Plasma hepcidin is associated with future risk of venous thromboembolism

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Key points

- Iron deficiency may explain the association between red cell distribution width and risk of venous thromboembolism (VTE).
- Contrary to the hypothesis, increasing plasma levels of hepcidin, a biomarker of iron stores, were associated with increased risk of VTE.

Abstract

Red cell distribution width (RDW) is associated with venous thromboembolism (VTE), but the underlying mechanism(s) is unsettled. 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 on 390 VTE-patients and 802 age- and sex-matched controls selected from a population-based cohort, the Tromsø Study. Physical measurements and blood samples were collected in 1994-95. 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 above the 90% percentile had an OR for VTE of 1.66 (95% CI 1.14-2.42) compared to the reference group (quartile 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 rejected the hypothesis that iron deficiency explains the association between RDW and VTE, and suggested, 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 1-2 per 1000 individuals each year,[1,2] with serious short- and long-term complications.[3,4] In contrast to arterial cardiovascular diseases, such as myocardial infarction and ischemic stroke, where the incidence has declined by 25-50% the last two decades,[5] the incidence of VTE has slightly increased during this time period.[6,7] It is assumed that the incidence of VTE will continue to rise in the coming years since the prevalence of major risk factors for VTE,[8-10] such as high age, obesity and cancer, are increasing in the population.[8,11,12] VTE has become a major challenge to public health and healthcare systems due to frequent hospitalizations, monitoring of treatment to avoid bleeding complications, severe complications and a high mortality rate.[13] In order 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,[14] including VTE,[15-17] but the underlying mechanisms remain unknown. RDW is traditionally used in a classification system for anemia,[18] and iron deficiency anemia is strongly associated with high RDW.[19,20] Moreover, iron deficiency anemia has been associated with risk of incident VTE,[21] VTE recurrence,[22] PE,[23] and cerebral venous thrombosis.[24] However, iron deficiency anemia may have several causes which may act as confounders for the association between iron deficiency and VTE.[25] Therefore, whereas the association between iron deficiency and high RDW as well as the association between high RDW and VTE risk seem well established, the association between iron deficiency and VTE remains to be proven.

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

The aims of the present nested case-control study with subjects recruited from a population-based cohort were: i) to investigate whether iron deficiency, assessed by hepcidin and FtL, was associated with risk of VTE, and ii) 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 in the municipality of Tromsø, Norway.[37] The fourth survey was conducted in 1994-95, and all inhabitants aged 25 years and older were invited. 27 158 subjects participated (77% of those invited), and were followed from the date of inclusion until an incident VTE, migration, death, or end of follow-up (September 1, 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 (UNN), which is the sole hospital in the Tromsø region. Trained personnel confirmed and recorded each VTE by extensive medical records review, as previously described [38]. A VTE was confirmed if presence of signs and symptoms of PE or DVT were combined with objective confirmation by radiological procedures, which resulted in treatment initiation (unless contraindications were specified). During the follow-up period (1994-2007), 462 individuals experienced a VTE event. For each VTE-case, two 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. 45 cases and 75 controls did not have available plasma samples of sufficient quality for the analyses. Moreover, five subjects were excluded due to missing values of hepcidin, FtL or RDW. Finally, participants with a cancer diagnosis prior to (n=57) or within one year from the inclusion date (n=12) were excluded, as active or occult cancer might influence 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 subjects gave their written consent to participate.

Measurements

Physical measurements and blood samples were collected in 1994/95. Height and weight were measured with subjects wearing light clothes and no shoes. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of height in meters (kg/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, UK). RDW was calculated by dividing the standard deviation of MCV by MCV and multiplying by 100 to express the result as a percentage.[20] Serum and citrated plasma were prepared by centrifugation (2000xg for 15 minutes) after one hour respite at room temperature, and frozen at -70ºC. 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 number: H00002512-AP49 and recombinant protein: H00002512-P01, Abnova, Taipei, Taiwan) and C-reactive protein (CRP, catalog number: DY1707, R&D Systems) in a 384 format using a combination of a CyBi SELMA (CyBio, Jena, Germany), EL406 washer/dispenser (Biotek, Winooski, VT, USA) and Synergy H2 microplate reader (Biotek). The intra- and inter-assay coefficients of variation were <10% for all assays.

Statistical analyses

Statistical analyses were performed with STATA version 14.0 (Stata corporation, College Station, TX, USA). Participants were categorized into quartiles based on the distribution of baseline hepcidin, FtL and RDW among the control population. Extra cut-off points were established at the 10^{th} and 90^{th} 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 (OR) with 95% confidence intervals (CI) for VTE by Hepcidin, FtL and RDW modelled as continuous and categorical variables. Quartiles 2 and 3 (i.e. 25-75^{th} percentile) were combined and used as 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). In general, there were only minor differences between the case and control groups. VTE-patients had slightly higher BMI and proportion of males, whereas the proportion of smokers and subjects 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. 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.

