRB approval for this study from the Vanderbilt University Medical Center and informed consent was obtained from all subjects. Both the groups had moderately different patient recruitment requirements. For study 1, exclusion criteria included pregnancy, prior occupational exposure to Mn (miners, welders), anemia, liver disease, diagnosis of neurological or psychiatric diseases, and subjects on Mn or Fe dietary supplementation. For study 2, the exclusion criteria remain the same—while the inclusion criteria for the RLS patients were altered. In study 2, only RLS patients with a family history of RLS were recruited, that is, having at least one immediate family member with RLS, and have a positive RLS diagnosis themselves. In study

2, control subjects (RLS negative) were excluded for having at least one immediate family member with RLS. For study 2, patients were either medication naïve or were tapered off all RLS treatments for at least 1 week and were medication free for an additional 1 week prior to the blood draw. These medications included benzodiazepines, dopamine agonists, opioids, and alpha 2 delta calcium channel blockers such as gabapentin and pregabalin. Medications that were also tapered included those which worsen RLS such as SSRI and SNRI antidepressants, antihistamines, and dopamine receptor blocking agents such as antipsychotics and GI medications such as metoclopramide. MEIS1 and BTBD9 were selected for genotyping analysis as they were previously established through GWAS studies to be highly associated with RLS [15–19]. One control subject (non-RLS) in cohort 2 withdrew from the study and is not counted in the numbers or data reported in the results.

There was no apparent imbalance in the medical problems usually associated as triggers for RLS in our final combined population for serum metal analysis. Specifically, no patients or controls had peripheral neuropathy, renal failure, nongestational diabetes, inflammatory or autoimmune disorders, or significant cardiovascular disease. Three patients and two controls had hypertension. One patient had irritable bowel syndrome. We excluded the presence of anemia from subjects at the beginning of the study both by specifically asking them if they had anemia and checking their medical charts to see if they had been given a diagnosis of anemia. We also examined both the hemoglobin and hematocrit values as part of routine clinical care for the combined studies on 23 patients and 14 controls, none of which showed evidence of anemia.

Lymphocyte isolation

Eight milliliters of blood were drawn up into a special preparation tube (BD Vacutainer CPT Cell Preparation Tube with sodium citrate). None of the patients were nutritionally deficient and all were on a normal diet. In addition, the blood samples were all drawn during the day within a 3–4 h range of each other. The blood sample was stored at room temperature prior to lymphocyte isolation (which must occur within 2 h). The tubes were inverted 7–8 times immediately prior to centrifugation and then spun at 2,800 rpm for 15 min. The lymphocyte/monocyte layer (buffy coat layer) was transferred to 50 mL tubes and washed with RPMI 1640 media twice. From this cell suspension, 0.1 mL was used to estimate cell count (live dead staining and lymphocyte cell count) and the remainder was spun at 1,200 rpm × 5 min; supernatant poured off and pellet resuspended in 1 mL freezing media (95% heat-inactivated FBS and 5% DMSO). The tubes were placed in a Nalgene freezing container (Sigma-Aldrich) in a freezer at –80°C.

DNA isolation and allele analysis

Isolated genomic DNA was used to determine the presence of specific alleles of MEIS1 and BTBD9, which are both single nucleotide polymorphisms (SNPs) within these two RLS genes, in each sample of the study. Primers were designed to flank the SNP MEIS1 (intron 9) and BTBD9 (intron 5) and the PCR product was purified and sent for Sanger sequencing to Genewiz. The sequences were analyzed for the presence of the respective

SNPs. SNPs were genotyped using the TaqMan allelic discrimination genotyping assay with a protocol recommended by the manufacturer (Applied Biosystems). The assay kit contained the forward target-specific PCR primer, the reverse primer, and the TaqMan MGB probes labeled with two special dyes for two alleles: FAM and VIC. Genotyping was performed in a 5- μ L PCR reaction which contained 25 ng/ μ L DNA, 2.5 μ L of TaqMan Universal PCR Master Mix and 0.25 μ L of TaqMan SNP genotyping assay. The PCR program was 95°C for 10 min, 60 cycles of 92°C for 15 s and 60°C for 1 min and, finally, 4°C for storage (Yang, Sleep Medicine 2011). Genotyping data were collected using an ABI PRISM 7900HT Sequence Detection System. Genotypes were called using Software SDS Version 2.1 with automatic allele calling. The undetermined genotype rates were 5% for rs2300478 (all were control cases) and 5% for rs9357271 (50% were control cases and 50% were RLS cases).

Inductively coupled plasma-mass spectrometry (ICPMS): zinc quantification

ICPMS. Zinc and other metals were quantified as previously described (Development, validation and application of an ICP-MS/MS method to quantify minerals and (ultra) trace elements in human serum) [20].

Laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) imaging of zinc in the substantia nigra of autopsy tissue

Substantia nigra sections from autopsy tissue from four RLS and four sex- and age-matched control subjects were used in this study. Samples were obtained from one of our coauthors, J.C. Imaging of element distribution was performed using an NWR213 laser ablation (LA) system (Elemental Scientific Lasers, NE, USA) and 8800 Series inductively coupled plasma-triple quadrupole-mass spectrometer (ICP-QQQ-MS; Agilent Technologies, Victoria, Australia), and methods were adapted from those previously described [21, 22]. The NWR213 emits a nanosecond UV laser pulse at 213 nm, and a standard two-volume cell (dimensions 100 mm $\times$ 100 mm) was used for all experiments. The LA system was hyphenated to the ICP-QQQ-MS by a ~50 cm length of Tygon tubing connected directly to the ICP torch via a custom-built airtight quartz ball-and-socket. The ICP-QQQ-MS operated in MS/MS mode, with hydrogen (2 mL/min) to remove potential polyatomic interferences, primarily [23] Ar, [16] O⁺ on ⁵⁶Fe⁺ [24]. The ICP-QQQ-MS was fitted with “cs” lenses for enhanced sensitivity. Spatial quantitative data was generated with external calibration with matrix-matched (homogenized sheep cortex) tissue standards [25].

