PRESS RELEASE

ORIGINAL ARTICLE

Low serum iron levels are associated with elevated plasma levels of coagulation factor VIII and pulmonary emboli/deep venous thromboses in replicate cohorts of patients with hereditary haemorrhagic telangiectasia

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ABSTRACT

Background Elevated plasma levels of coagulation factor VIII are a strong risk factor for pulmonary emboli and deep venous thromboses.

Objectives To identify reversible biomarkers associated with high factor VIII and assess potential significance in a specific at-risk population.

Patients/Methods 609 patients with hereditary haemorrhagic telangiectasia were recruited prospectively in two separate series at a single centre. Associations between log-transformed factor VIII measured 6 months from any known thrombosis/illness, and patient-specific variables including markers of inflammation and iron deficiency, were assessed in stepwise multiple regression analyses. Age-specific incidence rates of radiologically proven pulmonary emboli/deep venous thromboses were calculated, and logistic regression analyses performed.

Results In each series, there was an inverse association between factor VIII and serum iron that persisted after adjustment for age, inflammation and/or von Willebrand factor. Iron response elements within untranslated regions of factor VIII transcripts provide potential mechanisms for the association. Low serum iron levels were also associated with venous thromboemboli (VTE): the age-adjusted OR of 0.91 (95% CI 0.86 to 0.97) per 1 μmol/litre increase in serum iron implied a 2.5-fold increase in VTE risk for a serum iron of 6 μmol/litre compared with the mid-normal range (17 μmol/litre). The association appeared to depend on factor VIII, as once adjusted for factor VIII, the association between VTE and iron was no longer evident.

Conclusions In this population, low serum iron levels attributed to inadequate replacement of haemorrhagic iron losses are associated with elevated plasma levels of coagulation factor VIII and venous thromboembolic risk. Potential implications for other clinical populations are discussed.

Key messages

What is the key question?

- Can we find new risk factors for venous thromboemboli (VTE) that might allow the development of a strategy to prevent pulmonary emboli and deep venous thromboses?

What is the bottom line?

- By focusing on the known VTE risk factor, coagulation factor VIII, low serum iron levels are identified as a biomarker for high factor VIII levels, and clinical VTE.

Why read on?

- Low serum iron levels are treatable by increasing iron intake, and thus represent a potentially reversible risk factor for pulmonary emboli and deep venous thromboses.

INTRODUCTION

Pulmonary emboli and deep venous thromboses cause major morbidity and mortality.[1 2] The importance of venous thromboemboli (VTE) is emphasised by the Department of Health's mandatory VTE risk assessment data collection programme for NHS-funded acute care hospitals.

Patients with hereditary haemorrhagic telangiectasia (HHT)[3] represent a specific patient group with unexplained high rates of VTE.[4] Pulmonary emboli and anticoagulation carry particular hazards for these patients who exhibit sustained and chronic blood losses from nasal and gastrointestinal telangiectasia, and usually have arteriovenous malformations (AVMs) in pulmonary, hepatic and/or cerebral vascular beds.[3] Elevated levels of coagulation factor VIII (FVIII) at least 6 months from any acute illness, infection or thrombosis are a strong predictor of long-term VTE risk in HHT.[4] Similarly, elevated FVIII levels are a strong risk factor for VTE in the general population.[5 6]

In contrast to haemophilia A caused by mutations in the FVIII gene leading to severely reduced FVIII levels, to date, no unique genetic basis for elevated FVIII levels has been identified.[7] In the CHARGE (Cohorts for Heart and Aging Research in Genomic Epidemiology) genome-wide association study of 23 608 people of European descent, all five FVIII-associated loci were also associated with higher levels of von Willebrand factor (vWF),[7] the glycoprotein with which FVIII circulates in a non-covalent complex. General population studies have delineated environmental factors that elevate plasma FVIII, and these parallel clinical risk factors for VTE.[1] Thus FVIII levels are higher with increased age,[8] and are acutely elevated in the setting of an acute phase inflammatory response.[9]

Patients with HHT provide a good group to further study the association between high FVIII and VTE because inflammation is not a prominent disease feature, yet patients display high rates of thrombotic events at relatively young ages.[4] To identify novel biomarkers associated with elevated FVIII, stringently phenotyped HHT populations were examined. We hypothesised that this might facilitate a better understanding of why FVIII is elevated in patients with HHT, test the causal chain of biomarker-high FVIII-VTE, and importantly, allow the development of a strategy to reduce FVIII levels, and thereby also prevent pulmonary emboli and deep venous thromboses.

METHODS

The online supplement provides full details of patient assessments, power calculations and statistical methods.

Ethical approvals

A case notes review of patients with hereditary HHT was ethically approved by the Hammersmith, Queen Charlotte's, Chelsea, and Acton Hospital Research Ethics Committee (LREC 00/5792), and the approval remains valid. The study is also registered on the National Clinical Trials Database as NCT00230685 (PI Shovlin).

STUDY PARTICIPANTS

Patients were reviewed between 1 May 1999 and 7 January 2011 at the Hammersmith Hospital HHT/pulmonary AVM service in London, UK, a centre that receives nationwide referrals for these conditions. The sole eligibility criterion for this study was a definite diagnosis of HHT, assigned in the presence of at least three of four recognised international criteria of nosebleeds, mucocutaneous telangiectasia, visceral involvement and family history.[10] Series 1 consisted of the 309 consecutive patients with HHT reviewed between 1999 and 2006.[4] Series 2 ran from 2006 to 2011, consisting solely of all (n=300) patients with definite HHT who had not been part of series 1. Data are reported on all patients.

Patient histories recorded the presence or absence of HHT-related symptoms and complications, other medical pathologies, and all treatments received. Routine assessments included a complete blood count; coagulation screen with fibrinogen; and biochemical screens of electrolytes, liver function, C reactive protein (CRP), and iron status (serum iron and transferrin saturation index (T*f*SI), with ferritin measured routinely from 2006 after iron associations emerged in series 1 analyses (Kulinskaya and Shovlin, 2006, unpublished)). In 1999, the optimal timing for the measurement of iron levels had been considered carefully based on reported diurnal variation in iron levels,[11] and requirements to manage iron deficiency anaemia which is common in the population due to chronic nasal and/or gastrointestinal blood loss from HHT telangiectasia.[3] Due to clinic arrangements, it was not possible to take blood samples in the early morning as recommended,[11] and blood tests were taken in the late afternoon until September 2008, when sampling switched to lunchtime due to a change in clinic structures (for significance, see online supplementary figure 1). FVIII:Ag was included in routine blood tests from 2002, but not if it was within 6 months of a known confounding state such as VTE, infection, embolisation, surgery or pregnancy. vWF was included from 2006, after elevated FVIII levels were identified in series 1.[4] All patients underwent a screen for pulmonary AVMs that included standardised measurements of oxygen saturation in the erect posture, and for patients with pulmonary AVM undergoing subsequent embolisation, mean pulmonary artery pressure, measured routinely at angiography.[12] Pulmonary emboli and deep venous thromboses were included as VTE endpoints only if confirmed by Doppler ultrasound, CT pulmonary angiography, other contrast studies, or ventilation-perfusion scanning resulting in mismatched perfusion defects not explained by the presence of pulmonary AVMs. 'Community-restricted VTE' were defined as any spontaneous deep venous thromboses or pulmonary emboli that were not related to current or recent (within 6 weeks) hospitalisation.

Statistical methods

The distribution of patient-specific variables was assessed using one-way tables and data plots using Stata statistical software, release 11 (Statacorp, 2009, College Station, TX, USA). Identified outliers (prothrombin time>16 s; CRP >40 iu/ml) were excluded.

The distribution of FVIII:Ag was skewed and normalised by logarithmic transformation (data not shown). Log-transformed FVIII (lnFVIII) was used as the dependent variable for multiple regression analyses as previously.[4] Levels were compared with concurrent indices and other parameters of clinical status. For each series, automated and manual stepwise forward and backwards linear regression analyses were performed using Stata 11 (Statacorp, TX, USA).

Age-standardised VTE incidence rates were calculated by allocating VTE cases to the decade of life in which they occurred. Incidence rates in each decade were calculated using the total number of person years per decade provided by the cohorts, using (Stata 11). Incidence rates were compared with previously published rates for the general population,[2] and graphed using an exponential growth programme (GraphPad Prism 5.00, San Diego, California, USA[13]). Relationships between VTE and other patient-specific variables were assessed in stepwise logistic regression analyses using Stata 11. Interim analyses used the FVIII dataset for all iron indices, but these often differed between the time of FVIII measurement and VTE. For the final analyses, separate serum iron and T*f*SI measurements closest to VTE (interval 6 weeks to 60 months, mean 19 months), were used.

RESULTS

Details of populations

As demonstrated in online supplementary table 1, there were broad similarities among the 309 patients in series 1 and the 300 patients in series 2, with average ages of 49 and 46 years; a female bias of 62.7% and 60.3%; and two-thirds having pulmonary AVMs (67% and 72%). Approximately one-quarter of the patients in each series (29.6% and 24.7%) were using iron tablets for iron deficiency anaemia resulting from heavy iron losses as a result of nosebleeds and gastrointestinal bleeding. Similar proportions in each series were iron deficient.

