

# Plasma hepcidin is associated with future risk of venous thromboembolism

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## Key Points

- Iron deficiency may explain the association between RDW and risk of VTE.
- Contrary to the hypothesis, increasing plasma levels of hepcidin, a biomarker of iron stores, were associated with increased risk of VTE.

Red cell distribution width (RDW) is associated with venous thromboembolism (VTE), but the underlying mechanism(s) is unclear. Iron deficiency is associated with high RDW, and studies suggest an association between iron deficiency and VTE. To assess whether iron deficiency is a risk factor for VTE that explains the association between RDW and VTE, we conducted a nested case-control study of 390 patients with VTE and 802 age- and sex-matched controls selected from the population-based cohort of the Tromsø Study. Physical measurements and blood samples were collected from 1994 to 1995. Logistic regression models were used to calculate odds ratios (ORs) with 95% confidence intervals (CIs) for VTE by RDW, hepcidin, and ferritin light chain (FtL). RDW was inversely associated with hepcidin, FtL, and hemoglobin. The risk of VTE increased linearly across categories of higher plasma hepcidin levels. Participants with hepcidin in the highest quartile had an OR for VTE of 1.32 (95% CI, 1.00-2.42), and those in the >90% percentile had an OR for VTE of 1.66 (95% CI, 1.14-2.42) compared with the reference group (quartiles 2 and 3). The risk estimates remained similar after adjustment for C-reactive protein. The risk of VTE increased by categories of higher RDW and was strengthened after inclusion of hepcidin and FtL in the multivariable model. Our findings reject the hypothesis that iron deficiency explains the association between RDW and VTE and suggest, in contrast, that high body iron levels might increase the risk of VTE.

## Introduction

Venous thromboembolism (VTE) is a collective term for deep vein thrombosis (DVT) and pulmonary embolism (PE). VTE is a complex disease affecting 1 to 2 per 1000 individuals each year,<sup>1,2</sup> with serious short- and long-term complications.<sup>3,4</sup> In contrast to arterial cardiovascular diseases, such as myocardial infarction and ischemic stroke, where incidence has declined by 25% to 50% over the last 2 decades,<sup>5</sup> the incidence of VTE has slightly increased during this time period.<sup>6,7</sup> It is assumed that the incidence of VTE will continue to rise in the coming years because the prevalence of major risk factors for VTE,<sup>8-10</sup> such as older age, obesity, and cancer, is increasing in the population.<sup>8,11,12</sup> VTE has become a major challenge to public health and health care systems because of associated frequent hospitalizations, monitoring of treatment to avoid bleeding complications, severe complications, and a high mortality rate.<sup>13</sup> To diminish the health burden of VTE, it is crucial to identify novel biomarkers and unravel underlying disease mechanisms, which may improve risk prediction and guide decisions on targeted prevention and treatment.

Red cell distribution width (RDW) has in recent years been associated with several diseases,<sup>14</sup> including VTE,<sup>15-17</sup> but the underlying mechanisms remain unknown. RDW is traditionally used in a classification system for anemia,<sup>18</sup> and iron deficiency anemia is strongly associated with high RDW.<sup>19,20</sup> Moreover, iron deficiency anemia has been associated with risk of incident VTE,<sup>21</sup> VTE recurrence,<sup>22</sup> PE,<sup>23</sup> and cerebral venous thrombosis.<sup>24</sup> However, iron deficiency anemia may have several causes, which may act as confounders for the association between iron deficiency and VTE.<sup>25</sup> Therefore, whereas the association between iron deficiency and high RDW as well as the association between high RDW and VTE risk seems well established, the association between iron deficiency and VTE remains to be proven.