The material was ablated and transported to the ICP-QQQ-MSA square 50 μ m $\times$ 50 μ m beam operating with a constant fluence of 0.3 J/cm² via an argon carrier gas (1.1 L/min). The sample traversed the laser beam in the x-axis at 200 μ m/s, with the total integration time for each mass sweep set to 0.25 s, thus reproducing images with a true pixel area of 3,600 μ m² [26]. Data were collected in time-resolved analysis mode, with a single horizontal line of ablation exported as a comma-separated value file. Approximately 400 lines of ablation were used to construct the two-dimensional map of element distribution for each sample. Images were constructed using the in-house produced Biolite

software tool [27], built as an add-on module for the (now) commercial iolite (University of Melbourne, Victoria, Australia) data analysis resource [28]. A detailed visual tutorial for using Biolite can be found [29], and the Biolite add-on is freely available to iolite customers on request. Data were exported as tagged image file format (.TIFF) images for further interrogation. Differences in median values between groups were assessed using appropriate t-tests.

Lymphoblastoid cell culture and cell isolation

Lymphoblastoid cell lines [30, 31] from RLS patients and control subjects were maintained on Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin/glutamine, and 1% amphotericin B for two passages. After the second passage, cells were collected, washed twice with phosphate-buffered saline (PBS), and were resuspended in 500 μ L of PBS with protease inhibitors. The cells were lysed using sonication and centrifuged to remove cellular debris. Total protein was determined using a 2D Quant Kit (GE Healthcare).

Lymphoblastoid cell lysate digestion and atomic absorption analyses

Five mg of each lysate was resuspended and digested with 600 μ L of concentrated nitric acid for 18 h at 65°C. Afterward, the digested lysates were allowed to cool to room temperature and diluted with 600 μ L of distilled water. For analysis, each sample was diluted 1:10 and analyzed in triplicate using a Perkin Elmer Model: AAnalyst 800. Calibration standards (0.2–1.0 mg/L) were included with each analysis.

ELISA assay for ZIP8, ZIP14, and TRPM7

Lymphocytes were resuspended in phosphate-buffered saline and were sonicated for 30 s on ice. Lysed cells were centrifuged to remove cellular debris and protein amount was quantified using a 2D Quant kit as per manufacturer's recommendations (GE Healthcare, VWR). Quantification of zinc transporter (ZIP14, ZIP8, and TRPM7) was performed using ELISA assays against human ZIP8 (MBS9317558), ZIP14 (MBS9319878), and TRPM7 (MBS9322665) from MyBiosource (San Diego, CA). All samples were prepared and assays performed per the manufacturer's protocol and were run in duplicate. All quantification was determined against a standard curve that was run on the same plate.

C. elegans strains and zinc quantification

Nematodes were grown and maintained using standard procedures [32]. Wildtype N2 strain, and CB644 (*Meis1/unc-62* substitution mutant) were ordered from *C. elegans* genetic center (CGC), while the BTBD9/*hpo-9* mutant (allele *tm3719* with 761 bp deletion) was ordered from National BioResource Project: *C. elegans* in Japan. The nematodes were grown to the adult stage and their embryos were then synchronized at Larva 1 stage [33, 34]. Approximately 30,000 synchronized Larva 1 worms were pelleted and washed five times in 85 mM NaCl and resuspended in 500 μ L of 85 mM NaCl supplemented with 1% protease inhibitor

in an Eppendorf tube (1.5 mL). Samples were homogenized by sonication. An aliquot of 20 μ L was saved for protein quantification using the bicinchoninic acid (BCA) assay-kit (Thermo Scientific). The remaining samples were flash-frozen again in liquid nitrogen, then thawed and microwave digested. For microwave digestion, 450 μ L of the worm homogenate, 1230 μ L dH_2O , 200 μ L HNO_3 , 100 μ L H_2O_2 , and 20 μ L internal standard (100 $\mu\text{g/L}$ Rh) were transferred into 20 mL TFA microwave vessels. Digestion was carried out in closed vessels by heating to 200°C within 15 min applying 650 W and maintaining this temperature for another 20 min. After cooling down, samples were completely transferred into 15 mL polyethylene tubes. The concentration of ^{66}Zn was determined using ICP-MS (Agilent 8800 ICP-QQQ) [34]. Determinations of blank and reference material (certified fish reference material (ERM-BB422) (Joint Research Centre, European Commission, Geel, Belgium) were performed periodically.

Statistical analysis

Statistical tests were conducted in GraphPad Prism version 8 (GraphPad Software Inc.) and results were expressed as mean $\pm$ standard deviation. Differences were considered statistically significant if the p -value was <0.05 .

Results

Study cohort and genotype analysis

The study was conducted separately in two adult groups referred to as study 1 and study 2. Study 1 consisted of nine control (without a positive RLS diagnosis and no familial history of RLS) (six female and three male, average age 48.8 years, range 30–65 years) and 10 RLS patients (from the Vanderbilt University Medical Center seen by one of us (ASW) (6 female, 4 male, average age 53.1 years, range 39–65). Study 2, consisted of 20 control (without a positive RLS diagnosis and no familial history of RLS) (10 female and 10 male, average age 47.45 years, age range 26–76 years) and 20 RLS patients (with a family history of RLS) (11 female and 9 male, average age 48.65 years, range 29–71 years). We selected the single-nucleotide polymorphism (SNP) rs2300478 in MEIS1 and rs9357271 in BTBD9 for this study. These SNPs were chosen a priori to study initiation and based on the work of several groups [15–19].