An association between low serum iron and high plasma FVIII that is independent of inflammation

As expected, univariate analyses demonstrated that lnFVIII levels were higher in older patients, and in the setting of raised inflammatory markers (online supplementary table 2 and supplementary figure 2). Supplementary table 2 also demonstrates the expected association between higher levels of FVIII and raised levels of vWF. vWF, which was only routinely available in series 2, accounted for 15.7% of the variance in FVIII levels in that series ($p<0.0001$, data not shown).

Surprisingly, lnFVIII levels were also higher in patients in whom serum iron or T*f*SI values were low (supplementary table 2). Regression plots for FVIII with these commonly used markers of iron deficiency/overload are provided in figure 1A,B. In contrast, there was no relationship between FVIII and serum ferritin, another commonly used serum marker of iron status (online supplementary table 2; figure 1C). There was also no association between serum iron and vWF (N=213; r^2=0.009, p>0.17).

Both FVIII levels[4] and the severity of HHT telangiectasia[3] increase with age, but in both series, the inverse relationships between lnFVIII and serum iron (or T*f*SI) remained after adjustment for age and/or vWF (table 1).

Inflammation results in low iron levels[14] and is recognised to elevate FVIII.[9] Although the multiple regression analyses did not suggest that the low iron—high FVIII association was due to accompanying inflammation, we addressed this further. In keeping with a predominantly haemorrhagic cause of iron deficiency in patients with HHT, CRP and fibrinogen exhibited only weak relationships with serum iron or T*f*SI (figure 1D,E). In contrast, serum iron and T*f*SI were closely correlated as expected (figure 1F). Somewhat surprisingly, there was little correlation between serum iron (or T*f*SI) with serum ferritin (figure 1G). Ferritin is an acute phase protein, but the lack of association with serum iron/T*f*SI was not explained by concurrent confounding inflammatory responses (figure 1H,I). We concluded that the observed association between low serum iron/T*f*SI and high FVIII was independent of inflammation, and that despite a lack of diurnal variability (online supplementary figure 1), ferritin was not a robust means to evaluate the physiological state associated with low serum iron/T*f*SI in these cohorts (online supplementary figure 3).

Alternative mechanisms for the association between low serum iron/T*f*SI and high FVIII were considered. In conditions of low intracellular iron, more avid binding of iron reactive proteins to iron response elements (IREs) in untranslated regions (UTRs) of RNA transcripts inhibit protein translation, or enhance mRNA stability according to 5′ and 3′ UTR position respectively.[14 15] The FVIII protein is encoded by a 26-exon transcript, but in endothelial cells, the same gene locus also generates several shorter transcripts with alternate first exons and UTRs.[16] Examining FVIII transcript sequences[15] identified a 5′UTR IRE within the alternate first exon of alternate transcript variant 2 (NM_019863, exon 22B, nt 11-42), with a predicted free energy of −8.5 kCal, and a 3′ IRE sequence within the final exon of transcripts 1 (NM_000132, full length), 2 and 3. This pattern would be predicted to enhance FVIII full length transcript 1 production in the setting of iron deficiency.

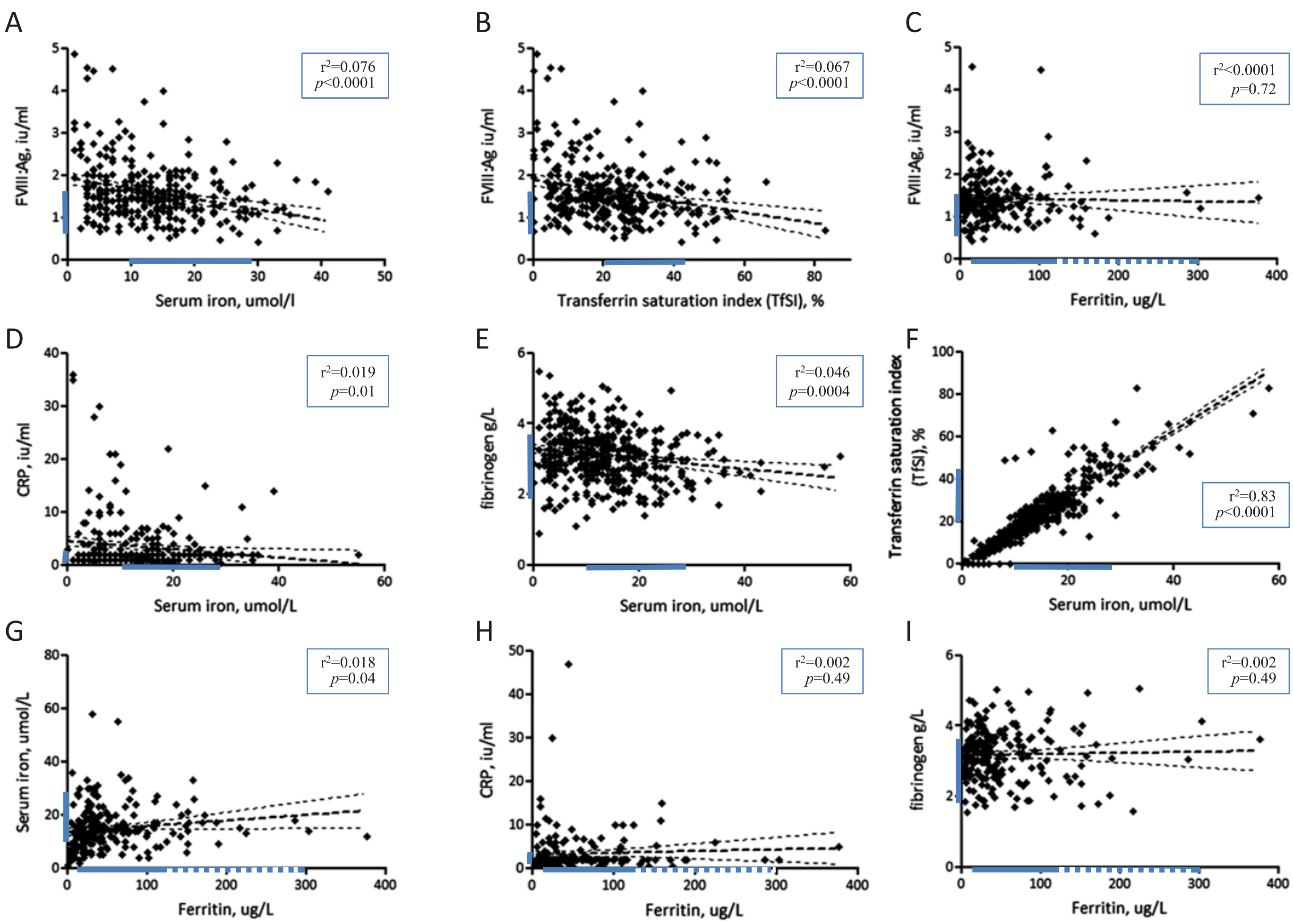

Figure 1 Factor VIII (FVIII) and associated regression plots. Scatter plots for key univariate associations in the combined series. The superimposed lines represent the linear regression line (bold) with 95% CIs. Boxes indicate the r^2 values and p value for goodness of fit for each regression line. Thick bars on x and y axes indicate normal ranges, with dotted bars for extended ferritin normal range in men and post menopausal women. Upper panel: linear regression of FVIII with the iron indices of serum iron (A), transferrin saturation index (T*f*SI) (B) and ferritin (C). Middle panel: linear regression of serum iron with C-reactive protein (CRP) (D), fibrinogen (E), and TfSI (F). Bottom panel: linear regression of ferritin with serum iron (G), CRP (H), and fibrinogen (I).

Table 1 Multiple regression of log-transformed factor VIII

	Regression coefficient	95% CI	p value
Series 1			
Age	0.0076	0.0026 to 0.013	0.003
Hypertension	0.24	0.04 to 0.44	0.017
Serum iron	−0.0086	−0.017 to 0.00033	0.059
Series 2			
Von Willebrand factor	0.37	0.27 to 0.46	<0.001
Serum iron	−0.0092	−0.015 to −0.0032	0.003

Multiple regression analyses for log-transformed factor VIII. For each model, the variables identified as making a significant contribution to the final model, once adjusted for the presence of other variables within the model, are presented. (Full model descriptive parameters are presented in online supplementary table 3.) Note that von Willebrand factor was only measured routinely for series 2, and therefore was not part of the series 1 model. There was no significant difference if transferrin saturation index was used instead of serum iron (data not shown). Higher order variables and interaction terms were not significant in either model. To enhance statistical power, we considered pooling the series, but this was not valid for these analyses, because in a combined stepwise regression model, the series indicator was significant.

VTE rates and HHT

High proportions of patients with HHT are iron deficient (figure 1), and FVIII levels are known to be associated with VTE risk in HHT, as in the general population. Patients with HHT would therefore be predicted to have higher rates of VTE than the general population. Online supplementary table 4 provides details of individual pulmonary emboli and deep venous thrombotic events in the cohorts. The overall VTE incidence rate was 138.3 per 100 000 patient years respectively. Age-standardised incidence rates were higher than for hospitalised patients from the general population (figure 2). The HHT data captured VTE occurring in patients currently or recently hospitalised with long-term inflammatory/immobility states, particularly following a pulmonary AVM-induced brain abscess.[4 17] However, age-standardised incidence rates for community-restricted VTE were also approximately twofold higher than rates for hospitalised patients from the general population (figure 2).