Assessment of body iron stores is challenging, especially in healthy individuals.<sup>26</sup> The human body has no known active iron excretion, and intestinal iron absorption and macrophage iron recycling regulate iron homeostasis.<sup>27</sup> Hepcidin has, since its discovery in 2001, been identified as a key regulator of iron metabolism.<sup>28</sup> Ferroportin transports iron from duodenal enterocytes, macrophages, and hepatocytes to plasma and is the only known iron exporter in humans.<sup>29</sup> Hepcidin causes endocytosis and degradation of ferroportin, resulting in a rapid decrease in plasma iron.<sup>30</sup> Additionally, hepcidin might inhibit apical iron uptake in enterocytes.<sup>31</sup> Hepcidin expression is regulated by iron availability, inflammatory cytokines, and erythropoietic demand.<sup>28,32</sup> Iron deficiency results in a swift reduction in hepcidin levels,<sup>33</sup> and hepcidin might be a useful biomarker of iron deficiency in healthy individuals.<sup>34</sup> Ferritin light chain (FtL), part of the major iron metabolism protein ferritin, is especially important for iron uptake and storage,<sup>35</sup> and FtL is less sensitive to inflammation than ferritin.<sup>36</sup>

The aims of the present nested case-control study with participants recruited from a population-based cohort were: to investigate whether iron deficiency, assessed by hepcidin and FtL, was associated with risk of VTE and to investigate whether the apparent association between RDW and VTE could be explained by underlying iron deficiency as assessed by these markers.

## Material and methods

### Study population

The Tromsø Study is a single-center prospective cohort study with repeated health surveys of the inhabitants of the municipality of Tromsø, Norway.<sup>37</sup> The fourth survey was conducted from 1994 to 1995, and all inhabitants age  $\geq 25$  years were invited to participate. A total of 27 158 individuals participated (77% of those invited) and were followed from the date of inclusion until an incident VTE, migration, death, or end of follow-up (1 September 2007). All first lifetime VTE events were identified using the hospital discharge diagnosis registry, the autopsy registry, and the radiology procedure registry from University Hospital of North Norway, which is the sole hospital in the Tromsø region. Trained personnel confirmed and recorded each VTE by extensive medical record review, as previously described.<sup>38</sup> A VTE was confirmed if presence of signs and symptoms of PE or DVT were combined with objective confirmation by a diagnostic procedure (ie, compression ultrasonography, venography, spiral computed tomography, perfusion-ventilation scan, pulmonary angiography, or autopsy), resulting in treatment initiation (unless contraindications were specified). The VTEs were classified as PE with or without concurrent DVT or as DVT only. During the follow-up period (1994-2007), 462 individuals experienced a VTE event. For each VTE case, 2 age-, sex-, and index date-matched controls were selected ( $n = 924$ ). The index date was defined as the

date of the VTE event, meaning that the controls had to be alive and without a VTE diagnosis at the time of the VTE event in the corresponding case. For 45 cases and 75 controls, plasma samples of sufficient quality for the analyses were unavailable. Moreover, 5 individuals were excluded because of missing values for hepcidin, FtL, or RDW. Finally, participants with a cancer diagnosis before ( $n = 57$ ) or within 1 year from the inclusion date ( $n = 12$ ) were excluded, because active or occult cancer might have influenced the hepcidin and/or FtL levels. In total, 390 VTE cases and 802 controls were included in our study. The regional committee of medical and health research ethics approved the study, and all participants provided written consent.

### Measurements

Physical measurements and blood samples were collected from 1994 to 1995. Height and weight were measured with participants wearing light clothes and no shoes. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters ( $\text{kg}/\text{m}^2$ ). Information on smoking habits was obtained from self-administered questionnaires. For measurement of blood-cell variables (including RDW), 5 mL of blood was drawn from an antecubital vein into a vacutainer tube containing EDTA as an anticoagulant and analyzed within 12 hours in an automated blood-cell counter (Coulter Counter; Coulter Electronics, Luton, United Kingdom). RDW was calculated by dividing the standard deviation (SD) of mean corpuscular volume by mean cell volume and multiplying by 100 to express the result as a percentage.<sup>20</sup> Serum and citrated plasma were prepared by centrifugation ( $2000 \times g$  for 15 minutes) after 1-hour respite at room temperature and frozen at  $-70^\circ\text{C}$ . Plasma samples were thawed, and enzyme-linked immunosorbent assays were used to measure levels of hepcidin (catalog #DY8307-05; R&D Systems, Stillwater, MN), FtL (catalog #H00002512-AP49 and recombinant protein H00002512-P01; Abnova, Taipei, Taiwan), and C-reactive protein (CRP; catalog #DY1707; R&D Systems) in a 384 format using a combination of a CyBi SELMA (CyBio, Jena, Germany), EL406 washer/dispenser (Biotek, Winooski, VT), and Synergy H2 microplate reader (Biotek). The intra- and interassay coefficients of variation were  $<10\%$  for all assays.

