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Original Article

No association between plasma hepcidin levels and restless legs syndrome - results from the Danish Blood Donor Study

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ABSTRACT

Background: Restless Legs Syndrome (RLS) is a neurological sensorimotor disorder that occurs in the evening and night, thereby often impacting quality of sleep in sufferers. The aetiology of RLS is not completely understood although iron dysregulation has been suggested as a likely pathway. The relationship between RLS and the iron regulatory protein hepcidin has not been studied in large cohorts. We aimed to assess whether an association between plasma hepcidin variation and RLS exists in a large cohort of healthy individuals.

Methods: Plasma hepcidin levels were measured in 9708 Danish blood donors from the Danish Blood Donor Study all of whom correctly completed the validated Cambridge–Hopkins RLS-questionnaire for RLS assessment.

Results: A total of 466 blood donors were determined as current RLS cases in the sample (4.8%). RLS cases had a significantly higher proportion of females (56.7% vs 46.7%; $P < 0.001$) and were older (median age [IQR] 40.6 years vs 38.0 years; $P = 0.010$) than controls. RLS cases were also more frequent smokers ($P = 0.004$). No significant differences were found in body mass index (BMI), alcohol consumption, time of donation and donation history between cases and controls. No difference in plasma hepcidin levels was observed between RLS cases and controls (median concentration [IQR]: 10.5 ng/ml [6.3–16.4] in RLS cases vs 10.5 ng/ml [6.0–16.5] in controls). Using a logistic regression model, we found that hepcidin levels were not associated with RLS after adjusting for age, sex, alcohol consumption, smoking status, donation time and donation history (OR = 1.00 [0.99–1.02] per 1 ng/ml increase of hepcidin; $P = 0.429$).

Conclusion: Our study in Danish blood donors did not find an association between RLS and plasma hepcidin levels. Our findings suggest that plasma hepcidin's role as a potential diagnostic biomarker of RLS is inadequate.

1. Introduction

Restless Legs Syndrome (RLS) is a sensorimotor disorder with a prevalence ranging from 5 to 18.8% in European populations [1–3]. The irresistible urge to move one's legs is a predominant symptom of RLS and either exclusively occurs or worsens during the evening and night, thereby typically affecting sleep [4]. Many health risk factors are associated with RLS including obesity, smoking, high alcohol intake and low physical activity [1,5,6]. Sufferers of RLS report worse sleep quality and are more likely to experience poor health-related quality of life and depressive symptoms [5,7]. Despite the high prevalence and the associated negative impacts, the pathophysiology of RLS is not completely understood, although current accepted pathways include genetic predisposition, iron dysregulation in the central nervous system and dopaminergic dysfunction [8,9]. A link between inflammation and RLS has been suspected due to many of the RLS-associated conditions having inflammatory associations [10]. Inflammatory biomarkers such as serum C-reactive protein (CRP), interleukin-6, CRP/albumin ratio

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and neutrophil-to-lymphocyte ratio have previously been associated with RLS [11–14]. A proposed link between RLS, iron dysregulation and inflammation may be found in the iron regulatory protein hepcidin. Hepcidin is the primary hormone responsible for the regulation of systemic iron levels and acts by binding to the iron-exporter ferroportin, inhibiting cellular iron efflux and resulting in decreased iron availability [15,16]. Inflammation leads to the production of pro-inflammatory cytokines such as interleukin-6 (IL-6), which are known to induce the synthesis of hepcidin [17]. Increased levels of hepcidin have also previously been associated with RLS-associated conditions including obesity and rheumatoid arthritis [18,19]. To date, the relationship between hepcidin and RLS has not been studied in larger cohorts, however in a small case–control study (102 RLS-cases and 73 controls), increased serum hepcidin levels were associated with RLS patients, regardless of treatment and history of augmentation [20]. Similarly in another small study (36 RLS-cases and 36 controls), increased serum hepcidin levels were associated with increased probability of RLS in chronic haemodialysis patients [21]. Therefore, the aim of our study was to assess the association between plasma hepcidin variation and RLS in a large cohort of otherwise healthy individuals, using data from the Danish Blood Donor Study cohort (N = 9708).