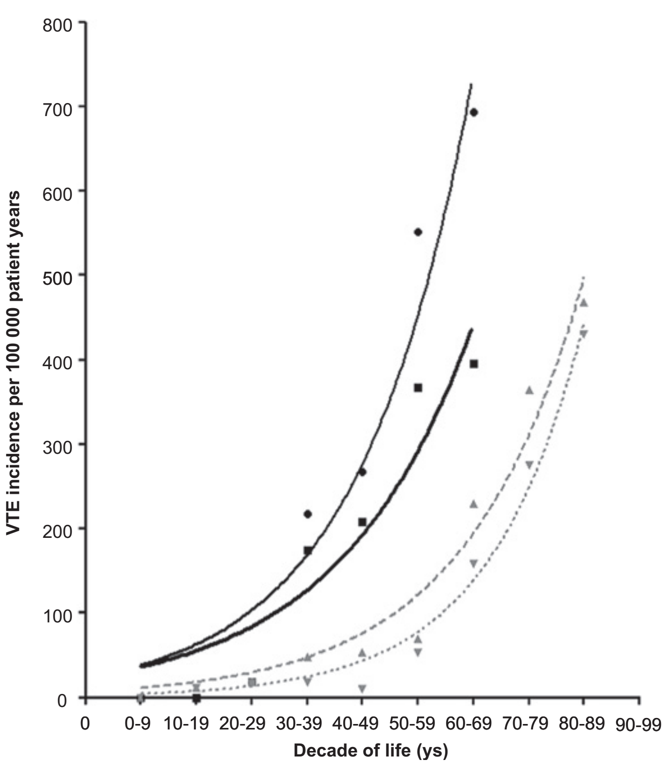

Figure 2 Comparison of age-specific venous thromboemboli (VTE) incidence rates for patients with haemorrhagic telangiectasia (HHT) and hospitalised patients from the general population. Circles, 1 pt line: all HHT VTE; squares, 2 pt line: 'community-restricted' HHT VTE. Grey symbols and lines from general population data: grey triangles, grey dashed line: hospitalised male patients[2]; grey dotted line: hospitalised female patients.[3]

An association between low serum iron and VTE risk that is independent of inflammation

We examined whether low iron levels, or any other marker of HHT haemorrhage, were associated with VTE in the population. We separately analysed all VTE (which included inpatient events), and community-restricted VTE. As demonstrated in table 2, in age, and age/inflammation-adjusted figures, only serum iron or serum T*f*SI were significantly associated with VTE risk. The age-adjusted OR for serum iron of 0.91 (95% CIs 0.86 to 0.97 for all VTE; 0.84 to 0.99 for community-restricted VTE) per 1 μmol/litre increase in serum iron implied that a serum iron of 6 μmol/litre would increase VTE risk approximately 2.5-fold compared with 17 μmol/litre, the midpoint of the normal range. We concluded that the VTE association was with a status identified by low levels of serum iron/TfSI and not by other haemorrhage-related variables.

The association between low serum iron and VTE depends on high FVIII

To determine more complete VTE risk profiles, logistic regression analyses using all available patient variables were performed. For VTE events occurring in any setting ('All VTE'), and for VTE events only occurring in the community, FVIII levels were the strongest univariate predictor of VTE risk. Once adjusted for FVIII, neither iron/T*f*SI nor any other variable was significant at the 5% significance level in either setting (table 3).

DISCUSSION

Elevated plasma levels of FVIII are emerging as one of the strongest risk factors for VTE in the general population.[5 6] In health, genetic determinants of FVIII levels are primarily dependent on levels of its carrier protein vWF.[7] The key findings from the current study are the identification of low serum iron levels as a potentially reversible biomarker for high FVIII levels and clinical VTE. These associations appear to operate independently to levels of vWF, or the inflammatory precipitants that are known to be associated with elevated FVIII and thromboembolic risk. Although the data were obtained in a specific patient group, they are supported by limited data from the general population literature that link iron deficiency or haemorrhage-associated anaemia with venous thromboses.[18–20]

The major strength of our study was the consistent timing of blood samples to late afternoon or lunchtime, capturing the time of daytime 'peaks' of serum iron and T*f*SI (online supplementary figure 1). This is important because the temporal variation of serum iron is complex,[21 22] which makes interpretation of serum iron levels more difficult than is generally assumed. Serum ferritin levels are often considered a better marker of iron deficiency, but these too are difficult to interpret in the presence of coexisting pathologies[23]: in the current study, ferritin values appeared to be disproportionately high in patients with severe hepatic AVM disease and iron deficiency, or those requiring weekly transfusions/iron infusions (online supplementary figure 3). The replicate HHT cohorts were statistically powerful due to the high prevalence of iron deficiency and high VTE rates. Additional strengths were the homogeneous populations, limited number of

Table 2 ORs for associations between venous thromboemboli (VTE) and haemorrhage-associated variables

	N	Age-adjusted OR	N′	Age and fibrinogen-adjusted OR
All VTE				
Serum iron, at VTE	493	**0.91 (0.86 to 0.97)**	449	**0.90 (0.84 to 0.97)**
Serum T*f*SI, at VTE	494	**0.95 (0.92 to 0.99)**	450	**0.95 (0.91 to 0.99)**
Ferritin	243	1.00 (0.98 to 1.01)	226	0.99 (0.98 to 1.01)
Haemoglobin	543	0.94 (0.81 to 1.05)	487	0.94 (0.80 to 1.11)
Platelets	550	1.00 (1.00 to 1.01)	495	1.00 (1.00 to 1.01)
On iron treatment (oral)	593	1.52 (0.73 to 3.20)	496	1.93 (0.89 to 4.20)
Ever transfused	599	0.93 (0.31 to 2.84)	494	1.00 (0.32 to 3.14)
Using hormones	591	0.92 (0.31 to 2.70)	487	1.11 (0.37 to 3.34)
Using tranexamic acid	592	1.02 (1.00 to 1.05)	489	1.30 (0.27 to 6.05)
Community restricted				
Serum iron, at VTE	493	**0.91 (0.84 to 0.99)**	449	**0.90 (0.82 to 0.99)**
Serum T*f*SI, at VTE	494	**0.95 (0.91 to 0.996)**	450	**0.94 (0.89 to 0.99)**
Ferritin	243	1.00 (0.98 to 1.01)	226	1.00 (0.98 to 1.01)
Haemoglobin	543	0.89 (0.72 to 1.09)	487	0.89 (0.72 to 1.10)
Platelets	550	1.00 (1.00 to 1.01)	495	1.00 (0.99 to 1.01)
On iron treatment (oral)	599	0.90 (0.32 to 2.490)	496	1.07 (0.37 to 3.10)
Ever transfused	599	0.94 (0.20 to 4.35)	494	1.00 (0.21 to 4.74)
Using hormones	591	1.80 (0.58 to 5.54)	487	1.55 (0.43 to 5.64)
Using tranexamic acid	593	0.94 (0.12 to 7.40)	489	1.25 (0.15 to 10.23)

ORs for all VTE occurring in series 1 and 2 combined, with ORs significantly different to 1.00 denoted in bold. A series indicator variable was used to confirm the validity of series pooling. N, number of observations for age-adjusted figures; N′, number including fibrinogen, selected as acute phase markers because available in 449 patients compared with 320 for C-reactive protein. Intravenous iron could not be part of any model due to the low frequency of use. T*f*SI, transferrin saturation index.

confounding diseases and immediate relevance to HHT. Furthermore, in many patients in the series, a polycythaemic stimulus due to pulmonary AVM-induced hypoxaemia masked the fall in haemoglobin due to iron deficiency.[24] This poses difficult issues in HHT management, but importantly for this article, allowed iron deficiency to be distinguished from low haemoglobin/anaemia which would be more difficult in the general population. These factors may help explain why the iron deficiency—VTE associations have not been identified clearly in large general population epidemiological studies.

There are clear rationales why prothrombotic disease endpoints may be affected by iron deficiency, as evolutionary fitness would be enhanced by the capacity to augment coagulation (to limit blood loss at sites of vascular injury) when iron stores are depleted and the capacity to restore circulating blood haemoglobin is impaired. When iron deficiency prothrombotic risks have been reported previously, speculative mechanistic comments have focused on high platelet counts or inflammation.[18–20] Our data did not identify such associations. The assumption that iron deficiency is associated with increased platelet counts has also been challenged elsewhere.[25] The current study points to an alternative mechanism by which iron deficiency may promote thromboses via elevation of plasma levels of coagulation factor FVIII. Mechanisms governing the regulation of plasma FVIII levels are not well understood, and so it is relevant that bioinformatic searches[15] of endothelial FVIII transcripts[16] predict the presence of IREs that suggest FVIII full length transcript 1 production may be enhanced in the setting of iron deficiency. We can therefore propose a plausible mechanism linking low serum iron to elevated FVIII.

Table 3 Logistic regression of venous thromboemboli (VTE)

	OR (95% CI)	p value
All VTE		
FVIII	3.09 (1.95 to 4.90)	<0.001
Community VTE		
FVIII	2.88 (1.71 to 4.83)	<0.001

Relationships between the binary dependent outcome variable of VTE with other patient-specific variables assessed by logistic regression analyses for all VTE (pseudo r^2 for final model 0.13, $p<0.0001$) and community-restricted VTE (pseudo r^2 for final model 0.12, $p=0.0002$). Stepwise regression based on the likelihood-ratio method was used to construct the models. Note that in each setting, factor VIII (FVIII) emerged as most significant in the first step, and no other variable was significant, once adjusted for FVIII levels in the 343 patients. In contrast to the FVIII regression analyses, in neither model was the series indicator variable significant, confirming the validity of pooling the series to enhance statistical power.

