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 When iron associations emerged in Series 1 multiple regression analyses (Shovlin and Kulinskaya, 2006 unpublished), measurements of iron status were considered further.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

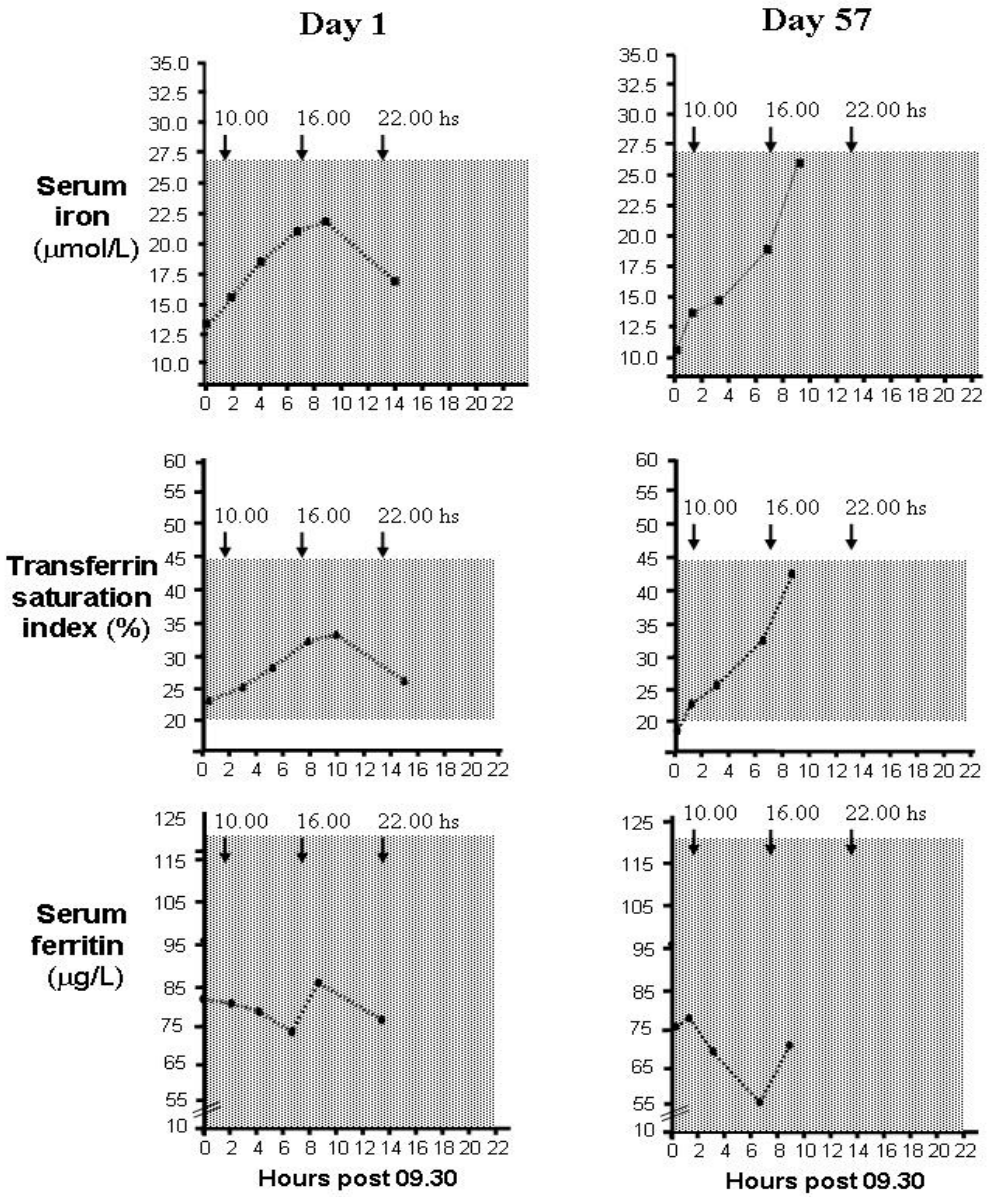
Legend: Multiple regression analyses for lnFVIII. For each model, the variables identified as making a significant contribution to the final model, once adjusted for the presence of other variables within the model, are presented. Full model descriptive parameters are presented in C). N, number of observations.

