Iron deficiency responses and integrated compensations in patients according to hereditary hemorrhagic telangiectasia *ACVRL1*, *ENG* and *SMAD4* genotypes

Severe hemorrhage and anemia in hereditary hemorrhagic telangiectasia (HHT) is a major focus of drug development and repurposing. HHT is not a single disorder, but molecularly heterogenous, and most commonly caused by a single loss-of-function DNA variant in *ACVRL1*, *ENG* or *SMAD4*. As for all individuals in the general population, HHT patients are at risk of developing iron deficiency, anemia and sequelae if iron lost through hemorrhage is not adequately replaced, but as we have published, quantitative examination in over two decades of HHT care emphasizes that exact responses vary between individuals. We hypothesized that one element of variability may reflect the underlying HHT genotype. Here we demonstrate subtle differences that may be important to recognize when designing randomized controlled trials of new HHT therapies, and also for existing management strategies. We show that where. HHT patients were becoming anemic and iron deficient, those with a pathogenic variant in *SMAD4* displayed different patterns of compensations compared to those with an *ACVRL1* or *ENG* pathogenic variant. While the study is limited by the rarity of the *SMAD4* genotype (currently, only 2.5% of HHT causal variants on the HHT Mutation Database), we explore reasons that may explain a distinctive phenotypic cluster in *SMAD4* HHT patients. In order to further stimulate future prospective studies, we outline the potential relevance to HHT symptom burden and complications, rationales for differing iron treatment regimes by HHT genotype, and more broadly suggest that the cohorts provide an opportunity to further clarify relationships involved in iron and circulatory homeostasis.

To provide further detail, HHT is a complex, heritable vasculopathy that is estimated to affect between one in 3,000-8,000 people, and is characterized by nosebleeds (epistaxis), mucocutaneous telangiectasia and visceral arteriovenous malformations (AVM).[1,2] In hematological circles, the greatest concern is the management of iron deficiency anemia secondary to HHT bleeding. This was a focus of recent International HHT Guidelines, and is an active area of pharmaceutical development.[1] HHT patients develop iron deficiency when dietary iron is inadequate to provide both their usual iron requirements, and to replace additional iron losses due to bleeding from the nose, the gastrointestinal tract, menstruation, blood donation, and other losses as quantified by the hemorrhage-adjusted iron requirement.[3] Iron deficiency anemia can have significant consequences for HHT patients and is one of the strongest predictors of mortality in HHT.[4] As for any anemia, iron deficiency anemia reduces arterial oxygen content necessitating higher cardiac output to maintain tissue oxygenation, and this is a particular problem in HHT where visceral AVM lower the systemic vascular resistance, also resulting in higher cardiac outputs.[2,5] Additional complications associated with iron deficiency and its treatment are reported in HHT, in particular patients with hepatic or pulmonary AVM.[1,2] Furthermore, in HHT, ongoing bleeds mean that conventional transfusional support algorithms need to be modified.[1] Pan-HHT genotyping is identifying more variable expressivity in all genotypes than previously expected, including paucity of clinical signs in patients presenting by less conventional routes (e.g., organ-specific AVM rather than clinical genetics, hematology or ENT surgery; AVM/HHT rather than juvenile polyposis gastroenterology).[6] That said, there is clear evidence that particular AVM distribution patterns differ between the major HHT genotypes,[7] and that *SMAD4*$^{+/-}$ patients can have additional phenotypes including juvenile polyposis and aortopathy.[1,2,7]

In order to take a first look at whether there were distinctive iron deficiency indices and responses between the HHT genotypes, with ethical approval (LREC 2000/5764), serial anonymized data were analyzed retrospectively from all genotyped patients with clinical HHT reviewed at a single institution between May 1999 and August 2021 where serum iron, transferrin saturation index (T*f*SI) and supine/erect pulse were measured as standard of care. The 426 patients had a median age of 50 (interquartile range [IQR], 39-62) years at the time of their measurements, and 264 (62%) were female (*Online Supplementary Table S1*). They provided 686 measurements in 246 *ENG*$^{+/-}$ patients, 166 measurements in 102 *ACVRL1*$^{+/-}$ patients, 32 measurements in 11 *SMAD4*$^{+/-}$ patients, and 118 measurements in patients who tested negative for variants in known HHT causal genes (*Online Supplementary Table S1*). Analyses were conducted using all available measurements, and also in the smaller dataset of first measurements only per patient, where the small number of *SMAD4* cases (n=11) impeded statistical comparisons.

First, we examined iron indices across the three genotypes. Overall serum iron ranged from 6-18 μmol/L (median 11), transferrin saturation index (T*f*SI) from 9-28% (median 18) and ferritin from 14-67 μg/L (median 28). As shown in Figure 1A, serum ferritin was similar in *ACVRL1*$^{+/-}$, *ENG*$^{+/-}$ and *SMAD4*$^{+/-}$ patients (median values 31, 25 and 26 μg/L, respectively).