For study 2, we genotyped one risk allele for MEIS1 (rs2300478) and BTBD9 (rs9357271), respectively, in the lymphocytes derived from both the control and RLS patients. For either risk allele, it is uncertain if they act via a dominant, recessive, or additive manner [19]. For rs2300478, we considered the presence of either one or two risk alleles “G” to be “at risk” given their being a minor allele in the population and thus anticipated a low probability of detecting G/G homozygous subjects (indeed only one was observed across all 40 subjects, who happened to be a control) (Table 1). Whereas for BTBD9 the risk SNP T is the major allele in our population, where C is the minor allele in the population. Therefore, given the low probability of detecting C/C homozygous subjects, the BTBD9 risk genotype was considered the presence of a homozygous T/T genotype (Table 1). As expected, we observed only 2 of 20 control subjects as homozygous C/C, and 1 of 20 RLS patients as homozygous C/C. The study 2 cohort was divided first based on the sex and then compared for the enrichment of risk genotypes for BTBD9 (T/T) or MEIS1 (G/T or G/G) using a chi-square test. We found that there was no significant enrichment of either of the risk genotypes by sex (Figure 1A, B, Table 1). Interestingly, when we tested the risk alleles based on disease status, we found that there was a significant enrichment for the presence ($p = 0.0252$) of BTBD9 risk genotype in the RLS group as compared to the control (Figure 1C), but this would not retain statistical significance if adjusted for multitesting. For MEIS1, there was no significant increased presence of the risk genotypes in the RLS patients (Figure 1D). Further, we tested for the enrichment of either risk genotype in the RLS and control groups, to test if combining the two risk genotypes would increase enrichment for RLS disease status. We found that there was a significant increase ($p = 0.0181$) in the presence of either or both of the risk genotypes in RLS patients versus control subjects (Figure 1E). Genetic analysis corroborated prior studies that carrying either one, or both risk genotypes was associated with RLS (Figure 1C–E). Details of the genotyping data are included in Table 1.

Analysis of metal concentrations in the serum

The concentrations of several metals were measured from the patients’ serum. Both the study 1 and study 2 cohorts were pooled to give a total of 29 controls (16 female and 13 male, average age 47.86 years, range 26–76 years) and 30 RLS (17 female and 13 male, average age 50.13 years, range 29–71 years).

Table 1. Chi-square analysis of genotype associations by sex and RLS status

Alleles	Group comparison (group 1 vs. group 2)	Number of genotype carriers	p value	Odds ratio for risk allele (95% CI)
MEIS (rs2300478)	Females vs. males	GT/GG : 9 vs. 8 TT: 11 vs. 12	0.749	1.23 (0.36–4.4)
BTBD9 (rs9357271)	Females vs. males	TT : 8 vs. 9 CT/CC: 12 vs. 11	0.749	0.81 (0.22–2.8)
MEIS (rs2300478)	RLS cases vs. healthy controls	GT/GG : 9 vs. 8 TT: 11 vs. 12	0.749	1.23 (0.36–4.4)
BTBD9 (rs9357271)	RLS cases vs. healthy controls	TT : 12 vs. 6 CT/CC: 8 vs. 14	0.0252*	4.50 (1.2–18)
MEIS (rs2300478) & BTBD9 (rs9357271)	RLS cases vs. healthy controls	GT/GG&TT : 17 vs. 10 TT&CT/CC: 3 vs. 10	0.0181*	5.67 (1.3–22)

Risk genotypes are highlighted in bold.

Figure 1. Genotype study of risk SNPs for MEIS1 (rs2300478) and BTBD9 (rs9357271). For rs2300478, the risk allele is the presence of G instead of a T. We defined MEIS1 risk in our cohort as either heterozygous G/T alleles or homozygous G/G. Similarly, for BTBD9 the risk SNP is T instead of a C, where C is the minor allele in the population. In our study sample, BTBD9 risk is the presence of homozygous T/T alleles at the locus. A&B, risk allele enrichment based on the sex for BTBD9 (T/T) (A) and Meis1 (G/T or G/G) (B). (C–E), the enrichment of risk genotypes for BTBD9 ($p = 0.0252$), MEIS1 and either BTBD9/Meis1 ($p = 0.0181$). A chi-square test was used to compare the genotypes.

Metals were analyzed in study cohorts 1 and 2 individually and in the pooled dataset. We found that there is a significant increase in the measured zinc in the RLS patients, while the other metals (including magnesium, calcium, manganese, iron, and copper) remained unchanged. This trend was consistent in the individual (Figure 2A, $p = 0.0458$; Figure 2B, $p = 0.0139$) and the combined sample (Figure 2C, $p = 0.0003$) data (Figure 2A–C; corresponding Tables 2–4). There was no correlation between the severity of RLS at the time of the blood draw as measured by the IRLS and the levels of serum zinc in either cohort 1, cohort 2, or the pooled data set. In this combined cohort, 5 controls were taking a multivitamin and six RLS patients were taking a multivitamin. To exclude the possibility that zinc was contained in the multivitamins and that zinc intake may have influenced the results, we did a subanalysis of those subjects not taking multivitamins and the results were similar, showing a statistically significant increase in zinc levels in RLS patients compared to controls ($p = 0.001416$). In addition, we also measured zinc levels

in lymphoblastoid cells derived from RLS and control subjects [30, 31]. All samples were analyzed by atomic absorption analysis. Unlike the serum measurements, the lymphoblastoid cell lines derived from subjects with RLS showed no significant difference in zinc content compared to cell lines derived from control subjects (Figure 2D). Taken together, the findings suggest that the underlying basis for elevated serum Zn in RLS patients may not be due to cell autonomous changes in zinc homeostasis of lymphocytes.