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

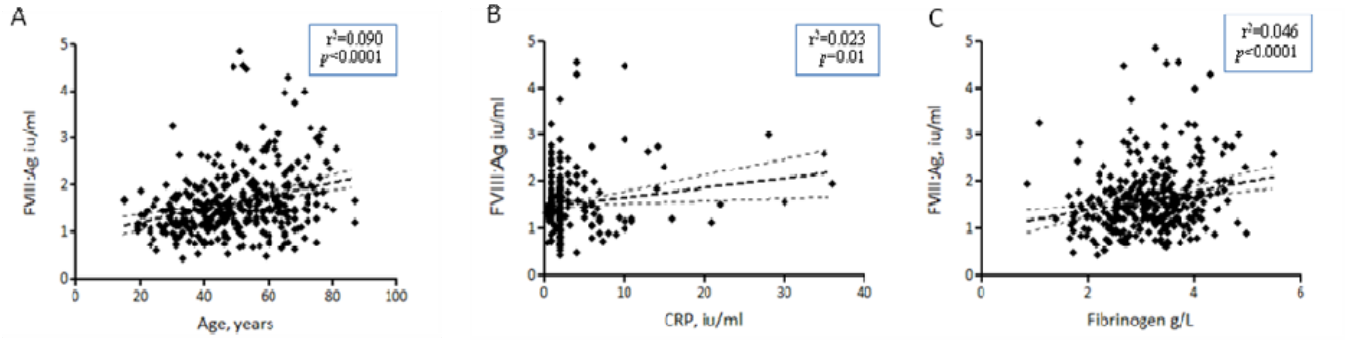
Legend: ^ include two cases of cerebral vein thrombosis. ± brain abscess, a common complication for HHT patients with pulmonary AVMs; * systemic lupus erythematosis (SLE), gout, liver infarction/failure. # Of these individuals, three were using hormones for HHT bleeding (hormone replacement; tranexamic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

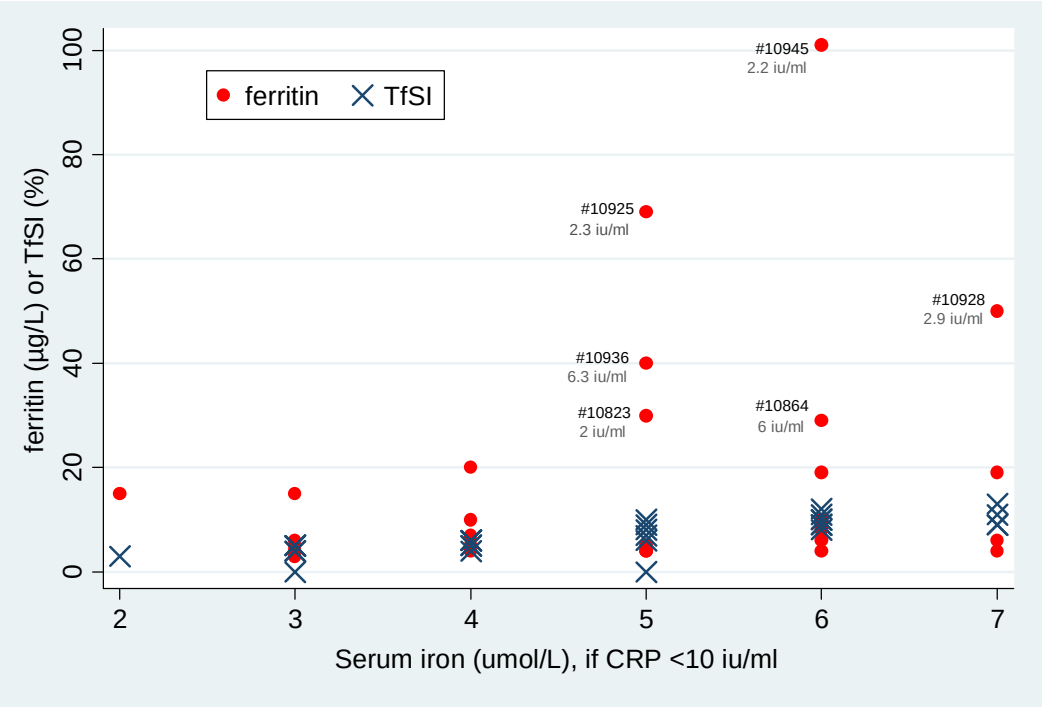


Legend:

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).

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