2. Methods

2.1. Study population

The individuals that participated in this study are part of The Danish Blood Donor Study (DBDS), a nationwide research platform utilizing the existing infrastructure in the Danish blood banks by including blood donors when they show up to donate [22]. Participants are between the ages of 18 and 67 years and must be generally healthy and not on medication to be eligible as blood donors. Upon enrolment, participants gave informed consent, whole blood, plasma, and answered a comprehensive questionnaire. So far, approximately 135,000 participants have been enrolled. The project is approved by the Research Ethics Committee (M–20090237) and by the Danish Data Protection Agency under the combined approval for health care research at The Capital Region of Denmark (P-2019-99).

2.2. RLS assessment

RLS-status was determined using the Cambridge–Hopkins RLS-questionnaire (CH-RLSq), which is a self-completed written questionnaire containing 10 items, including items that exclude mimics of RLS such as positional discomfort and leg cramps, and has been validated in several population settings, including English blood donors (diagnostic sensitivity 87.2% and specificity 94.4%) [23,24]. The questionnaire was translated from English to Danish using the back-translation method as previously described [5]. A total of 53,239 DBDS participants have answered the CH-RLSq as well as other health- and life-style related questions such as smoking and alcohol consumption. For this study, the blood donor must have experienced the RLS symptoms within the past 12 months to be considered an RLS case. As part of the questionnaire, symptom severity was also assessed using two additional questions. To be classified as a severe RLS case in the study, the blood donor must answer “moderately” or “extremely distressing” to the question “When you actually experience the feelings in your legs, how distressing are they?“, as well answer either “2–3 days per week”, “4–5 days per week” or “every day” to the question “In the past 12 months, how often did you experience these feelings in your legs?“. Blood donors with correctly completed CH-RLSq, complete covariate data and hepcidin measurements available were used in the analysis.

2.3. Hepcidin measurement

Hepcidin was assessed by a validated high-sensitivity hepcidin ELISA kit (EIA-5782, DRG Instruments, Marburg, Germany) and was performed as described by the manufacturers' instructions. The assay is a competitive ELISA that uses biotinylated hepcidin to compete with endogenous test sample hepcidin for binding with a monoclonal anti-hepcidin antibody which has been precoated on to the wells of microtiter plates. Samples were assayed in an automated setup by use of Hamilton MicroLab STAR liquid-handling platform for all the liquid handling steps and all washing steps were done by an automated plate washer. For the initial validation four plasma samples were measured 8 times in non-adjacent positions in one plate, and then CV% was calculated for each sample to determine intra-assay variation (CV% ranged from 6 to 11%). Samples were also tested in singlicate wells in two identical positions in two plates. The inter-assay correlation coefficient was 0.96 and 0.97.

Blood samples were collected in standard tubes, from which plasma aliquots were added to 2D barcoded Matrix tubes and stored at −20 °C until analysis. Plasma samples (N = 10,029) were measured in singlicates and hepcidin concentrations were derived from standard curves obtained using the manufacturers' supplied standards (range 0.153 ng/ml − 81 ng/ml) and calculated using 4-parameter curve fit using GraphPad Prism Software v. 6.03 (La Jolla, California, USA). Day-to-day kit reproducibility and performance was assessed by Statistical Process Control. Compliance with the DRG upper and lower thresholds for both “Low” and “High” controls was also assessed and all were within the manufacturers' stated limits. All CV% were below 15% for both controls and standards. The optical densities of the zero standards were all ≥1.20 (manufacturers stated minimum).

Plasma ferritin was measured in a subgroup of DBDS (N = 30,903) of which N = 4544 overlapped with the same samples used in the hepcidin cohort (ie, same donor on same donation date (matching sample ID numbers)). Ferritin was measured on fresh EDTA-anticoagulated plasma samples using the commercially available assay Ortho Vitros 5600 (Ortho Clinical Diagnostics, Rochester, NY, USA).