Given the significant association of the BTBD9 risk genotype with RLS patients in study 2 (Figure 1C), we examined whether the BTBD9 or MEIS1 risk genotype (versus nonrisk genotype) were associated with differences in serum metal levels. We observed no significant differences across any of the serum metals analyzed between risk versus non-risk genotypes (data not shown), these comparisons were performed using a two-tailed t-test, assuming equal variances without multi-testing correction to maximize sensitivity for detecting a genotype by

Figure 2. The levels of the individual metals in the serum and blood cells. The levels of magnesium (Mg), calcium (Ca), manganese (Mn), iron (Fe), copper (Cu), and zinc (Zn) were measured using ICPMS. (A–C) metals levels in the serum derived from study cohorts 1 (A), 2 (B), and the combined group (C). (D) Zinc concentrations in the lymphoblast cell lines were derived from RLS and control subjects. The data were analyzed by Multiple t-test with a Holm–Sidak correction (A, * $p = 0.0458$; B, * $p = 0.0139$; C, *** $p = 0.0003$).

Table 2: Study cohort 1 only (9 control/10 RLS) samples with serum metals levels measured by ICPMS

Metal	Control mean (mg/L)	Control std. dev. (mg/L)	RLS mean (mg/L)	RLS std. dev. (mg/L)	Holm–Sidak corrected p value
Mg	18.01	2.089	19.793	2.456	0.4247
Ca	93.66	6.141	97.005	3.870	0.4247
Mn	0.001081	0.001254	0.0007813	0.0002776	0.4700
Fe	1.174	0.3042	1.398	0.3614	0.4247
Cu	1.347	0.4000	1.102	0.2001	0.4247
Zn	0.9089	0.09307	1.096	0.1635	0.04580*

Table 3. Study cohort 2 only (20 control/20 RLS) serum metal levels as measured by ICPMS

Metal	Control mean (mg/L)	Control std. dev. (mg/L)	RLS mean (mg/L)	RLS std. dev. (mg/L)	Holm–Sidak corrected p value
Mg	19.40	1.966	19.35	2.002	0.9746
Ca	103.3	7.594	102.5	7.517	0.9746
Mn	0.00072	0.00036	0.00068	0.00028	0.9746
Fe	1.318	0.463976	1.559	0.4296	0.3948
Cu	0.9849	0.160837	1.016	0.2161	0.9746
Zn	0.8565	0.165477	1.024	0.1576	0.01390*

Table 4. Combined study cohort 1 and study cohort 2 (cohort 1: 9 control/10 RLS, study 2: 20 control/20 RLS) serum metal levels as measured by ICPMS

Metal	Control mean (mg/L)	Control std. dev. (mg/L)	RLS mean (mg/L)	RLS std. dev. (mg/L)	Holm-Sidak corrected <i>p</i> value
Mg	18.97	2.074	19.50	2.132	0.8080
Ca	100.3	8.404	100.7	6.857	0.8611
Mn	0.00083	0.00075	0.00071	0.00028	0.8080
Fe	1.273	0.4208	1.505	0.4092	0.1666
Cu	1.097	0.3038	1.045	0.2114	0.8080
Zn	0.8727	0.1472	1.048	0.1605	0.000316***

Figure 3. Zinc levels in the substantia nigra of autopsy tissue by LA-ICP-MS. The substantia nigra sections from these autopsy tissues of RLS and control subjects (age and sex-matched) were imaged by LA-ICP-MS. Representative elemental maps for zinc (⁶⁶Zn) (A) and quantitation of average zinc concentration (B). The data were analyzed by Student's *t*-test (*n* = 4; **p* = 0.0413).

serum-zinc association. Thus, despite a significant association of RLS risk with the established BTBD9 risk genotype in this cohort; the risk genotypes themselves had no significant influence on serum zinc or other metal levels.

Zinc levels in the brain of RLS patients

As blood zinc level was increased in RLS patients, we expected that the brain zinc levels may also be increased. To assess brain zinc levels, substantia nigra autopsy samples from four RLS and four control subjects (age- and sex-matched) were analyzed using LA-ICP-MS (Figure 3A). Our results show a statistically significant (*p* = 0.0413) increase in zinc levels for the RLS patients (5.24 ± 0.40 µg/g tissue) versus the controls (4.06 ± 0.22 µg/g tissue) (Figure 3B).

Zinc quantification in *C. elegans* RLS models

Worm strains *hpo-9(tm3719)* and *unc-62(e644)* were selected as the RLS risk alleles of BTBD9 and MEIS1, respectively. *hpo-9(tm3719)* has a deletion in exon 2 of the *hpo-9* gene, resulting in almost complete removal of exon 2. Although *unc-62(e644)* is a substitution mutant, it results in defects in both development and behavior, and is widely used to study *unc-62/MEIS1* function in *C. elegans*. The levels of the individual metals in these mutant strains were measured by ICP-MS and compared with wild-type N2 worms. Consistent with the human blood result, we found zinc levels were significantly increased (*p* = 0.0227) in *hpo-9(tm3719)* mutants, while levels of the other metals (including magnesium, manganese, iron, and copper) were statistically indistinguishable from controls (Figure 4).

Figure 4. The levels of the individual metals in the nematodes. The Control and BTBD9/*hpo-9* (*tm3719*) mutant worms were synchronized at larva stage 1, washed and collected for ICPMS study. The levels of Mg, Mn, Fe, Cu, and Zn were measured. The data were analyzed by two-way ANOVA with Sidak's multiple comparisons test (*n* = 4; **p* = 0.0227).