Associations cannot however indicate direction or causality, particularly when these include potentially codependent variables. Thus we cannot rule out the possibility that high plasma FVIII levels (with accompanying VTE risk) somehow lower circulating levels of iron, although it is difficult to postulate a potential mechanism. Similarly, we cannot exclude chronic haemorrhage, or some component of HHT vascular pathology, causing both low serum iron and high FVIII. This has theoretical attractions because the transforming growth factor β signalling pathways perturbed in HHT[3] are related to pathways involved in hepcidin and iron regulation[14] However, no associations of FVIII with other parameters of HHT haemorrhage were identified, nor was there evidence of iron deficiency in patients without major blood losses due to nosebleeds or gastrointestinal bleeding. Thus we predict that any contribution to the serum iron—FVIII relationship due to HHT-specific vascular pathology will be at best modest.

The crucial clinical question is whether low serum iron levels are provoking a prothrombotic state that could be reversed.

For patients with HHT, it is important to recognise that, as in the general population, provision of iron supplements can correct low serum iron levels in the face of ongoing blood loss. As illustrated by recent HHT guidelines,[26] current practice is generally not to seek and treat iron deficiency, but instead to focus on identification and treatment of iron deficiency anaemia. The data within this manuscript would support the additional use of earlier corrective interventions before anaemia develops.

Case studies and small comparative series link iron deficiency or haemorrhage-associated anaemia with venous thromboses in the general population, though FVIII levels were not reported.[18–20] Management of iron deficiency anaemia is integral to virtually all medical and surgical disciplines including obstetric medicine where pulmonary emboli remain a relatively common cause of maternal mortality.[27] Iron deficiency anaemia is estimated to affect at least 1 billion people worldwide,[28] and is treatable by increasing iron intake to exceed total body losses.[29] Thus the question of whether iron deficiency provides a potentially reversible prothrombotic stimulus is of wide general relevance.

There are also specific pulmonary vasculature implications, not least because pulmonary artery and microvascular endothelial cells synthesise and secrete FVIII.[16 30 31] Elevated plasma levels of FVIII are unusual among general thrombotic risk factors, as they are not only a risk factor for VTE, but are also associated with chronic thromboembolic and pulmonary arterial hypertension, for which pathology includes intrapulmonary microvascular thromboses.[32 33] A prothrombotic potential for the recently observed unexplained iron deficiency in patients with pulmonary arterial hypertension[34–36] is therefore intriguing.

In summary, data from replicate cohorts of patients with hereditary haemorrhagic telangiectasia strongly link low serum iron levels to VTE, with excess risk attributable to elevation of plasma levels of the prothrombotic coagulation factor FVIII. Further mechanistic and clinical examination in general population studies is warranted.

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Competing interests None.

Ethics approval Hammersmith, Queen Charlotte's, Chelsea, and Acton Hospital Research Ethics Committee.

Contributors JAL generated the series 2 database to mid 2010; performed interim statistical analyses using Stata, performed the SIRES searches; and wrote the first manuscript draft. RAM and MAL made all factor VIII (FVIII) measurements, and advised on thrombotic and FVIII concepts. JM advised on iron measurements, and measured iron indices in the patients and in the diurnal study. JEJ reviewed patients with pulmonary arteriovenous malformations, and performed all angiography with associated measurements including pulmonary artery pressure. EK performed initial statistical analysis of series 1; advised on final statistical methodology; and contributed to final statistical interpretation. CLS designed the study including diurnal assessments; reviewed the patients; generated the series 1 database; validated and extended the series 2 database; performed all statistical analyses presented; generated the figures; and wrote the final manuscript. All authors contributed to manuscript review, and approved the final version.

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REFERENCES

1. **Kyrle PA,** Rosendaal FR, Eichinger S. Risk assessment for recurrent venous thrombosis. *Lancet* 2010;**376**:2032–9.
2. **Anderson FA Jr,** Wheeler HB, Goldberg RJ, *et al*. A population-based perspective of the hospital incidence and case-fatality rates of deep vein thrombosis and pulmonary embolism. The Worcester DVT Study. *Arch Intern Med* 1991;**151**:933–8.
3. **Shovlin CL.** Hereditary haemorrhagic telangiectasia: pathogenesis, diagnosis and treatment. *Blood Rev* 2010;**24**:203–19.
4. **Shovlin CL,** Sulaiman NL, Govani FS, *et al*. Elevated factor VIII in hereditary haemorrhagic telangiectasia (HHT): association with venous thromboembolism. *Thromb Haemost* 2007;**98**:1031–9.
5. **Kraaijenhagen RA,** in't Anker PS, Koopman MM, *et al*. High plasma concentration of factor VIIIc is a major risk factor for venous thromboembolism. *Thromb Haemost* 2000;**83**:5–9.
6. **Kyrle PA,** Minar E, Hirschl M. High plasma levels of FVIII and the risk of recurrent venous thromboembolism. *N Engl J Med* 2000;**343**:457–62.
7. **Smith NL,** Chen MH, Dehghan A, *et al*. Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor: the CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. *Circulation* 2010;**121**:1382–92.
8. **Luxembourg B,** Schmitt J, Humpich M, *et al*. Intrinsic clotting factors in dependency of age, sex, body mass index, and oral contraceptives: definition and risk of elevated clotting factor levels. *Blood Coagul Fibrinolysis* 2009;**20**:524–34.
9. **Begbie M,** Notley C, Tinlin S, *et al*. The factor VIII acute phase response requires the participation of NFkappaB and C/EBP. *Thromb Haemost* 2000;**84**:216–22.
10. **Shovlin CL,** Guttmacher AE, Buscarini E, *et al*. Diagnostic criteria for hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome). *Am J Med Genet* 2000;**91**:66–7.
11. **Burtis CA,** Ashwood ER, Bruns DE, eds. *Tietz Textbook of Clinical Chemistry and Molecular Diagnostics*. 4th edn. St Louis: Elsevier, 2006.
12. **Shovlin CL,** Jackson JE, Bamford KB, *et al*. Primary determinants of ischaemic stroke/ brain abscess risks are independent of severity of pulmonary arteriovenous malformations in hereditary haemorrhagic telangiectasia. *Thorax* 2008;**63**:259–66.
13. **Motulsky HJ,** Christopoulos A. Fitting models to biological data using linear and non linear regression. *A practical guide to curve fitting*. San Diego CA: GraphPad Software Inc., 2003. www.graphpad.com
14. **Ganz T.** Hepcidin and iron regulation, 10 years later. *Blood* 2011;**117**:4425–33.
15. **Campillos M,** Cases I, Hentze MW, *et al*. SIREs: searching for iron-responsive elements. *Nucleic Acids Res* 2010;**38**(Suppl. W):360–7.
16. **Shovlin CL,** Angus G, Manning RA, *et al*. Endothelial cell processing and alternatively spliced transcripts of factor VIII. Potential implications for coagulation cascades and pulmonary hypertension. *PLOS One* 2010;**5**:e9154.
17. **Shovlin CL,** Wilmshurst P, Jackson JE. Pulmonary arteriovenous malformations and other pulmonary aspects of HHT. *Eur Respir Mon* 2011;**54**:218–45.
18. **Stolz E,** Valdueza JM, Grebe M, *et al*. Anemia as a risk factor for cerebral venous thrombosis? An old hypothesis revisited. Results of a prospective study. *J Neurol* 2007;**254**:729–34.
19. **Sundström A,** Seaman H, Kieler H, *et al*. The risk of venous thromboembolism associated with the use of tranexamic acid and other drugs used to treat menorrhagia: a case–control study using the General Practice Research Database. *BJOG* 2009;**116**:91–7.
20. **Adoh A,** Kouame AN, Kouassi YF, *et al*. Etiological factors of deep vein thromboses in the limbs of Black African subjects. *Med Trop (Mars)* 1992;**52**:131–7.
21. **Malhotra S,** Garg SK, Khullar GK, *et al*. Kinetics of two different iron formulations and their effect on diurnal variation of serum iron levels. *Methods Find Exp Clin Pharmacol* 2004;**26**:417–20.
22. **Hutchinson C,** Conway RE, Bomford A, *et al*. Post-prandial iron absorption in humans: comparison between HFE genotypes and iron deficiency anaemia. *Clin Nutr* 2008;**27**:258–63.
23. http://cks.nhs.uk/anaemia_iron_deficiency/management/scenario_diagnosis/investigations/interpreting_ferritin_levels (accessed 4 Nov 2011).
24. **Roked F,** Jackson JE, Fuld J, *et al*. Pulmonary thromboemboli modifying the natural history of pulmonary arteriovenous malformations. *Am J Respir Crit Care Med* 2011;**183**:828–9.
25. **Kuku I,** Kaya E, Yologlu S, *et al*. Platelet counts in adults with iron deficiency anemia. *Platelets* 2009;**20**:401–5.
26. **Faughnan ME,** Palda VA, Garcia-Tsao G, *et al*. International guidelines for the diagnosis and management of hereditary hemorrhagic telangiectasia. *J Med Genet* 2011;**48**:73–87.
27. **Confidential enquiry into maternal deaths (CEMACH).** *Saving Mothers Lives 2003–2005 (Full Report)*. http://cemach.interface-test.com (accessed 29 Nov 2011).
28. **McLean E,** Cogswell M, Egli I, *et al*. Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993–2005. *Public Health Nutr* 2009;**12**:444–54.
29. **Centers for Disease Control and Prevention.** Recommendations to prevent and control iron deficiency in the United States. *MMWR Recomm Rep* 1998;**47**:1–36.
30. **Jacquemin M,** Neyrinck A, Hermanns MI, *et al*. FVIII production by human lung microvascular endothelial cells. *Blood* 2006;**108**:515–17.
31. **Shahani T,** Lavend'homme R, Luttun A, *et al*. Activation of human endothelial cells from specific vascular beds induces the release of a FVIII storage pool. *Blood* 2010;**115**:4902–9.
32. **Bonderman D,** Turecek PL, Jakowitsch J, *et al*. High prevalence of elevated clotting factor VIII in chronic thromboembolic pulmonary hypertension. *Thromb Haemost* 2003;**90**:372–6.
33. **Wolf M,** Boyer-Neumann C, Parent F, *et al*. Thrombotic risk factors in pulmonary hypertension. *Eur Respir J* 2000;**15**:395–99.
34. **Ruiter G,** Lankhorst S, Boonstra A, *et al*. Iron deficiency is common in idiopathic pulmonary arterial hypertension. *Eur Respir J* 2011;**37**:1386–91.
35. **Soon E,** Treacy CM, Toshner MR, *et al*. Unexplained iron deficiency in idiopathic and heritable pulmonary arterial hypertension. *Thorax* 2011;**66**:326–32.
36. **Rhodes CJ,** Howard LS, Busbridge M, *et al*. Iron deficiency and raised hepcidin in idiopathic pulmonary arterial hypertension: clinical prevalence, outcomes, and mechanistic insights. *J Am Coll Cardiol* 2011;**58**:300–9.