### Statistical analyses

Statistical analyses were performed with STATA software (version 14.0; Stata Corporation, College Station, TX). Participants were categorized into quartiles based on the distribution of baseline hepcidin, FtL, and RDW among the control population. Extra cutoff points were established at the 10th and 90th percentiles, respectively.

Pearson's correlation coefficients were estimated for RDW, hepcidin, FtL, hemoglobin, and CRP. Unconditional logistic regression models were used to calculate odds ratios (ORs) with 95% confidence intervals (CIs) for VTE by hepcidin, FtL, and RDW modeled as continuous and categorical variables. Quartiles 2 and 3 (ie, 25th-75th percentiles) were combined and used as a reference group in the categorical analysis. The multivariable model included age, sex, BMI, and CRP. For the analysis of VTE risk by RDW, hepcidin and FtL were included in an extra multivariable model.

## Results

### Baseline characteristics and correlations

Among the 390 VTE cases, 42.6% ( $n = 166$ ) were unprovoked events, and 38.7% ( $n = 151$ ) were diagnosed with a PE (Table 1).

**Table 1. Characteristics of VTE cases and controls**

Characteristic	VTE Cases (n = 390)	Controls (n = 802)
Age, y	59.8 ± 13.9	59.7 ± 13.8
Male sex	48.5 (189)	47.4 (380)
BMI, kg/m <sup>2</sup>	27.1 ± 4.6	26.0 ± 4.1
Smoker	30.8 (120)	32.3 (262)
Hemoglobin, g/dL	14.3 ± 1.2	14.1 ± 1.1
Trc, ×10 <sup>9</sup> /L	246.9 ± 56.1	244.1 ± 52.5
RDW, %	13.2 ± 1.1	13.0 ± 0.8
Hepcidin, ng/mL	55.9 ± 36.6	50.2 ± 33.5
FtL, ng/mL	11.2 ± 12.4	9.9 ± 10.0
CRP, mg/L	1.7 ± 1.4	1.6 ± 1.4
Anemia*	2.1 (8)	4.2 (33)
Unprovoked VTE	42.6 (166)	—
PE	38.7 (151)	—
DVT	61.3 (239)	—

Values are means ± 1 SD or percentages with absolute numbers in parentheses.

Trc, thrombocyte.

\*Anemia defined as hemoglobin levels <12.0 g/dL in women and <13.0 g/dL in men.

In general, there were only minor differences between the case and control groups. Patients with VTE had slightly higher BMI, and a slightly higher proportion were men, whereas the proportion of smokers and participants with anemia was higher in the control group. CRP, hemoglobin, and platelet levels were similar in cases and controls (Table 1).

The correlations between the studied markers are shown in Table 2 and supplemental Figure 1. RDW was inversely correlated with plasma levels of hepcidin, FtL, and hemoglobin and positively correlated with plasma CRP. As expected, hepcidin correlated positively with FtL and hemoglobin, and FtL correlated with hemoglobin. In contrast to the correlation with RDW, only weak correlations were found between CRP and hepcidin and between CRP and FtL.

### Association of hepcidin and FtL and future risk of VTE

The risk of VTE increased with higher plasma levels of hepcidin (Table 3). In crude analysis, study participants with hepcidin in the highest quartile had a 32% higher risk of VTE than those with hepcidin in the 2 middle quartiles (OR, 1.32; 95% CI, 1.00-2.42). The risk estimates increased further for participants with hepcidin >90th percentile, with an OR of 1.66 (95% CI, 1.14-2.42). Moreover, a trend toward a lower risk of VTE with lower hepcidin levels was noted. Participants with hepcidin in the lower quartile had an OR for VTE of 0.92 (95% CI, 0.67-1.25) compared with the reference group, whereas those with hepcidin levels <10th percentile had an OR of 0.82 (95% CI, 0.52-1.29). Adjustment for age, sex, BMI, and CRP did not significantly alter the risk estimates. In the linear model, 1 SD increase in hepcidin was associated with a 17% higher risk of VTE (OR, 1.17; 95% CI, 1.04-1.31). The risk estimate was essentially similar in the fully adjusted model (OR, 1.15; 95% CI, 1.02-1.30).