2.4. Statistical analyses

Differences between RLS cases and controls were first compared using descriptive statistics including median with interquartile ranges (IQR) for non-normally distributed quantitative variables, and count number with percentages for categorical variables. Comparisons between RLS cases and controls were made using the Chi-square test for categorical variables and Kruskal–Wallis rank test for continuous variables. Logistic regression models were then used to assess the effect of hepcidin levels on the probability of having RLS. Demographic and lifestyle variables that are known to be associated with RLS and hepcidin were included in an adjusted logistic regression model, including time of blood donation due to known diurnal hepcidin level variability [25,26], and number of donations within the last 3 years as a proxy for iron deficiency [27]. A p-value <0.05 was defined as being statistically significant. All statistical analyses were performed using R (version 3.5).

3. Results

A total of 9708 Danish blood donors were available for the study, of which 466 were determined as current RLS cases (4.8%). The proportion of females in the RLS cases group was higher than in controls (56.7% vs 46.7%, P < 0.001), and RLS cases were also older (median age 40.6 years in RLS cases vs 38.0 years in controls; P = 0.010). There was a difference in smoking between the two groups, with 13.3% of RLS cases smoking more than one cigarette a day compared to only 8.9% in the control group (P = 0.004). Body mass index (BMI), alcohol consumption, time of donation and donation history did not differ between RLS cases and the control group (Table 1). Plasma ferritin measurements were available for a subgroup (N = 4544). Ferritin levels did not differ between RLS cases and controls (median concentration: 52.3 ng/ml [33.2–77.6] in RLS cases vs 53.8 ng/ml [35.3–82.4] in controls; P = 0.258).

Plasma hepcidin levels in our sample ranged between 0.1 and 69.1 ng/ml; however, one individual in the control group had a hepcidin level below the detection limit, and was recoded with a hepcidin level of 0.1 ng/ml. No difference in plasma hepcidin levels was observed between RLS cases and controls (median concentration [IQR]: 10.5 ng/ml [6.3–16.4] in RLS cases vs 10.5 ng/ml in controls [6.0–16.5]). Using a logistic regression model with RLS-status as the dependent variable, we found no association between hepcidin levels and RLS in blood donors both in the crude analysis (crude OR = 1.00 [0.99–1.01] per 1 ng/ml increase of hepcidin; P = 0.669) and after adjusting for age, sex, alcohol consumption, smoking status, time of donation, BMI and number of donations within the last three years (multivariable OR = 1.00 [0.99–1.02] per 1 ng/ml increase of hepcidin; P = 0.429). Similarly, when dividing hepcidin levels into tertiles, we observed no statistically significant association between hepcidin levels and RLS (Table 2). We additionally performed the same analyses in the subgroup where ferritin measurements were available, however adjusting for ferritin did not improve the model (OR = 1.00 [0.98–1.02] per 1 ng/ml increase of hepcidin; P = 0.997) (Table 2).

We additionally examined whether an association could be found between hepcidin levels and severe RLS. Of the 466 RLS cases in the sample, 21 were classified as severe RLS cases (blood donors reporting the experience of having either moderately or extremely distressing RLS symptoms AND experiencing RLS symptoms at least 2–3 days per week in the last 12 months). Plasma hepcidin levels did not differ between severe RLS cases and controls (OR = 1.00 [95%CI 0.95–1.05], P = 0.916 in unadjusted model; OR = 1.01 [0.95–1.06], P = 0.789 in model adjusting for age, sex, alcohol consumption, smoking status, time of donation, BMI and donations within last three years) (Table 3).

4. Discussion

Our study examined the plasma hepcidin levels in a cohort of 9708 otherwise healthy Danish blood donors with known RLS status. No difference in plasma hepcidin levels was observed between RLS cases and controls and no associations were found in neither crude nor adjusted logistic regression models, suggesting that hepcidin's potential as a biomarker of RLS is limited.

Our findings are in contrast to results reported in previous studies. Chenini et al. [20] found higher hepcidin levels in RLS patients than in controls, and Tufekci and Kara [21] reported a significant association between RLS and hepcidin in chronic haemodialysis patients. A clear strength of our study is the sample size of N = 9,708, which surpasses the size of the previous studies

Table 1
Demographic and donation descriptive statistics of RLS cases and controls in the Danish Blood Donor Study (N = 9708), including a subgroup of the cohort with ferritin measurements available (N = 4544). P-values in bold denote significant differences between RLS cases and controls (P < 0.05).