Low serum iron levels are associated with elevated plasma levels of coagulation factor VIII and pulmonary emboli/deep venous thromboses in replicate cohorts of hereditary haemorrhagic telangiectasia patients.

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ON LINE DATA SUPPLEMENT

SUPPLEMENTARY METHODS:

Patient evaluations:

Patient histories recorded the presence or absence of HHT-related symptoms and complications; other medical pathologies, and all treatments received. Of specific relevance to this study, to assist clinical management of potential or existing iron deficiency, from May 1999, standardised histories recorded blood losses (such as HHT nosebleeds, gastrointestinal and menstrual/post partum bleeds), and iron intake (dietary iron intake, use of pharmaceutical iron tablets or supplements, intravenous iron or blood transfusions). Also recorded were strategies to limit HHT-related bleeding, such as dedicated ENT or endoscopic treatments, use of female hormones, or other agents used in the treatment of HHT-bleeding. [1] Of these, tranexamic acid and aminocaproic acid were being used by a proportion of patients at the time of at least one review; a very small number of non-VTE patients had previously used thalidomide for a few months, and no-one in the series ever received bevazicumab. Routine assessments included a complete blood count; coagulation screen with fibrinogen; and biochemical screens of electrolytes, liver function, C-reactive protein (CRP), and iron status (serum iron and transferrin saturation index (T*f*SI)). All patients underwent a screen for pulmonary AVMs that included standardized validated measurements of oxygen saturation (SaO_2) in the erect posture [2], and for pulmonary AVM patients undergoing subsequent embolization, mean pulmonary artery pressure (mPAP), was measured routinely at angiography [3].

In 1999, the optimal timing for the measurement of iron levels was considered carefully. Morning measurements were recommended based on a reported evening dip, [4] but this was not feasible due to clinic arrangements; blood tests were taken in the late afternoon. When iron associations emerged in Series 1 multiple regression analyses (Shovlin and Kulinskaya, 2006 unpublished), measurements of iron status were considered further. Contrary to textbook suggestions, [4] iron and T*f*SI (but not ferritin) demonstrated spontaneous daytime rises in non fasted individuals. In our studies, for iron and T*f*SI, normal daily variation could span 75-95% of the normal range (Supplementary Figure 1), whereas diurnal variability of ferritin accounted for 10-20% (Supplementary Figure 1). Ferritin had not been measured as an iron status marker in Series 1 because of its status as an acute phase protein, and potential perturbation in HHT patients with hepatic AVMs (which affect 30-70% of HHT patients, but are not screened for routinely [1]). However, in view of the limited diurnal variability, ferritin was included for routine assessment of iron status in Series 2. In September 2008, due to a change in clinic structures, blood sampling switched to lunchtime.

Factor VIII:Ag (FVIII) was included in routine blood tests from 2002 (but not if the individual was within six months of a known confounding state such as VTE, infection, embolization, surgery or pregnancy). Von Willebrand Factor, which is recognised to influence FVIII levels that were elevated in Series 1 [5], was measured routinely from 2006. PE and DVT were included as VTE endpoints only if confirmed by doppler ultrasound, CT-pulmonary angiography, other contrast studies, or ventilation-perfusion scanning resulting in mismatched perfusion defects not explained by the presence of pulmonary AVMs.

Statistical methods:

Missing data were recorded as (.). An indicator variable was assigned according to the series of origin. The distribution of patient-specific variables was assessed using one way tables and data plots (Stata Statistical Software Release 11, StataCorp 2009, College Station, TX, USA). Identified outliers (prothrombin time

>16 seconds; C-reactive protein >40 iu/ml) were excluded as follows: Where prothrombin time (PT) values exceeded 16 seconds due to warfarin therapy, APTT and TT values were also excluded. Where C-reactive protein (CRP) values exceeded 40, all thrombotic, coagulation and inflammatory data in the row were excluded. The distribution of FVIII:Ag distributions was skewed, and normalized by logarithmic correction. In contrast, in this population in which data of patients with high CRP had been excluded, the distribution of fibrinogen approximated to normality (data not shown).

FVIII levels were compared to concurrent blood indices and other parameters of clinical status. For each Series, automated stepwise forward linear regression, and backwards linear regression were performed. Results were confirmed by separately regressing each individual potential predictive variable with lnFVIII, and the single variable explaining the largest proportion of lnFVIII variability used as the base for the next step. This was continued until no further statistically significant variables could be added.

Relationships between the binary dependent outcome variable of VTE with other patient-specific variables were assessed in logistic regression analyses. Interim analyses used one the FVIII dataset for all iron indices, but these often differed between the time of FVIII measurement and VTE. For the final analyses, separate serum iron and T*f*SI measurements closest to VTE (interval 6 weeks to 60 months, mean 19 months), were used. The use of iron tablets at the time of FVIII measurement or VTE was also separated in final analyses. Use of transfusions, intravenous iron, female hormones, tranexamic acid or aminocaproic acid did not differ between the time of FVIII measurement and VTE, and effectively these variables were recorded as positive if used at any time by the patient. Two separate sets of logistic regression models were constructed, examining ”all VTE” and ”community-restricted VTE” (any spontaneous DVT or PE that was not related to current or recent hospitalization). In each case, models were built from the most significant variable(s) on post-estimation likelihood ratio testing from the preceding set of models. For both VTE outcomes, FVIII emerged as most significant in the first step, so all variables were therefore tested with FVIII in the second step, with steps to be repeated until the strongest final linear model was identified. Models were constructed separately without FVIII to capture a higher proportion of cases, but the strength of such models was substantially lower than those utilising FVIII.

In order to identify non-linear relationships, associated variables were also tested as squared variables to detect higher order associations, and examined for interactions.

Power calculations:

To assess when to halt Series recruitments, power calculations were performed comparing two groups, those that had experienced a particular complication, and those who had not. It was recognised that for any complication, the two groups would not be equal. In 1999, there were no data regarding VTE prevalence in HHT, but power calculations could be performed for the complication of paradoxical embolic stroke, for which there were literature data providing a rate of approximately 10% in pulmonary AVM patients [6], the majority of whom had underlying HHT. An Altman nomogram [7] was then used, recognising that compared to equal sized groups, the numbers needed for equivalent power would increase by approximately 1.56 for a complication rate of 20%, 2.8 fold for a complication rate of 10%, and 5.26 fold for a complication rate of 5%. These considerations suggested that a total series of 200 patients would provide acceptable power for complication rates of 5-10% or greater. During the post-recruitment one year follow-up required for the pulmonary AVM series [8], HHT patients continued to be accrued into Series 1, thus HHT Series 1 ran from 1999-2006. Series 2 was originally powered in the same manner, and interim analyses performed in the summer of 2010. However, recognising the importance of the interdependency of important candidate VTE predictors, the cohort was then extended to include all patients with definite HHT reviewed by January 2011, with a final cohort study size of 300.