However, we did not observe any correlation between zinc levels and the presence or absence of the *unc-62* alleles (data not shown).

Quantification of zinc transporter proteins

Given that zinc levels were elevated in serum and autopsy brain specimens of RLS patients, we wonder whether this consequence was due to alteration of zinc transporters in RLS patients. Thus, we quantified the protein levels of three major zinc transporters (ZIP8, ZIP14, and TRPM7) in the lymphocytes derived from RLS and control subjects using ELISA assays. However, no significant differences were observed in these transporters between RLS patients and controls (Figure 5).

Discussion

The main findings of this study are that zinc levels are elevated in the serum and the brain of RLS patients compared to controls. The fact that the changes in serum zinc levels did not correlate with the severity of RLS at the time of the blood draw suggests that the changes in zinc are intrinsic to underlying pathophysiologic mechanism of RLS itself rather than a transitory change due to changes in symptom severity. Although changes in iron, calcium, and magnesium metabolism have been implicated in RLS [1–13], we did not observe changes in the serum levels of iron, calcium, and magnesium levels in this study, compatible with some of the previous literature [1, 7, 13]. Indeed, changes in serum iron levels in RLS have been an inconsistent finding in the literature. The most consistent finding in serum in RLS

Figure 5. Quantification of zinc transporter protein levels in RLS and Control peripheral lymphocytes. The levels of zinc transporter (ZIP8, ZIP14, and TRPM7) were determined with ELISA assays. No significant difference was observed in the protein levels of three zinc transporters. (A) ZIP8 (RLS: $n=17$, Control: 18); (B) ZIP14 (RLS: $n=15$, Control: 17); (C) TRPM7 (RLS: $n=15$, Control: 17). The box edges denote the upper and lower quartile range, with the box itself denoting the interquartile range, whiskers denote the full data range, and the horizontal line denotes the median data value for each group. The data were analyzed by Student's t-test. For each experiment, $n > 15$ samples measured, and each sample was run in duplicate.

is a decrease in serum ferritin, the iron-binding protein, which we did not study. In addition, changes in iron parameters in RLS are more consistently found in CSF, MRI, and autopsy studies as opposed to serum [2–4].

For this study, we addressed MEIS1 (rs2300478) and BTBD9 (rs9357271) risk alleles as possible factors in the etiology of familial RLS. The MEIS1 risk allele, an SNP “G,” has a reported minor allele frequency of 0.255 in controls and 0.395 in RLS cases [19]. In our cohort, we observed a minor allele frequency (MAF) of 0.225 in both the control and RLS cases. For BTBD9, the risk allele, an SNP “T,” is the major allele in the population, while the most prevalent minor allele is an SNP “C.” The reported frequency of the rs9358271 major allele T is 0.762 for controls and 0.817 for RLS cases [19]. In our study 2 cohort, we find that the allele frequency for the risk genotype is 0.600 in controls and 0.775 in RLS cases. Even with a very small population of 40 subjects, we find that the risk allele distribution is similar to the reported populations that had between 100 and 1,000 RLS patients [15–19], with estimated odds ratios for BTBD9 very similar to what we observed in this smaller population. Further, we find that the combined enrichment of the presence of both risk alleles in all RLS cases is highly significant ($p < 0.02$), even in a relatively small, and narrowly defined population, reinforcing the role of genetics in the etiology of RLS (Figure 1E). When looking at risk alleles independently we find that only the BTBD9 risk SNP is significantly enriched in our population, but not MEIS1 (Figure 1C, D). However, given the small sample size, the relative lower abundance of the MEIS1 risk allele in our population, and the clear improved enrichment when the two alleles are combined (Table 1), we do not think this negative result should be interpreted as evidence against MEIS1 playing a role in this population rather just that its impact may be weaker than BTBD9 due to either statistical power, biological effect size, or both (it is not possible to distinguish these from our data here). We also found no evidence of sex bias for either MEIS1 or BTBD9 risk alleles (Figure 1A, B, Table 1), though RLS occurrence is more prevalent in women.

From the analysis of the blood metal levels for the entire study samples (study 1 and study 2), we find that there is no significant difference in the levels of iron or manganese in the RLS patients. We find that there is an effect on zinc levels, such that RLS patients have significantly ($p < 0.05$) higher zinc levels than their controls (Figure 2C–E and Tables 2–4). This trend is

consistently seen in both the individual and the pooled studies. Consistent with these findings, our autopsy study of brain metals by LA-ICP-MS showed a significant increase of zinc level in the substantia nigra of RLS patients (Figure 3). There is no prior indication of an association of zinc changes with RLS, however, prior studies have shown that iron deficiency anemia is accompanied by zinc deficiency [35]. A known symptom of iron deficiency anemia is secondary RLS, and pregnancy-related RLS, both of which are ameliorated by iron administration. Further, zinc and iron are both cofactors of dopamine metabolic enzymes, thus supporting a possible role for zinc in RLS related to dopamine neurobiology.

In *C. elegans*, both BTBD9 and Meis1 have corresponding homologs known as *hpo-9* and *unc-62*, respectively. In the presence of *hpo-9*/BTBD9 mutation, zinc levels were elevated (Figure 4). The results are consistent with our findings in human serum samples (Figure 2). No difference was seen in the *unc-62*/MEIS1 mutant, which might be due to the mild nature of the substitution mutation, that is, the single nucleotide change in *unc-62* may not alter function sufficiently to impact metal levels in the context of the whole worm. Due to technical challenges, we were unable to determine zinc levels in the nematode head. However, given the significant results seen in the human substantia nigra, we postulate that zinc levels could also be upregulated in the nervous system of the nematode. Thus, although we do not see an association of the BTBD9 genetic risk factor in all subjects (RLS and control) for serum zinc levels, this observation in the worm model leaves open the possibility that BTBD9 related biology directly or indirectly leads to RLS and changes in zinc homeostasis.