	Full DBDS hepcidin cohort (N = 9708)					Subgroup with ferritin measurements (N = 4544)				
	Controls N = 9242		RLS Cases N = 466		P value	Controls N = 4330		RLS Cases N = 214		P value
	N	%	N	%		N	%	N	%	
Sex										
Male	4928	53.3	202	43.3	**<0.001**	2157	49.8	92	49.5	0.051
Female	4314	46.7	264	56.7		2173	50.2	122	50.5	
Age										
median (IQR)	38.0 (27.6–50.0)		40.6 (29.3–50.8)		**0.010**	33.9 (26.0–47.3)		36.1 (27.5–46.6)		0.195
BMI										
median (IQR)	24.66 (22.50–27.17)		24.51 (22.64–27.16)		0.929	24.17 (22.16–26.54)		24.05 (22.24–26.38)		0.854
<18.5	52	0.6	<5	<1	0.659	34	0.8	<5	<1	0.296
18.5–25	5044	54.6	262	56.2		2594	59.9	132	61.7	
25–30	3123	33.8	155	33.3		1312	30.3	69	32.2	
30–35	788	8.5	41	8.8		289	6.7	12	5.6	
35–40	180	1.9	5	1.1		73	1.7	<5	<1	
>40	55	0.6	<5	<1		28	0.6	<5	<1	
Smoking status										
Non-smoker	7987	86.4	380	81.5	**0.004**	3697	85.4	179	83.6	0.560
<1 cigarette per day	434	4.7	24	5.2		259	6.0	12	5.6	
>1 cigarette per day	821	8.9	62	13.3		374	8.6	23	10.7	
Alcohol consumption										
Never/almost never	1209	13.0	47	10.1	0.052	21	9.8	21	9.8	0.588
A couple of times a month	4786	51.8	230	49.4		109	50.9	109	50.9	
A couple of times a week	2851	30.8	163	35.0		71	33.2	71	33.2	
Daily/almost daily	396	4.3	26	5.6		13	6.1	13	6.1	
Time of donation										
Median hour (IQR)	12 (10–14)		12 (10–14)		0.929	12 (10–14)		11 (10–13)		0.518
Morning (before noon)	4383	47.4	211	47.4	1.000	2099	48.5	108	50.5	0.320
Early Afternoon (after noon; before 4pm)	4163	45.0	210	45.1		2028	46.8	92	43.0	
Late Afternoon (after 4pm)	696	7.5	35	7.5		203	4.7	14	6.5	
Number of donations						**Ferritin (ng/ml)**; median (IQR)				
In previous 3 years, median (IQR)	4 (3–6)		4 (3–6)		0.686	53.8 (35.3–82.4)		52.3 (33.2–77.6)		0.258

Table 2
Logistic Regression Models for hepcidin levels in RLS cases and controls in the Danish Blood Donor Study.

N = 9708	Controls N = 9242		RLS Cases N = 466		Model 0 (Crude association)		Model 1 (adjusted for: age, sex, alcohol, smoking + donation time + BMI + donations within last 3 years)	
	median (IQR)		median (IQR)		OR [95% CI]	P value	OR [95% CI]	P value
Hepcidin, ng/ml	10.5 (6.0–16.5)		10.5 (6.3–16.4)		1.00 [0.99–1.01]	0.669	1.00 [0.99–1.02]	0.429
Hepcidin tertiles	n	%	n	%				
<7.6 ng/ml	3,114	33.7	146	31.3	1		1	
7.6–14.0 ng/ml	3,055	33.1	166	35.6	1.16 [0.92–1.46]	0.205	1.21 [0.96–1.53]	0.113
>14.0 ng/ml	3,073	33.3	154	33.0	1.07 [0.85–1.35]	0.573	1.12 [0.88–1.44]	0.362
Subgroup with ferritin measurements available								
N = 4544	Controls N = 4330		RLS Cases N = 214		Model 0 (Crude association)		Model 2 (adjusted for: age, sex, alcohol, smoking + donation time + BMI + ferritin)	
	median (IQR)		median (IQR)		OR [95% CI]	P value	OR [95% CI]	P value
Hepcidin, ng/ml	10.3 (6.0–16.1)		10.1 (6.0–15.3)		1.00 [0.98–1.01]	0.821	1.00 [0.98-1.02]	0.997

Table 3
Logistic regression models for hepcidin levels in severe RLS cases vs controls in the Danish Blood Donor Study.