Similar, although less pronounced and not statistically significant, results were found for FtL, with increased risk of VTE by higher plasma levels of FtL and a modest trend toward decreased risk of VTE by lower levels of FtL (Table 3). Compared with the reference

**Table 2. Correlation matrix for RDW, hepcidin, FtL, hemoglobin, and CRP**

	RDW	Hepcidin	FtL	Hemoglobin	CRP
RDW	1.00				
Hepcidin	-0.17*	1.00			
FtL	-0.11*	0.50*	1.00		
Hemoglobin	-0.17*	0.15*	0.25*	1.00	
CRP	0.12*	0.08*	0.09*	0.08*	1.00

\*P < .05.

group, participants with FtL in the highest quartile had a 1.1-fold increased risk of VTE (OR, 1.10; 95% CI, 0.82-1.47), and those with FtL levels >90th percentile had an OR of 1.21 (95% CI, 0.81-1.80). The risk estimates were attenuated in the multivariate model, with ORs of 1.01 and 1.11, respectively. In the linear model, 1 SD increase in FtL yielded an OR for VTE of 1.10 (95% CI, 0.99-1.22), and the risk estimate was slightly attenuated in the multivariable model (OR, 1.06; 95% CI, 0.95-1.19).

### Association of RDW and future risk of VTE with and without adjustment for hepcidin and FtL

In accordance with previous findings, the risk of VTE increased with categories of higher RDW (Table 4). Individuals with RDW in the upper quartile had 46% higher odds of VTE (OR, 1.46; 95% CI, 1.11-1.93) compared with the reference group. Adjusting for age, sex, BMI, and CRP did not alter the risk estimates. Further adjustment for hepcidin and FtL strengthened the risk estimates slightly (fully adjusted OR, 1.58; 95% CI, 1.18-2.11).

## Discussion

In this nested case-control study, we investigated whether iron deficiency was a risk factor for VTE that could explain the association between RDW and VTE. However, we found a dose-dependent association between plasma levels of hepcidin and risk of VTE that was independent of CRP. Similar, although not statistically significant, results were found for FtL. Our results suggest that high body iron stores may increase the risk of VTE. In agreement with previous studies,<sup>16,17</sup> we demonstrated a dose-response relationship between RDW and risk of VTE. Adjusting for hepcidin and FtL increased rather than reduced the risk estimates for VTE by RDW, suggesting that these parameters may, at least partly, reflect different pathways in VTE development. Therefore, it is unlikely that the association between RDW and VTE is explained by underlying iron deficiency.

In contrast to our findings, a history of iron deficiency anemia was associated with VTE in a case-control study including 2522 patients with VTE and 12 610 randomly selected controls from Taiwan.<sup>21</sup> The OR for previous iron deficiency anemia was 1.43 (95% CI, 1.10-1.87) for patients with VTE compared with controls. Possible confounders, such as malignancy and inflammatory bowel disease, were included in the multivariable model. However, information on treatment was not available. Because both oral and IV iron therapies may induce oxidative stress,<sup>39</sup> the observed association could have been driven by the use of iron supplements among patients with VTE with a history of iron deficiency. To explore the effect of iron deficiency on the risk of VTE recurrence, Potaczek et al<sup>22</sup>