Lymphoblastoid cell lines derived from subjects with RLS versus unaffected subjects had no significant differences in zinc content (Figure 2D). Thus, the basis for serum zinc increases in RLS patients is unclear. In our previous manuscript, where we examined iron regulation in lymphocytes we reported that ferroportin levels are upregulated in RLS [36]. In addition, recently Mitchell et al. reported that in addition to iron both zinc and cobalt are able to be exported by ferroportin [37]. We propose that the aberrant ferroportin expression levels that exist in RLS lymphocytes may result in decreased intracellular zinc concentrations and contribute to the corresponding increase in serum and brain zinc concentrations observed in the RLS patients. Alternatively, the immortalization or developmental

immaturity of the lymphoblastoid cell lines may not fully reproduce changes in zinc biology that occur in vivo.

Zinc homeostasis is regulated by cognate transporter proteins. As zinc levels were increased in serum and brain of RLS patients, one possible explanation is that zinc transporters are dysregulated. Three major zinc transporters, including ZIP8, ZIP14, and TRPM7, were selected to test the hypothesis. ZIP8 (SLC39A8) and ZIP14 (SLC39A14) are originally described as zinc uptake proteins [38], but have also demonstrated to play a role in other divalent metal uptake, such as iron, cadmium, and manganese [23,39,40]. ZIP8 is highly expressed on T cells [38] and has been shown to be inducible in models of inflammation, suggesting it regulates the cytoplasmic zinc levels which ties ZIP8 directly to the T-cell receptor (TCR)-mediated T cell activation process [41]. Although less is known about ZIP14 in peripheral blood monocytes, Ryu et al. reported the presence of ZIP14 message in adult human peripheral blood monocytes [42]. TRPM7 (transient receptor potential cation channel subfamily M member 7) conducts divalent and monovalent cations and contributes to labile cytosolic and nuclear Zn^{2+} concentrations [43]. TRPM7 has been demonstrated to signal many cellular processes including gene expression, mitosis, and cell survival [44]. TRPM7 expression has been demonstrated in peripheral blood monocytes and it is upregulated in models of high-glucose induced oxidative stress [45]. In the peripheral lymphocytes, these zinc transporter did not show a significant difference between patients and controls (Figure 5), but, arguably, this result is via comparable mechanisms to that described above for zinc levels in lymphoblastoid cell lines (Figure 2D). As the increase in zinc level is modest (only ~20%), it is also possible that a significant alteration of zinc transporters is not necessary to achieve a slight increase. Furthermore, zinc export may be somehow suppressed in RLS patients, rather than zinc import gets elevated. In either case, it would be important in the future to investigate the protein levels of these zinc exporters.

Zinc interacts with other metals and neurotransmitters thought to play a role in RLS. For example, zinc interacts with iron regulatory systems. As aforementioned, iron-deficiency is the most well-studied link to RLS [1–4] and the prevalence of RLS in iron deficiency anemia is nine times higher than in the general population [46]. Zinc functions as a catalyst in iron metabolism through the activity of alpha-aminolevulinic acid and serum zinc levels are decreased in iron deficiency anemia [35]. In RLS, there are changes in the dopaminergic and serotonergic monoamine system as well as the glutamatergic and endogenous opioid systems [47–50]. Zinc is present at high levels in glutamatergic synaptic vesicles and the GPR39 zinc receptor has been implicated in monoaminergic and glutamatergic neurotransmission [51, 52]. Zinc can inhibit the binding of endogenous opioid enkephalin analogs to their receptors throughout the brain implying that zinc is an important modulator of endogenous opioid function [53].

Current evidence links RLS to hypertension, heart disease, and stroke and its attendant hypoxia as well as to inflammatory and possibly autoimmune mechanisms [54–57]. Zinc also interacts with hypoxic and inflammatory mechanisms as well [58, 59]. The exact role that zinc plays in RLS needs further exploration although the modest increases seen in this study suggest that changes in zinc are not the primary cause of RLS but rather are downstream of the principal cause.

Acknowledgments

We thank the patients and families for their participation in this study. We thank Wendi Welch and Caroline Dodson as research assistants for this study. We also thank the *Caenorhabditis* Genetics Center for strain CB644 (*unc-62(e644)*) and the National Bioresource Project for the nematode for strain *hpo-9(tm3719)*.

Author Contributions

KB, ABB, and ASW for human research study design, clinical characterization, and sampling. P-Chen, MM, and MA designed and performed the *C. elegans* study. LX and GAR for the lymphoblastoid cell lines. ABB, RN, SP for the lymphocyte genotyping analysis. ABB, RN, JB, TS, MA for serum ICP-MS. DJH, KK, P-Crouch for LA-ICP-MS imaging of autopsy samples. JC and SP for lymphocyte study and zinc transporter study. P-Chen, RN, ABB, and AWS performed acquisition and analysis of data. P-Chen, RN, MA, ABB, and AWS were responsible for drafting the manuscript and figure preparation. All authors read and approved the final manuscript.

Funding

This study is supported by the National Institute of Environmental Health Sciences (NIEHS) (grants R01ES10563, NIEHS R01ES07331, and NIEHS R21ES031315), the ViCTER award 3R01ES010563-13S1 and “Manganese levels in patients with Restless Legs Syndrome versus controls.” Internal Vanderbilt funding to Dr. Walters—“Sleep Research in Neurology.” We thank further the German Research Foundation (DFG) for the financial support of the DFG Research Unit TraceAge (FOR 2558).