	Controls N = 9242		Severe RLS Cases N = 21		Model 0		Model 1 (adjusted for: age, sex, alcohol, smoking + donation time + BMI + donations within last 3 years)	
	median (IQR)		median (IQR)		OR [95% CI]	P value	OR [95% CI]	P value
Hepcidin, ng/ml	10.5 (6.0–16.5)		12.1 (6.4–16.5)		1.00 [0.95–1.05]	0.916	1.01 [0.95–1.06]	0.789
Hepcidin tertiles	n	%	N	%				
<7.6 ng/ml	3,114	33.7	8	38.1	1		1	
7.6–14.0 ng/ml	3,055	33.1	5	23.8	0.64 [0.19–1.91]	0.429	0.69 [0.20–2.10]	0.517
>14.0 ng/ml	3,073	33.3	8	38.1	1.01 [0.37–2.76]	0.979	1.10 [0.37–3.21]	0.866

(N = 175 and N = 72 respectively). The validity of our study is additionally strengthened by using a uniformly healthy study population. Blood donors are thoroughly screened at every visit to the blood bank and are required to be healthy to be eligible as blood donors. Persons diagnosed with chronic diseases such as cancer and hypertension are permanently excluded. Therefore, the requirement of being generally healthy and not on medication reduces the presence of comorbidities that may confound true hepcidin-RLS associations. RLS can be categorised as either primary/idiopathic or secondary, where iron deficiency, pregnancy and kidney disease among other diseases may be the underlying comorbidity in secondary RLS [28]. We therefore assume that RLS cases in the DBDS are predominantly idiopathic and this may also explain the discrepant findings with the previous studies. Although blood donors may potentially have reduced iron levels, we have previously found that RLS is not associated with a reduced ferritin level in Danish blood donors [1]. The frequency of blood donations a blood donor has undertaken over a three year period can also be used as a proxy measure for iron deficiency, as this has previously been found to be the strongest predictor of iron deficiency in the DBDS population [27]. However, the number of blood donations three years prior to RLS assessment in RLS cases and controls in this sample did not differ (Table 1); a finding which has also been reported previously in Danish blood donors with RLS [1]. Finally, for a subgroup of the cohort, plasma ferritin levels were also available and we did not find a significant difference between ferritin levels in RLS cases and controls. Given this, as well as the larger sample size and their representation of a particularly healthy subset of the population, we remain confident that the use of blood donors was suitable for investigating the possible association between RLS and hepcidin in healthy individuals.

Despite finding no association between plasma hepcidin levels and RLS in our study, it is possible that hepcidin may still play a role in RLS pathophysiology. As discussed by Dauvilliers et al. [29], serum hepcidin levels and brain hepcidin concentration may not be correlated. Evidence of low brain iron levels in individuals with RLS have been found in several studies as summarised in recent reviews [9,30]. These studies include Magnetic Resonance Imaging (MRI) brain scans of RLS patients [31], cerebrospinal fluid (CSF) measurements [32], autopsy-based histological studies in the brains of RLS patients [33,34] as well as in numerous animal models. Although the majority of these studies focused on the iron markers ferritin and transferrin, a study specifically measured the autopsy-based brain expression and CSF concentration of pro-hepcidin (the inactive precursor to hepcidin) in RLS patients (12 with early-onset RLS, 13 with late-onset RLS, and 14 control subjects) [35]. The study found significantly increased pro-hepcidin expression in the brain parenchyma and within the neuromelanin cells of the substantia nigra of RLS patients compared to controls, as well as decreased pro-hepcidin levels in the CSF of early-onset RLS patients. Dopamine-based therapies have shown to be generally effective in treating RLS which is believed to be due to RLS patients' impaired re-uptake of synaptic dopamine and reduced D2 receptor density [36]. In line with our findings, a recent small study (18 RLS cases; 15 controls) found no significant differences in serum hepcidin levels between RLS cases and controls at diagnosis, but interestingly reported a significant hepcidin decrease in the same RLS cases after 12 weeks of dopaminergic treatment [37]. However, hepcidin levels were not different between the two groups in a similar setup after 13.8 months dopaminergic treatment [20].