**Table 3. ORs with 95% CIs for VTE by categories and per SD of hepcidin and FtL**

Percentile	Range, ng/mL	VTE cases, n	Controls, n	OR (95% CI)*	OR (95% CI)†	OR (95% CI)‡
<b>Hepcidin</b>						
<10th	2.0-10.2	30	80	0.82 (0.52-1.29)	0.84 (0.53-1.34)	0.84 (0.53-1.34)
<25th	2.0-22.0	84	200	0.92 (0.67-1.25)	0.93 (0.67-1.27)	0.93 (0.68-1.28)
25th-75th	22.0-65.2	184	401	Referent	Referent	Referent
>75th	65.2-192.0	122	201	1.32 (1.00-1.76)	1.29 (0.97-1.72)	1.27 (0.95-1.70)
>90th	95.9-192.0	61	80	1.66 (1.14-2.42)	1.62 (1.11-2.37)	1.61 (1.10-2.37)
Per 1 SD increase	—	—	—	1.17 (1.04-1.31)	1.16 (1.03-1.30)	1.15 (1.03-1.30)
<b>FtL</b>						
<10th	0.3-2.0	37	80	0.95 (0.62-1.46)	0.91 (0.59-1.41)	0.92 (0.59-1.42)
<25th	0.3-3.8	88	200	0.91 (0.67-1.23)	0.93 (0.68-1.27)	0.93 (0.68-1.39)
25th-75th	3.8-11.9	195	402	Referent	Referent	Referent
>75th	11.9-108.9	107	200	1.10 (0.82-1.47)	1.04 (0.77-1.41)	1.03 (0.76-1.39)
>90th	18.4-108.9	47	80	1.21 (0.81-1.80)	1.17 (0.77-1.76)	1.13 (0.75-1.71)
Per 1 SD increase	—	—	—	1.10 (0.99-1.22)	1.08 (0.97-1.21)	1.08 (0.96-1.21)

\*Model 1: crude OR.

†Model 2: adjusted for age, sex, and BMI.

‡Model 3: model 2 plus CRP.

conducted a prospective study of 229 patients with incident, unprovoked VTE. The exclusion criteria included, among others, iron administration, known cancer, and chronic inflammatory diseases. The authors did not report the number of patients with a VTE recurrence during follow-up, but the 24-month recurrence-free survival probability was 89.5%. Patients with iron deficiency ( $n = 47$ ), defined as serum ferritin levels  $<30 \mu\text{g/L}$ , had a threefold increased risk of VTE recurrence compared with patients without iron deficiency ( $n = 182$ ) during a mean follow-up of 13 months. The association was independent of CRP levels. However, the study sample was relatively small, the definition of iron deficiency may be questionable, and the findings in patients with VTE are not necessarily applicable to a predominantly healthy population.

Iron is an essential trace element required for several crucial physiologic functions. However, excess free iron is toxic. Humans have no known regulated pathway for iron excretion, and iron balance is maintained by the tight regulation of iron absorption from the intestine. When the amount of iron in the body decreases, the iron uptake must increase and vice versa.<sup>30,40</sup> Growing evidence suggests hepcidin is the primary regulator of iron stores. Hepcidin gene expression is induced by iron loading and suppressed by

anemia and hypoxia,<sup>28</sup> and it is a promising tool in assessing iron status.<sup>34,41</sup> Moreover, hepcidin gene expression is induced by inflammatory processes, and hepcidin is suggested to be the driver of anemia in chronic diseases.<sup>32</sup> To date, ferritin is among the most used measurements of iron status.<sup>26</sup> However, its ability to assess iron stores is reduced in individuals without anemia,<sup>42</sup> and the interpretation of ferritin values is challenging in the presence of concurrent inflammation.<sup>43</sup> Ferritin consists of 2 subunits, FtL and ferritin heavy chain. FtL is especially important for iron uptake and storage<sup>35</sup> and is less sensitive to inflammation than ferritin heavy chain,<sup>36</sup> and the combined use of hepcidin and FtL, as in the present study, may be an accurate tool to define iron deficiency.

In contrast to our hypothesis and previous studies, our results suggest that high body iron levels increase the risk of VTE. The underlying mechanism(s) is unclear, but the relationship between iron and oxidative stress may contribute to the association. Both oral and IV iron therapies may induce oxidative and nitrosative stress.<sup>39</sup> Chronic iron dextran administration (15 mg over 6 weeks) was associated with accelerated thrombus formation after photochemical carotid artery injury in mice,<sup>44</sup> and administration of a reactive oxygen species scavenger revoked the effect. Excess iron-induced oxidative