Conflict of interest statement: We declare no conflicts of interest.

References

1. Allen RP, et al.; International Restless Legs Syndrome Study Group (IRLSSG). Evidence-based and consensus clinical practice guidelines for the iron treatment of restless legs syndrome/Willis-Ekbom disease in adults and children: an IRLSSG task force report. *Sleep Med.* 2018;41:27–44.
2. Allen RP, et al. MRI measurement of brain iron in patients with restless legs syndrome. *Neurology.* 2001;56(2):263–265.
3. Earley CJ, et al. Abnormalities in CSF concentrations of ferritin and transferrin in restless legs syndrome. *Neurology.* 2000;54(8):1698–1700.
4. Connor JR, et al. Neuropathological examination suggests impaired brain iron acquisition in restless legs syndrome. *Neurology.* 2003;61(3):304–309.
5. O’Keefe ST, et al. Iron status and restless legs syndrome in the elderly. *Age Ageing.* 1994;23(3):200–203.
6. Högl B, et al. Restless legs syndrome: a community-based study of prevalence, severity, and risk factors. *Neurology.* 2005;64(11):1920–1924.
7. Collado-Seidel V, et al. Clinical and biochemical findings in uremic patients with and without restless legs syndrome. *Am J Kidney Dis.* 1998;31(2):324–328.
8. Lim LL, et al. Restless legs syndrome associated with primary hyperparathyroidism. *Sleep Med.* 2005;6(3):283–285.

9. Patton SM, et al. Proteomic analysis of the cerebrospinal fluid of patients with restless legs syndrome/Willis-Ekbom disease. *Fluids Barriers CNS*. 2013;**10**(1):20.
10. Wali S, et al. The effect of vitamin D supplements on the severity of restless legs syndrome. *Sleep Breath*. 2015;**19**(2):579–583.
11. Hornyak M, et al. Magnesium therapy for periodic leg movements-related insomnia and restless legs syndrome: an open pilot study. *Sleep*. 1998;**21**(5):501–505.
12. Bartell S, et al. Intravenous magnesium sulfate may relieve restless legs syndrome in pregnancy. *J Clin Sleep Med*. 2006;**2**(2):187–188.
13. Walters AS, et al. Magnesium not likely to play a major role in the pathogenesis of Restless Legs Syndrome: serum and cerebrospinal fluid studies. *Sleep Med*. 2007;**8**(2):186–187.
14. Fitsanakis VA, et al. Manganese (Mn) and iron (Fe): interdependency of transport and regulation. *Neurotox Res*. 2010;**18**(2):124–131.
15. Winkelmann J, et al. Genome-wide association study of restless legs syndrome identifies common variants in three genomic regions. *Nat Genet*. 2007;**39**(8):1000–1006.
16. Kemlink D, et al. Replication of restless legs syndrome loci in three European populations. *J Med Genet*. 2009;**46**(5):315–318.
17. Schormair B, et al. MEIS1 and BTBD9: genetic association with restless leg syndrome in end stage renal disease. *J Med Genet*. 2011;**48**(7):462–466.
18. Stefansson H, et al. A genetic risk factor for periodic limb movements in sleep. *N Engl J Med*. 2007;**357**(7):639–647.
19. Yang Q, et al. Association studies of variants in MEIS1, BTBD9, and MAP2K5/SKOR1 with restless legs syndrome in a US population. *Sleep Med*. 2011;**12**(8):800–804.
20. Meyer S, et al. Development, validation and application of an ICP-MS/MS method to quantify minerals and (ultra-) trace elements in human serum. *J Trace Elem Med Biol*. 2018;**49**:157–163.
21. Bishop DP, et al. Elemental bio-imaging using laser ablation-triple quadrupole-ICP-MS. *J Analyt At Spectrom*. 2016;**31**(1):197–202.
22. Hare DJ, et al. Laser ablation-inductively coupled plasma-mass spectrometry imaging of white and gray matter iron distribution in Alzheimer's disease frontal cortex. *Neuroimage*. 2016;**137**:124–131.
23. Liuzzi JP, et al. Zip14 (Slc39a14) mediates non-transferrin-bound iron uptake into cells. *Proc Natl Acad Sci USA*. 2006;**103**(37):13612–13617.
24. Lear J, et al. High-resolution elemental bioimaging of Ca, Mn, Fe, Co, Cu, and Zn employing LA-ICP-MS and hydrogen reaction gas. *Anal Chem*. 2012;**84**(15):6707–6714.
25. Hare DJ, et al. Protocol for production of matrix-matched brain tissue standards for imaging by laser ablation-inductively coupled plasma-mass spectrometry. *Analyt Methods*. 2013;**5**(8):1915–1921.
26. Lear J, et al. Improving acquisition times of elemental bio-imaging for quadrupole-based LA-ICP-MS. *J Analyt At Spectrom*. 2012;**27**(1):159–164.
27. Paul B, et al. Visualising mouse neuroanatomy and function by metal distribution using laser ablation-inductively coupled plasma-mass spectrometry imaging. *Chem Sci*. 2015;**6**(10):5383–5393.
28. Paton C, et al. Iolite: freeware for the visualisation and processing of mass spectrometric data. *J Analyt At Spectrom*. 2011;**26**(12):2508–2518.
29. Hare DJ, et al. Imaging metals in brain tissue by laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS). *J Visual Exp: JoVE*. 2017;**119**:55042.
30. Xiong L, et al. MEIS1 intronic risk haplotype associated with restless legs syndrome affects its mRNA and protein expression levels. *Hum Mol Genet*. 2009;**18**(6):1065–1074.
31. Catoire H, et al. A direct interaction between two Restless Legs Syndrome predisposing genes: MEIS1 and SKOR1. *Sci Rep*. 2018;**8**(1):12173.
32. Brenner S. The genetics of *Caenorhabditis elegans*. *Genetics*. 1974;**77**(1):71–94.
33. Leyva-Illades D, et al. SLC30A10 is a cell surface-localized manganese efflux transporter, and parkinsonism-causing mutations block its intracellular trafficking and efflux activity. *J Neurosci*. 2014;**34**(42):14079–14095.
34. Bornhorst J, et al. The effects of pdr1, djr1.1 and pink1 loss in manganese-induced toxicity and the role of [small alpha]-synuclein in *C. elegans*. *Metallomics*. 2014;**6**(3):476–490.
35. Kelkitli E, et al. Serum zinc levels in patients with iron deficiency anemia and its association with symptoms of iron deficiency anemia. *Ann Hematol*. 2016;**95**(5):751–756.
36. Earley CJ, et al. Altered iron metabolism in lymphocytes from subjects with restless legs syndrome. *Sleep*. 2008;**31**(6):847–852.
37. Mitchell CJ, et al. Functional properties of human ferroportin, a cellular iron exporter reactive also with cobalt and zinc. *Am J Physiol Cell Physiol*. 2014;**306**(5):C450–C459.
38. Begum NA, et al. *Mycobacterium bovis* BCG cell wall and lipopolysaccharide induce a novel gene, BIGM103, encoding a 7-TM protein: identification of a new protein family having Zn-transporter and Zn-metalloprotease signatures. *Genomics*. 2002;**80**(6):630–645.
39. Dalton TP, et al. Identification of mouse SLC39A8 as the transporter responsible for cadmium-induced toxicity in the testis. *Proc Natl Acad Sci USA*. 2005;**102**(9):3401–3406.
40. He L, et al. ZIP8, member of the solute-carrier-39 (SLC39) metal-transporter family: characterization of transporter properties. *Mol Pharmacol*. 2006;**70**(1):171–180.
41. Aydemir TB, et al. Zinc transporter ZIP8 (SLC39A8) and zinc influence IFN-gamma expression in activated human T cells. *J Leukoc Biol*. 2009;**86**(2):337–348.
42. Ryu MS, et al. Genomic analysis, cytokine expression, and microRNA profiling reveal biomarkers of human dietary zinc depletion and homeostasis. *Proc Natl Acad Sci USA*. 2011;**108**(52):20970–20975.
43. Krapivinsky G, et al. The TRPM7 channel is cleaved to release a chromatin-modifying kinase. *Cell*. 2014;**157**(5):1061–1072.
44. Vallee BL, et al. The biochemical basis of zinc physiology. *Physiol Rev*. 1993;**73**(1):79–118.
45. Wuensch T, et al. High glucose-induced oxidative stress increases transient receptor potential channel expression in human monocytes. *Diabetes*. 2010;**59**(4):844–849.
46. Allen RP, et al. The prevalence and impact of restless legs syndrome on patients with iron deficiency anemia. *Am J Hematol*. 2013;**88**(4):261–264.
47. Allen RP, et al. Thalamic glutamate/glutamine in restless legs syndrome: increased and related to disturbed sleep. *Neurology*. 2013;**80**(22):2028–2034.
48. Connor JR, et al. Altered dopaminergic profile in the putamen and substantia nigra in restless leg syndrome. *Brain*. 2009;**132**(Pt 9):2403–2412.
49. Jhoo JH, et al. Availability of brain serotonin transporters in patients with restless legs syndrome. *Neurology*. 2010;**74**(6):513–518.