Though there are clear strengths, we note some limitations to the present study. Hepcidin levels are subject to diurnal rhythms in which hepcidin increases during the day [25,38], and to avoid this, blood sampling should ideally be collected in the early morning after overnight fasting or at the same time during the day for all participants. This was not possible for our study as our samples are blood donors who donate their blood throughout the day. Additionally, blood donors are not allowed to donate when fasting to

minimize the risk of vasovagal events. To account for diurnal hepcidin variability, we used the time of blood donation as an additional covariate in the adjusted model. We adjusted for several covariates based on statistically significant differences found in the descriptive statistics as well as in prior literature; however, we cannot exclude residual confounding. We also note that hepcidin was measured in plasma samples, not serum samples in our study and likewise used a different (though validated and high-sensitivity) hepcidin ELISA kit than the smaller previous studies [20,21], although it is unknown whether this may explain our discrepant results.

RLS-status for the blood donors was determined using the CH-RLS questionnaire which lacks a quantitative scale for symptom severity that the International Restless Legs Syndrome Study Group (IRLSSG) rating scale provides. Both of the previously mentioned hepcidin-RLS studies used the IRLSSG rating scale for their analyses and Chenini et al. [20] excluded mild RLS cases, allowing only moderate to very severe RLS cases. Likely due to the healthiness of the blood donors, only 21 (0.22%) of Danish blood donors in our study were diagnosed as having severe RLS according to the CH-RLSq. It is unknown whether the limited number of severe cases in our study prevented us from replicating the smaller studies' RLS-hepcidin association. It has been reported that repeated blood donations has induced or perpetuated RLS due to iron deficiency [39], which may then force RLS-sufferers to stop donating blood, thereby biasing the blood donor population against those with RLS and severe RLS. Our particularly healthy population of blood donors may therefore not be generalizable to the general population, despite the healthiness of the donors also reduces presence of comorbidities and the possibility of secondary RLS. However, a study in English blood donors (N = 2005) found no evidence that the frequency or number of blood donations up to the maximum of three times a year would increase the risk of RLS [40]. Regardless, our study demonstrated that plasma hepcidin was not associated with RLS-cases when determined by the validated CH-RLSq in an otherwise healthy population of blood donors.

In conclusion, using a large cohort of healthy blood donors, our study is unable to replicate findings that increased plasma hepcidin levels are associated with RLS. Our results suggest that plasma hepcidin's potential role as a diagnostic biomarker for RLS is inadequate, however differentiating between idiopathic and secondary RLS in future studies may explain these discrepant findings. Further studies focusing on hepcidin in the central nervous system and the effect of dopaminergic treatment on hepcidin levels in larger cohorts may also clarify hepcidin's potential role in the pathophysiology of RLS.

Credit Author Statement

Joseph Dowsett: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing writing the original draft and editing. Maria Didriksen: Conceptualization, Funding acquisition, Data curation and Writing – review & editing reviewing and editing the draft. **Margit Hørup Larsen:** Methodology, Formal analysis, Investigation, Data curation and Writing – review & editing reviewing and editing the draft. **Kristoffer Sølvsten Burgdorf:** Supervision, Project administration and Writing – review & editing reviewing and editing the draft. **Lise Wegner Thørner:** Project administration and Writing – review & editing reviewing and editing the draft. **Erik Sørensen:** Project administration and Writing – review & editing reviewing and editing the draft. **Christian Erikstrup:** Project administration and Writing – review & editing reviewing and editing the draft. **Ole Birger Pedersen:** Project administration and Writing – review & editing reviewing and editing the draft. **Sisse Rye Ostrowski:** Supervision, Project administration and Writing – review & editing reviewing and editing the draft. **Henrik Ullum:** Supervision, Project administration and Writing – review & editing reviewing and editing the draft. All authors approved the final version of the manuscript.

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Conflict of interest

None declared.

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: https://doi.org/10.1016/j.sleep.2021.10.008.

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