The association of hepicin 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 32% higher risk of VTE than those with hepcidin in the two middle quartiles (OR 1.32, 95% CI 1.00-2.42). The risk estimates increased further for subjects with hepcidin above the 90th percentile, with an OR of 1.66 (95% CI 1.14-2.42). Moreover, a trend towards a lower risk of VTE with lower hepcidin levels was noted. Subjects with hepcidin in the lower quartile had an OR of VTE of 0.92 (95% CI 0.67-1.25) compared to the reference group, while those with hepcidin levels below the 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, one standard deviation (SD) increase in hepcidin was associated with 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, albeit 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 towards decreased risk of VTE by lower levels of FtL (Table 3). Compared to the reference group, subjects 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 above the 90th percentile had an OR of 1.21 (95% CI0.81-1.80). The risk estimates were attenuated in the multivariate model, with ORs of 1.01 and 1.11, respectively. In the linear model, one SD increase in FtL yielded an odds ratio 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).

The association of RWD and future risk of VTE with and without adjustment for hepicin 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 to 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. Contrary, we found a dose-dependent association between plasma levels of hepcidin and risk of VTE which was independent of CRP. Similar, albeit 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,[16,17] we demonstrated a dose-response relationship between RDW and risk of VTE. Adjusting for hepcidin and FtL did increase rather than reduce the risk estimates for VTE by RDW suggesting that these parameters may, at least partly, reflect different pathways in VTE development. Thus, 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 VTE patients and 12610 randomly selected controls from Taiwan.[21] The odds ratio for previous iron deficiency anemia was 1.43 (95% CI: 1.10-1.87) for VTE patients compared to controls. Possible confounders, such as malignancy and inflammatory bowel disease, were included in the multivariable model. However, information on treatment was not available. As both oral and intravenous iron therapies may induce oxidative stress,[39] the observed association could be driven by the use of iron supplements among VTE patients with a history of iron deficiency. To explore the effect of iron deficiency on the risk of VTE recurrence, Potaczeck et al conducted a prospective study on 229 patients with incident, unprovoked VTE.[22] The exclusion criteria included, among others, iron administration, known cancer and chronic inflammatory diseases. The authors do 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 µg/L, had a three-fold increased risk of VTE recurrence compared to 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 VTE patients 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.[30,40] Growing evidence suggest hepcidin as the primary regulator of iron stores. Hepcidin gene expression is induced by iron loading and suppressed by anemia and hypoxia,[28] and it is a promising tool in assessing iron status.[34,41] Moreover, hepcidin gene expression is induced by inflammatory processes, and hepcidin is suggested as the driver for anemia of chronic diseases.[32] To date, ferritin is among the most used measurements of iron status.[26] However, its ability to assess iron stores is reduced in subjects without anemia,[42] and the interpretation of ferritin values is challenging in the presence of concurrent inflammation.[43] Ferritin consists of two sub-units, the ferritin light chain (FtL) and ferritin heavy chain (FtH). FtL is especially important for iron uptake and storage,[35] and is less sensitive to inflammation than FtH,[36] and the combined use of hepidin 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) are unsettled, but the relationship between iron and oxidative stress may contribute to the association. Both oral and intravenous iron therapies may induce oxidative and nitrosative stress.[39] Chronic iron dextran administration (15 mg over 6 weeks) was associated with accelerated thrombus formation

after photochemical carotid artery injury in mice,[44] and administration of a reactive oxygen species scavenger revoked the effect. Excess iron-induced oxidative stress may also decrease the nitric oxide bioavailability and cause endothelial dysfunction and platelet activation.[45,46] Fibrinogen is particularly susceptible to oxidation, and exposure of fibrinogen to Fe^{3+} promotes fibrin formation, enhances platelet aggregation, and supports less efficient plasminogen activation by tissue-type plasminogen activator.[47,48] Although there was an association between high hepcidin levels and risk of VTE, we cannot necessarily conclude that these individuals have iron excess. Nonetheless, it is tempting to hypothesize that this association reflect 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 the iron metabolism.[28-33,35] However, their abilities to assess body iron stores among healthy individuals remain to be evaluated in relation to a gold standard (i.e. stainable bone marrow iron). The analysis of hepcidin, FtL and CRP were performed in blood samples drawn in 1994/95 and stored at -70°C for up to 23 years. This long storage time could potentially influence the plasma levels of hepcidin, FtL and CRP. Long-term storage of samples at -70ºC have displayed a minor lowering in serum levels of hepcidin,[49] but it is unlikely that it would affect the risk of VTE as long as the expected storage-effect is 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 due to the long time from blood sampling to outcome. This type of non-differential misclassification generally leads to underestimation of

the true association.[50] Finally, the observed association between hepcidin and VTE might be confounded by underlying inflammation. Among 51 critically ill patients with anemia, the sensitivity of hepcidin for detection of iron deficiency was only 50%,[51] and hepcidin was a poor predictor of bone marrow iron deficiency in 207 anemic children with high incidence of acute or chronic infections.[52] Nevertheless, inclusion of CRP in the multivariable model did not attenuate our results significantly, and CRP was only weakly correlated to hepcidin and FtL. Even though potential confounders were carefully checked, it is not possible to completely rule out residual confounding by unrecognized or unmeasured factors in observational studies.