50. Walters AS, et al. Does the endogenous opiate system play a role in the Restless Legs Syndrome? A pilot post-mortem study. *J Neurol Sci.* 2009;279(1-2):62–65.
51. Młyniec K, et al. Investigation of the GPR39 zinc receptor following inhibition of monoaminergic neurotransmission and potentialization of glutamatergic neurotransmission. *Brain Res Bull.* 2015;115:23–29.
52. Vergnano AM, et al. Zinc dynamics and action at excitatory synapses. *Neuron.* 2014;82(5):1101–1114.
53. Stengaard-Pedersen K. Inhibition of enkephalin binding to opiate receptors by zinc ions: possible physiological importance in the brain. *Acta Pharmacol Toxicol.* 1982;50(3):213–220.
54. Boulos MI, et al. Periodic limb movements and white matter hyperintensities in first-ever minor stroke or high-risk transient ischemic attack. *Sleep.* 2017;40(3):zsw080.
55. Ferri R, et al. Silent cerebral small vessel disease in Restless Legs Syndrome. *Sleep.* 2016;39(7):1371–1377.
56. Patton SM, et al. Hypoxia-inducible factor pathway activation in restless legs syndrome patients. *Eur J Neurol.* 2011;18(11):1329–1335.
57. Walters AS, et al. Review of the relationship of restless legs syndrome and periodic limb movements in sleep to hypertension, heart disease, and stroke. *Sleep.* 2009;32(5):589–597.
58. Bonaventura P, et al. Zinc and its role in immunity and inflammation. *Autoimmun Rev.* 2015;14(4):277–285.
59. Wang WM, et al. The zinc ion chelating agent TPEN attenuates neuronal death/apoptosis caused by hypoxia/ischemia via mediating the pathophysiological cascade including excitotoxicity, oxidative stress, and inflammation. *CNS Neurosci Ther.* 2015;21(9):708–717.