Supplementary Table 1: Descriptive Statistics of the Individual and Combined Series

	Number			*Median (Q1,Q3)*		
Continuous variables	*Series 1*	*Series 2*	*Total*	*Series 1*	*Series 2*	*Total*
Age (yr)	309	300	609	49 (36, 60)	46 (34, 60)	47 (35,60)
Haemoglobin (g/dl)	271	274	545	14.4 (12.6, 15.5)	13.75 (12.2, 14.9)	14 (12.4, 15.3)
Platelets ($x10^9$/dl)	276	273	549	266 (229, 325)	267 (230, 310)	266 (229, 317)
C-reactive protein (iu/ml)	94	247	339	1 (1, 3)	2 (2,3)	2 (2,2.9)
Fibrinogen (g/L)	250	255	502	3.0 (2.55, 3.46)	3.13 (2.62, 3.62)	3.10 (2.58, 3.53)
Serum iron, at time of FVIII (µmol/L)	237	256	493	11 (6, 16)	14 (8, 18)	12 (7, 18)
Transferrin saturation index, at FVIII (%)	238	256	494	16 (8, 26)	22 (13, 30)	20 (10, 28)
Serum iron, at time of VTE (µmol/L)	236	257	493	10.5 (5.5, 16)	14.5 (8, 18)	12 (7, 17)
Transferrin saturation index, at VTE (%)	237	257	494	16 (8, 26)	22 (13, 30)	19 (10, 28)
Ferritin (µg/L)	15	228	243	33 (21, 72)	34 (16.5, 69.5)	34 (18, 70)
Factor VIII:Ag (iu/ml)	125	220	343	1.77 (1.52, 2.22)	1.37 (1.09, 1.63)	1.48 (1.17, 1.86)
von Willebrand Factor (iu/ml)	78	199	278	1.04 (0.88, 1.37)	1.04 (0.82, 1.41)	1.04 (0.83, 1.39)
Oxygen saturation, SaO_2 (%)	296	273	569	95 (92, 97)	96 (94, 97)	95.5 (93, 97)
Pulmonary artery pressure (mean), mmHg	131	97	228	13 (11, 17)	14 (12, 17)	14 (12, 17)
Prothrombin time (s)	253	252	506	10.6 (10.4, 11.1)	10.7 (10.3, 11.1)	10.7 (10.4, 11.1)
Activated partial thromboplastin time (s)	248	249	497	25.8 (24, 27)	26.3 (24.9, 28.1)	26 (24.5, 34.7)
Thrombin time (s)	241	244	494	15 (12, 16)	14 (13,15)	14 (13, 16)

	Number			*%*		
Binary variables	*Series 1*	*Series 2*	*Total*	*Series 1*	*Series 2*	*Total*
Gender (% female)	309	300	609	62.7	60.3	61.6
Smoking (%)	300	290	590	45.3	30.7	38.1
Pulmonary AVMs (%)	309	300	609	67	72	69.4
Brain abscess (%)	309	292	601	9.06	4.1	6.66
Ischemic stroke (%)	309	293	602	10.68	8.87	9.8
Transfused (%)	308	291	599	12.3	5.5	9
Hormone use (%)	309	282	591	18.5	5.6	12.4
Iron use, at time of VTE (%)	308	291	599	29.2	27.8	28.5
Iron use, at time of FVIII (%)	308	291	599	29.6	24.7	27.2
Intravenous iron (%)	309	282	591	3.56	2.8	3.2
Tranexamic acid/aminocaproic acid (%)	309	284	593	7.1	2.4	4.89
Ever iron deficient, single variable (%)	308	297	605	47.1	52.1	49.6
Ever iron deficient, two or more variables (%)	309	297	606	30.7	39.7	35.1
Hypertension (%)	304	261	565	15.8	8.81	0.125
Migraines (%)	280	288	568	34.3	21.5	27.8

Supplementary Table 2: Univariate regressions with lnFVIII in Series 1 and Series 2

A) Series 1	*Regression coefficient (95% confidence interval)*	***p***	*adjusted r^2*	*N*
Age (per yr)	0.0096 (0.0053, 0.138)	**<0.001**	0.14	124
Gender (for female)	-0.0022 (-0.14, 0.14)	0.98	-0.008	124
Pulmonary AVMs (if present)	0.21 (-0.28, 0.45)	0.082	0.017	124
Ever transfused (if yes)	0.14 (-0.42, 0.32)	0.13	0.011	124
Current iron use (if yes)	0.14 (-0.047, 0.27)	0.058	0.021	124
Serum iron at FVIII (per µmol/)	- 0.0088 (-0.18, 0.0003)	0.059	0.023	112
Current transferrin saturation index (per %)	- 0.0034 (-0.0086, 0.0017)	0.19	0.0069	113
Current ferritin (per µg/L)	0.00092 (-0.012, 0.014)	0.79	-0.43	4
Ever on tranexamic acid (if yes)	0.148 (-0.14, 0.43)	0.31	0.0004	124
Ever on hormones (if yes)	-0.033 (-0.20, 0.14)	0.7	-0.007	124
Current haemoglobin (per g/dl)	-0.020 (-0.046, 0.005)	0.12	0.02	119
Current C-reactive protein (per iu/ml)	0.0038 (-0.0094, 0.17)	0.569	-0.01	64
Current platelets (per 10^9/dl)	0.00026 (-0.00062, 0.0011)	0.564	-0.0056	120
Prothrombin time (per s)	- 0.056 (-0.14, 0.29)	0.2	0.0055	123
Current von Willebrand Factor (per iu/ml)	-0.063 (-0.40, 0.27)	0.7	-0.037	25
Current fibrinogen (per g/L)	0.09 (0.0081, 0.17)	**0.032**	0.029	124
Oxygen saturation , SaO_2 (per %)	0.0023 (-0.0073, 0.12)	0.64	-0.0066	120
Brain abscess (if yes)	0.18 (-0.0066, 0.37)	0.059	0.021	124
Stroke (if yes)	0.14 (-0.43, 0.330	0.13	0.011	124
Migraines (if yes)	-0.026 (-0.16, 0.11)	0.71	-0.007	120
Smoking (if yes)	0.025 (-1.09, 0.16)	0.71	-0.007	122
Hypertension (if yes)	0.34 (0.16, 0.52)	**<0.001**	0.094	122
Pulmonary artery pressure, mean (per mmHg)	0.016 (-0.0036, 0.35)	0.108	0.021	78
Activated partial thromboplastin time (per s)	-0.039 (-0.67, -0.12)	**0.005**	0.055	121
Thrombin time (per s)	0.028 (-0.0080, 0.063)	0.13	0.012	116

B) Series 2	*Regression coefficient (95% confidence interval)*	*p*	*Adjusted r^2*	*N*
Age (per yr)	0.0060 (0.0028, 0.009)	**<0.001**	0.054	220
Gender (for female)	- 0.080 (-0.18, 0.24)	0.13	0.006	220
Pulmonary AVMs (if present)	0.012 (-0.099, 0.12)	0.83	-0.044	219
Ever transfused (if yes)	0.048 (-0.18, 0.28)	0.68	-0.0039	214
Current iron use (if yes)	0.11 (-0.0066, 0.22)	0.065	0.011	216
Serum iron at FVIII (per µmol/L)	-0.011 (-0.018, -0.0048)	**0.001**	0.049	210
Current transferrin saturation index (per %)	-0.0074 (-0.011, -0.0038)	**<0.001**	0.07	210
Current ferritin (per µg/L)	-0.00013 (-0.0011, 0.0008)	0.78	-0.0048	196
Ever on tranexamic acid (if yes)	0.059 (-0.28, 0.40)	0.73	-0.0043	208
Ever on hormones (if yes)	0.014 (-0.24, 0.270	0.913	-0.0048	207
Current haemoglobin (per g/dl)	-0.032 (-0.057, -0.0060)	**0.016**	0.022	219
Current C-reactive protein (per iu/ml)	0.020 (0.010, 0.030)	**<0.001**	0.062	207
Current platelets (per 10^9/dl)	0.000076 (-0.00068, 0.00083)	0.84	-0.0044	218
Prothrombin time (per s)	-0.081 (-0.17, 0.0074)	0.072	0.011	211
Current von Willebrand Factor (per iu/ml)	0.39 (0.29, 0.48)	**<0.001**	0.24	197
Current fibrinogen (per g/L)	0.18 (0.12, 0.24)	**<0.001**	0.12	211
Oxygen saturation , SaO_2 (per %)	-0.011 (-0.26, 0.0048)	0.174	0.0042	207
Brain abscess (if yes)	0.082 (-0.16, 0.32)	0.51	-0.0026	218
Stroke (if yes)	0.11 (-0.054, 0.28)	0.18	0.0036	218
Migraines (if yes)	- 0.00073 (-0.12, 0.12)	0.99	-0.0047	214
Smoking (if yes)	0.045 (-0.060, 0.15)	0.4	-0.0013	216
Hypertension (if yes)	0.11 (-0.065, 0.29)	0.21	0.003	192
Pulmonary artery pressure, mean (per mmHg)	0.36 (0.16, 0.57)	**0.001**	0.13	75
Activated partial thromboplastin time (per s)	-0.57 (-0.075, -0.0390)	**<0.001**	0.15	209
Thrombin time (per s)	0.053 (0.017, 0.088)	**0.004**	0.036	203