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 (i.e., iron stores in the upper part of normal values) are associated with increased risk of incident VTE. 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

TSE analyzed the data and drafted the manuscript. JL interpreted the results and revised the manuscript. TU, PA, SKB and JBH designed the study, contributed with data collection, and revised the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest disclosure

The authors report no conflicts of interest.

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Table 1. Characteristics of VTE-cases and controls. Values are means ± one standard deviation or percentages with absolute numbers in parenthesis.

	VTE	**Controls**
N	390	802
Age	59.8 ± 13.9	59.7 ± 13.8
Sex (male, %)	48.5 (189)	47.4 (380)
BMI ($kg/m^{2)}$	27.1 ± 4.6	26.0 ± 4.1
Smoking (%)	30.8 (120)	32.3 (262)
Hemoglobin (g/dL)	14.3 ± 1.2	14.1 ± 1.1
Trc (10^9/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 (	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)	-

* Anemia defined as hemoglobin levels <12.0 g/dL in females and <13.0 g/dL in men
BMI: Body Mass Index; CRP: C-reactive protein; DVT: Deep vein thrombosis; FtL: Ferritin light chain; PE: Pulmonary embolism; RDW: Red cell Distribution Width; Trc: Thrombocytes; VTE: Venous thromboembolism; WBC: White Blood Cell count;

Table 2. Correlation matrix for red cell distribution width (RDW), hepcidin, ferritin light-chain (FtL), hemoglobin (Hb) and C-reactive protein (CRP).

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

*p<0.05

Table 3. Odds ratios (ORs) with 95% confidence intervals (CIs) for VTE by categories and per standard deviation (SD) of Hepcidin and ferritin light chain (FtL).

		VTE cases	OR (95% CI)*	OR (95% CI)**	OR (95% CI)***
Hepcidin (ng/mL)	< 10 percentile	30	0.82 (0.52-1.29)	0.84 (0.53-1.34)	0.84 (0.53-1.34)
	< 25 percentile	84	0.92 (0.67-1.25)	0.93 (0.67-1.27)	0.93 (0.68-1.28)
	25-75 percentile	184	Ref	Ref	Ref
	> 75 percentile	122	1.32 (1.00-1.76)	1.29 (0.97-1.72)	1.27 (0.95-1.70)
	> 90 percentile	61	1.66 (1.14-2.42)	1.62 (1.11-2.37)	1.61 (1.10-2.37)
	Per 1SD increase	-	1.17 (1.04-1.31)	1.16 (1.03-1.30)	1.15 (1.03-1.30)
FtL (ng/mL)	< 10 percentile	37	0.95 (0.62-1.46)	0.91 (0.59-1.41)	0.92 (0.59-1.42)
	< 25 percentile	88	0.91 (0.67-1.23)	0.93 (0.68-1.27)	0.93 (0.68-1.39)
	25-75 percentile	195	Ref	Ref	Ref
	> 75 percentile	107	1.10 (0.82-1.47)	1.04 (0.77-1.41)	1.03 (0.76-1.39)
	> 90 percentile	47	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 body mass index
*** Model 3: Model 2 + C-reactive protein

Table 4. Odds ratios (ORs) with 95% confidence intervals (CIs) for VTE by categories and per standard deviation (SD) of red cell distribution width (RDW).

RDW	VTE cases	OR (95% CI)*	OR (95% CI)**	OR (95% CI)***
< 10 percentile	29	0.90 (0.56-1.42)	0.92 (0.58-1.48)	0.89 (0.56-1.44)
< 25 percentile	71	0.90 (0.65-1.24)	0.94 (0.67-1.31)	0.92 (0.66-1.29)
25-75 percentile	185	Ref	Ref	Ref
> 75 percentile	134	1.46 (1.11-1.93)	1.48 (1.11-1.97)	1.58 (1.18-2.11)
> 90 percentile	68	1.55 (1.09-2.21)	1.53 (1.06-2.19)	1.66 (1.15-2.41)
Per 1SD 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, body mass index and C-reactive protein
*** Model 3: Model 2 + Hepcidin and ferritin L-chain