**Table 4. ORs with 95% CIs for VTE by categories and per SD of RDW**

Percentile	Range, %	VTE cases, n	Controls, n	OR (95% CI)*	OR (95% CI)†	OR (95% CI)‡
<10th	11.2-12.2	29	73	0.90 (0.56-1.42)	0.92 (0.58-1.48)	0.89 (0.56-1.44)
<25th	11.2-12.5	71	178	0.90 (0.65-1.24)	0.94 (0.67-1.31)	0.92 (0.66-1.29)
25th-75th	12.5-13.3	185	417	Referent	Referent	Referent
>75th	13.3-20.1	134	207	1.46 (1.11-1.93)	1.48 (1.11-1.97)	1.58 (1.18-2.11)
>90th	13.9-20.1	68	99	1.55 (1.09-2.21)	1.53 (1.06-2.19)	1.66 (1.15-2.41)
Per 1 SD increase	—	—	—	1.24 (1.11-1.38)	1.23 (1.10-1.37)	1.28 (1.14-1.44)

\*Model 1: crude OR.

†Model 2: adjusted for age, sex, BMI, and CRP.

‡Model 3: model 2 plus hepcidin and FtL.

stress may also decrease nitric oxide bioavailability and cause endothelial dysfunction and platelet activation.<sup>45,46</sup> Fibrinogen is particularly susceptible to oxidation, and exposure of fibrinogen to Fe<sup>3+</sup> promotes fibrin formation, enhances platelet aggregation, and supports less efficient plasminogen activation by tissue-type plasminogen activator.<sup>47,48</sup> Although there was an association between high hepcidin levels and risk of VTE, we cannot necessarily conclude that these individuals had iron excess. Nonetheless, it is tempting to hypothesize that this association reflects a pathogenic link between iron and oxidative stress that could promote VTE.

Major strengths of our study are the clear temporal sequence between exposure and outcome, the large number of participants recruited from a general population, and the well-validated VTE events. Some limitations merit consideration. The biological properties of hepcidin and FtL are well described, and there is a good biological rationale for their role in iron metabolism.<sup>28-33,35</sup> However, their role in assessment of body iron stores among healthy individuals remains to be evaluated in relation to a gold standard (ie, stainable bone marrow iron). The analyses of hepcidin, FtL, and CRP were performed in blood samples drawn from 1994 to 1995 and stored at  $-70^{\circ}\text{C}$  for up to 23 years. This long storage time could potentially have influenced the plasma levels of hepcidin, FtL, and CRP. Samples stored at  $-70^{\circ}\text{C}$  for long periods have displayed a minor lowering in serum levels of hepcidin,<sup>49</sup> but it is unlikely that this would affect the risk of VTE as long as the expected storage effect were similar in all study participants. Moreover, the median time from blood sampling to a VTE event was 7 years, and the individual levels of hepcidin and/or FtL might have changed during this period. A modifiable risk factor may introduce misclassification because of the long time from blood sampling to outcome. This type of nondifferential misclassification generally leads to underestimation of the true association.<sup>50</sup> Moreover, the observed association between hepcidin and VTE might have been confounded by underlying inflammation. Among 51 critically ill patients with anemia, the sensitivity of hepcidin for detection of iron deficiency was only 50%,<sup>51</sup> and hepcidin was a poor predictor of bone marrow iron deficiency in 207 anemic children with high

incidence of acute or chronic infections.<sup>52</sup> Nevertheless, inclusion of CRP in the multivariable model did not attenuate our results significantly, and CRP was only weakly correlated with hepcidin and FtL. Various medications and dietary supplements may influence iron metabolism, as may underlying conditions such as heart failure or inflammatory conditions. Unfortunately, we did not have baseline information on these conditions, and therefore, it is not possible to rule out residual confounding by unrecognized or unmeasured factors in the present study.

In conclusion, we found a dose-response relationship between plasma levels of hepcidin and risk of VTE. These results suggest that iron deficiency is not a risk factor for VTE and cannot explain the association between RDW and VTE. In contrast, our findings suggest that high body iron stores (ie, iron stores in the upper part of normal values) are associated with increased risk of incident VTE in a general, initially cancer-free, population. Future studies should confirm our findings and investigate underlying mechanisms for the effect of high iron stores on future risk of VTE.

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## Authorship

Contribution: T.S.E. analyzed the data and drafted the manuscript; J.L. interpreted the results and revised the manuscript; T.U., P.A., S.K.B., and J.-B.H. designed the study, contributed to data collection, and revised the manuscript; and all authors read and approved the final version of the manuscript.

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