C) Combined Series	*Regression coefficient$ (95% confidence intervals)*	***P value***	*Adjusted r^2*	*N*
Age (per yr)	0.008 (0.0053, 0.011)	**<0.001**	0.085	343
Gender (for female)	-0.024 (-0.115, 0.067)	0.6	-0.002	343
Pulmonary AVMs (if present)	0.14 (0.38, 0.25)	**0.008**	0.018	342
Ever transfused (if yes)	0.20 (0.051, 0.351)	**0.009**	0.018	337
Current iron use (if yes)	0.156 (0.61, 0.25)	**0.001**	0.028	339
Serum iron at FVIII (per µmol/L)	-0.14 (-0.20, -0.009)	**<0.001**	0.07	321
Current transferrin saturation index (per %)	-0.0075 (-0.011, -0.044)	**<0.001**	0.063	322
Current ferritin (per µg/L)	-0.00026 (-0.0013, 0.00072)	0.6	-0.0037	198
Ever on tranexamic acid (if yes)	0.18 (-0.052, 0.42)	0.13	0.0041	331
Ever on hormones (if yes)	0.11 (-0.035, 0.26)	0.135	0.0038	330
Current haemoglobin (per g/dl)	-0.18 (-0.038, 0.0016)	0.072	0.0067	336
Current C-reactive protein (per iu/ml)	0.014 (0.005, 0.022)	**0.001**	0.0341	271
Current platelets (per 10^9/dl)	0.00039 (-0.0002	0.202	0.0019	338
Prothrombin time (per s)	-0.52 (-0.12, 0.015)	0.131	0.0039	333
Current von Willebrand Factor (per iu/ml)	0.34 (0.24, 0.44)	**<0.001**	0.165	220
Current fibrinogen (per g/L)	0.137 (0.081, 0.19)	**<0.001**	0.063	336
Oxygen saturation , SaO_2 (per %)	-0.010 (-0.018, -0.0017)	**0.018**	0.014	326
Brain abscess (if yes)	0.24 (0.082, 0.40)	**0.003**	0.023	341
Stroke (if yes)	0.16 (0.028, 0.30)	**0.018**	0.014	341
Migraines (if yes)	0.062 (-0.034, 0.16)	0.2	0.0019	333
Smoking (if yes)	0.080 (-0.0089, 0.169)	0.078	0.0063	337
Hypertension (if yes)	0.24 (0.10, 0.38)	**0.001**	0.033	313
Pulmonary artery pressure, mean (per mmHg)	0.019 (0.0039, 0.034)	**0.02**	0.029	153
Activated partial thromboplastin time (per s)	- 0.061 (-0.077, -0.044)	**<0.001**	0.14	329
Thrombin time (per s)	0.058 (0.033, 0.084)	**<0.001**	0.056	327

Legend: Univariate regressions with lnFVIII in Series 1, Series 2 and the Combined series. Regression coefficients were calculated for the indicated variables per unit increase (continuous variables), or the difference between the presence and absence (binary variables). Values refer to the equation lnFVIII = constant + (regression coefficient*variable), with the 95% confidence limits for the coefficient presented. The presented p values were calculated by Stata, based on the Student t distribution. P values less than 0.05 are denoted in bold text.

Supplementary Table 3: Full model details for multiple regression of ln transformed FVIII (Summary results presented in Table 1)

	Regression coefficient	*95% confidence intervals*	*Standard error*	*T test*	*P value*
A) Series 1					
Age	0.0076	0.0026, 0.013	0.0025	3.01	0.003
Hypertension	0.24	0.04, 0.44	0.1	2.42	0.017
Serum iron	-0.0086	-0.017, 0.00033	0.0045	-1.91	0.059
B) Series 2					
Von Willebrand Factor	0.37	0.27, 0.46	0.05	7.37	<0.001
Serum iron	-0.0092	-0.015, -0.0032	0.003	-3.03	0.003

C) Model parameters:	***N***	***Sum of squares***	***Degrees of freedom***	***Mean square***	***Variance ratio (F)***	***Adjusted r^2***	***Model p value (P>F)***
Series 1	107	Series 1	16.04	106	0.15	9.19	0.19
Series 2	138	Series 2	23.3	179	0.13	33.12	0.26

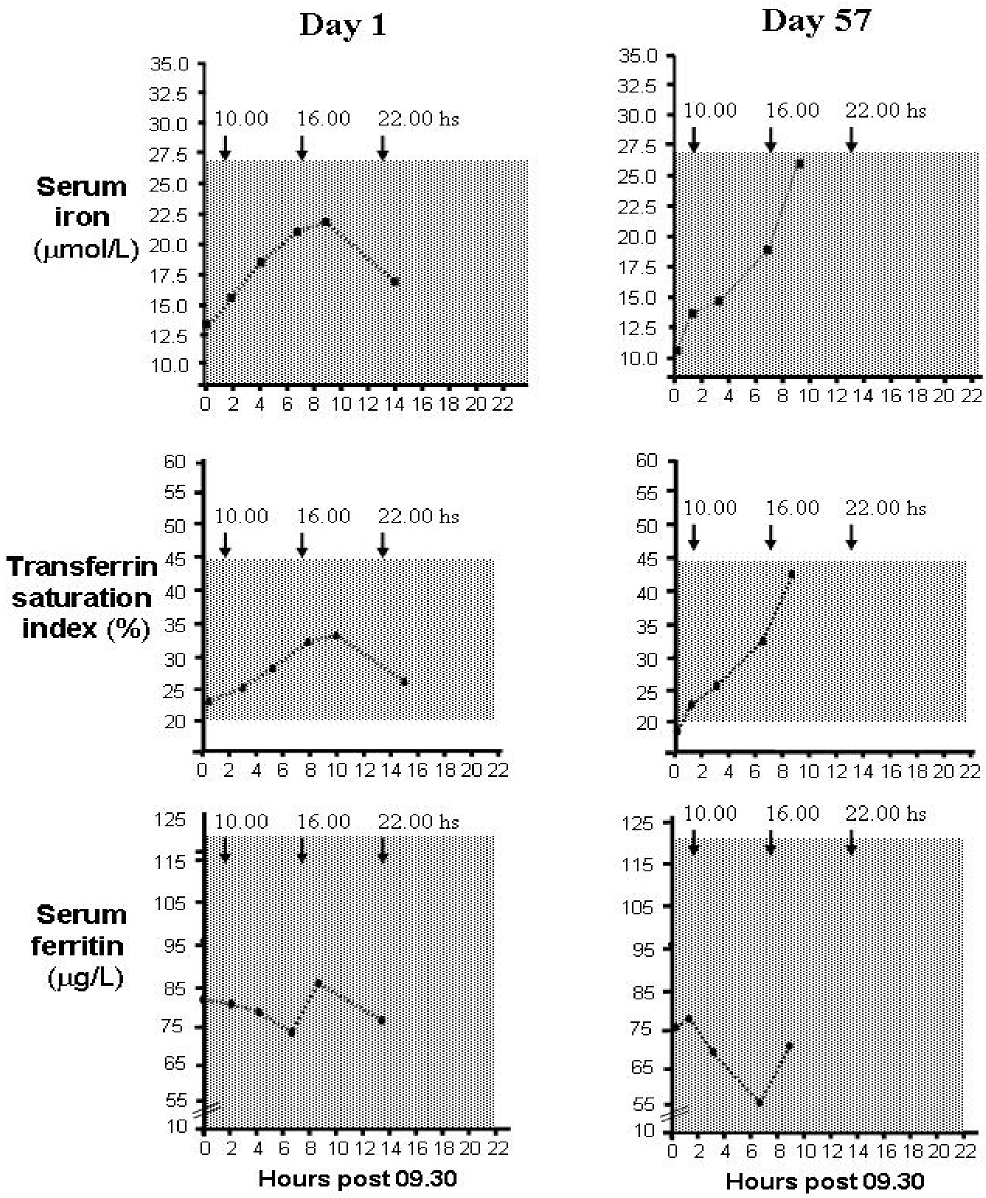
Legend: Multiple regression analyses for lnFVIII. For each model, the variables identified as making a significant contribution to the final model, once adjusted for the presence of other variables within the model, are presented. Full model descriptive parameters are presented in C). N, number of observations.

Supplementary Table 4: Details of venous thromboembolic events

	Series 1	**Series 2**	**Combined**
DVT/PE (events/patients)	**23 in 20**	**17 in 15**	**40 in 35**
Pulmonary enboli (+/- DVT)	9	9	17
Deep venous thromboses	14	9^	25^
Age at event (range [Q1, Q2, Q3])	28-70 (43, 52.5, 60)	30-65 (36.75, 50, 52)	28-71 (38.25, 50.5, 57.25)
Clinical setting			
Hospital or convalesence	**10**	**4**	**14**
Post brain abscess ±	7	1	8
Other inflammatory states*	2	2	4
Orthopaedic immobility, or intravenous line-related	1	1	2
Community	**13**	**13**	**24**
None (spontaneous)	8	8	16
Hormones +/-tranexamic acid/aminocaproic acid	4	0	4
Post flight	1	3	4
Pregnancy/post partum	0	2	2
Incidence rates			
All cases (per 100,000 patient yrs)	154.8	120.8	138.3
Community cases (per 100,000 patient yrs)	87.5	92.4	89.9

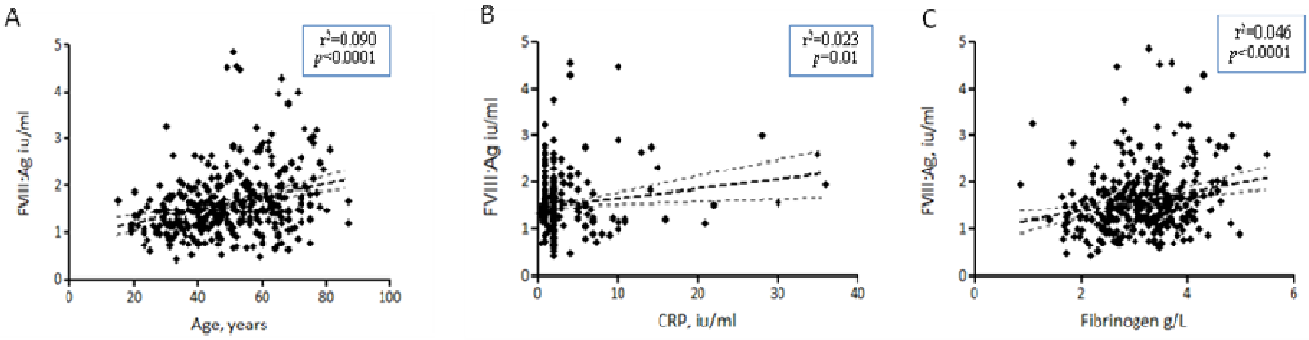
Legend: ^ include two cases of cerebral vein thrombosis. ± brain abscess, a common complication for HHT patients with pulmonary AVMs; * systemic lupus erythematosis (SLE), gout, liver infarction/failure. # Of these individuals, three were using hormones for HHT bleeding (hormone replacement; tranexamic acid or aminocaproic acid; hormones); and one for gynaecological purposes.

Supplementary Figure 1:



Legend: Reports of the pattern of variation of iron levels differed, with reports of daytime falls [4] and rises [9,10]. To facilitate optimisation of iron measurements for Series 2, diurnal variation was assessed on replicate test days, for a subject ingesting a replicate normal diet including meat. The normal ranges for each variable are shown stippled. Note that while total iron stores (ferritin) remained in the normal range throughout both days, there were substantial spontaneous rises in plasma iron (total iron, and transferrin saturation index (T*f*SI)). The mean variability in ferritin values was only 16% of the normal range, compared to 56% of the normal range for serum iron, and 69% of the normal range for serum T*f*SI. These data, and data published by others [9- 11] confirmed the need to standardise blood sampling times. They also led to routine ferritin measurements for Series 2, recognising that while it was a better marker in terms of hour-hour variability, it would be elevated by concurrent inflammatory or hepatic pathology.

Supplementary Figure 2: Additional FVIII regression plots

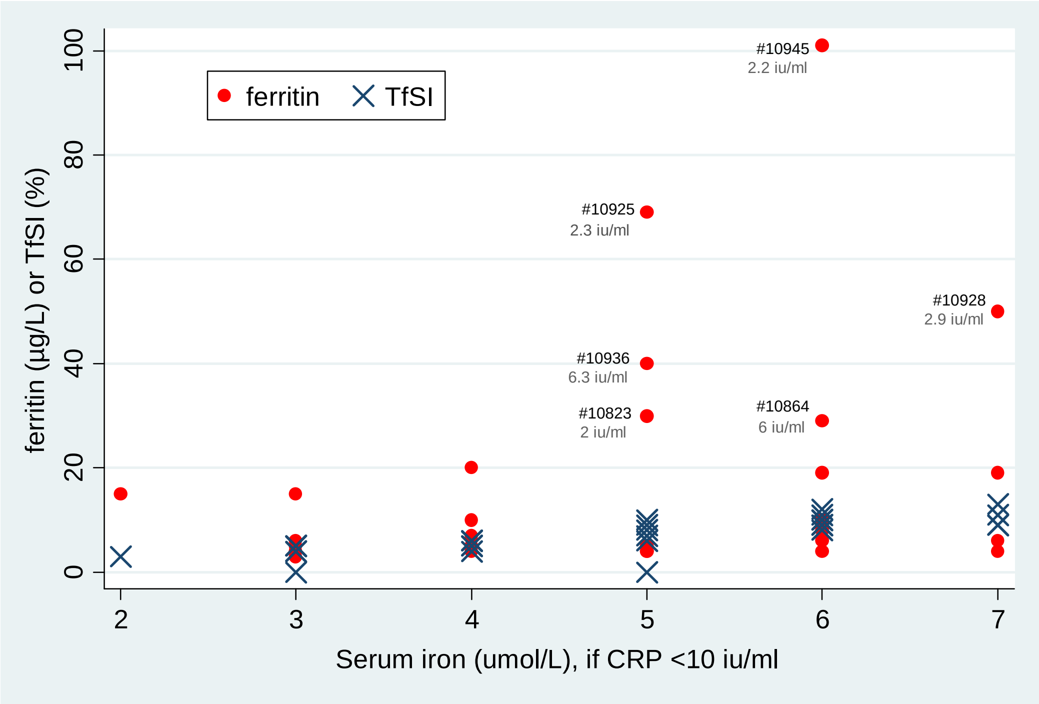


Legend:

Scatter plots for additional univariate associations in the Combined Series (presented data supplement those presented in Figure 1). Linear regression of FVIII with age **(A)** and the inflammatory markers CRP **(B)** and fibrinogen **(C).** The superimposed lines represent the linear regression line (bold) with 95% confidence intervals. Boxes indicate the r^2 values and *p* value for goodness of fit for each regression line.

Supplementary Figure 3:

High ferritin values in iron deficient individuals without an acute inflammatory response.



Legend:

Serum ferritin is widely regarded as the best serum marker of iron stores, and a low plasma ferritin level has a high predictive value for the diagnosis of uncomplicated iron deficiency anaemia. [12,13] In certain inflammatory diseases however, the ferritin can be raised above 100 µg/L even in the presence of iron deficiency anaemia.[13] Additional coexisting diseases in which ferritin levels may be misleading include liver or kidney disease, malignancy, rheumatoid disease, hyperthyroidism, or heavy alcohol intake. [13]

Further dissection of the relationship between serum iron and ferritin was therefore performed, using STATA to select the datasets where serum iron was in the lowest quartile (≤ 7µmol/l) and CRP was known to be less than 10 iu/ml, thus excluding individuals with confounding inflammatory stimuli. Note that all T*f*SI values (navy crosses) were ≤ 13% [normal range 20-40%]. Although 19/30 (63%) of ferritin values (red circles) were ≤10µg/L (the lower limit of normal for pre-menopausal women), and 24/30 (80%) were ≤20µg/L (lower limit of normal for men and post-menopausal women), the distribution was markedly skewed (range 3 -101 [Q_1 4; Q_3 19.25] µg/L). The identity and CRP values for the six outliers (three male [M], three female [F]) are indicated. The two highest ferritin values were in transfusion-dependent individuals receiving weekly intravenous iron (Cosmofer) preparations (#10945 F, #10925 M). The next highest value was in #10928 M, who, together with #10823 F, had severe hepatic AVM disease with a high output state. Individual #10936 F was using hormone replacement therapy and a statin, factors of uncertain relevance. No potential confounding state could be identified for the sixth individual (#10864 M).

REFERENCES

1. Shovlin CL. Hereditary haemorrhagic telangiectasia: Pathogenesis, diagnosis and treatment. Blood Reviews 2010;24:203-19.

2. Thompson RD, Jackson J, Peters AM, Doré CJ, Hughes JMB. Sensitivity and specificity of radioisotope right-left shunt measurements and pulse oximetry for the early detection of pulmonary arteriovenous malformations. Chest 1999;115:109-13

3. Shovlin CL, Tighe HC, Davies RJ, Gibbs JS, Jackson JE. Embolisation of pulmonary arteriovenous malformations: no consistent effect on pulmonary artery pressure. Eur Resp J. 2008;32:162-169

4. Burtis CA, Ashwood ER, Bruns DE, editors. Tietz textbook of clinical chemistry and molecular diagnostics. 4th ed. St Louis: Elsevier, 2006.

5. Shovlin CL, Sulaiman NL, Govani FS, Jackson JE, Begbie ME. Elevated Factor VIII in hereditary haemorrhagic telangiectasia (HHT): association with venous thromboembolism. Thromb Haemost 2007;98(5):1031-9.

6. Shovlin CL, Letarte M. Hereditary haemorrhagic telangiectasia and pulmonary arteriovenous malformations: issues in clinical management and review of pathogenic mechanisms. Thorax. 1999;54:714-29

7. Altman DG. Practical statistics for medical research. Chapman and Hall/CRC, London 1991

8. Shovlin CL, Jackson JE, Bamford KB, Jenkins IH, Benjamin AR, Ramadan H, Kulinskaya E. Primary determinants of ischaemic stroke/ brain abscess risks are independent of severity of pulmonary arteriovenous malformations in hereditary haemorrhagic telangiectasia. Thorax 2008;63:259-66.

9. Malhotra S, Garg SK, Khullar GK, Malhotra AS, Kondal A, Rana H, et al. Kinetics of two different iron formulations and their effect on diurnal variation of serum iron levels. Methods Find Exp Clin Pharmacol 2004;26:417-20.

10. Lin L, Valore EV, Nemeth E, Goodnough JB, Gabayan V, Ganz T. Iron transferrin regulates hepcidin synthesis in primary hepatocyte culture through hemojuvelin and BMP2/4 Blood. 2007;110:2182-9.

11. Hutchinson C, Al-Ashgar W, Liu DY, Hider RC, Powell JJ, Geissler CA. Oral ferrous sulphate leads to a marked increase in pro-oxidant nontransferrin-bound iron. Eur J Clin Invest. 2004;34:782-4.

12. Valberg LS. Plasma ferritin concentrations: their clinical significance and relevance to patient care. Can Med Assoc J. 1980 Jun 7;122(11):1240-8.

13.http://cks.nhs.uk/anaemia_iron_deficiency/management/scenario_diagnosis/investigations/interpreting_ferritin_levels# (accessed 07.11.2011)