Despite the similar serum ferritin, there were differenc-

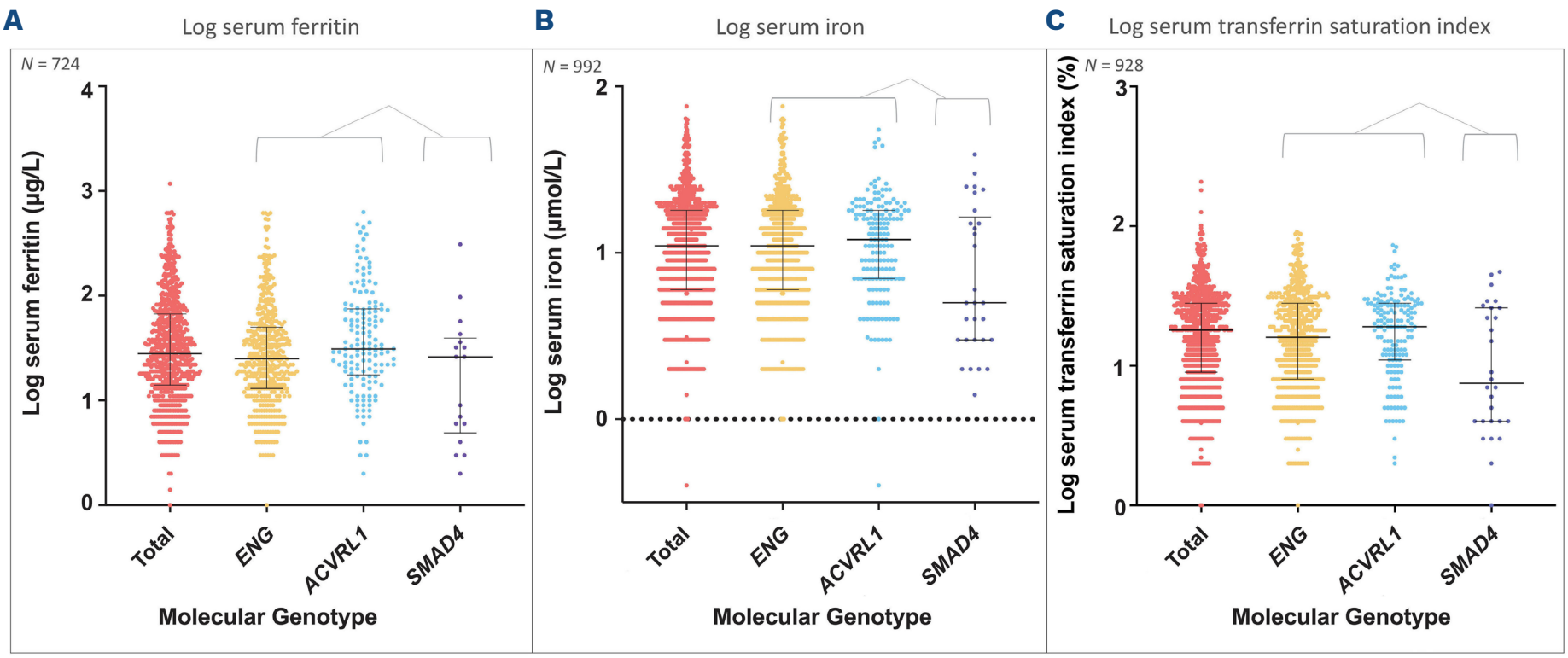


Figure 1. Comparison of iron indices between hereditary hemorrhagic telangiectasia molecular genotypes. All dataset comparisons of values for hereditary hemorrhagic telangiectasia (HHT) patients by molecular genotype. (A) Serum ferritin. (B) Serum iron. (C) Serum transferrin saturation index (T*f*SI). Error bars indicate median and interquartile range. Non-independent datasets unequal in number mean absolute *P* values for the indicated comparisons should be viewed with caution, though for relative comparisons, for ferritin, iron and T*f*SI, *P* values were 0.08, 0.02 and 0.007 by Spearman's rank correlation and Mann Whitney comparing *SMAD4* to *ENG* and *ACVRL1* combined.

es examining serum iron and T*f*SI. Serum iron was lower in *SMAD4*$^{+/-}$ patients than in *ACVRL1*$^{+/-}$ or *ENG*$^{+/-}$ patients (median values 5, 12 and 11 µmol/L respectively; Figure 1B). Similarly, T*f*SI was lower in *SMAD4*$^{+/-}$ than in A*CVRL1*$^{+/-}$ or ENG$^{+/-}$ patients (median values 7, 19 and 16% respectively; Figure 1C) with similar trends in first-visit measurements (*Online Supplementary Figure S2*).

Next, we examined distributions of red cell indices. As shown in Figure 2A, there were similar patterns in the relationship between hemoglobin and serum iron levels between the different HHT molecular genotypes. However, red blood cell mean corpuscular volume (MCV) was lower in *SMAD4*$^{+/-}$ than *ACVRL1*$^{+/-}$ or *ENG*$^{+/-}$ (medians 75.1; 89.7; 89.0 fL respectively (*Online Supplementary Table S1*) and visual comparison suggested this was not fully explained by iron status (Figure 2B). Similar patterns were seen for mean corpuscular hemoglobin concentration (MCHC) overall (*Online Supplementay Table S1*) and in relationship to serum iron (Figure 2C). Hemoglobin was maintained in the *SMAD4*$^{+/-}$ patients despite lower hemoglobin content per red cell, by a higher total red cell count compared to *ACVRL1*$^{+/-}$ and *ENG*$^{+/-}$ patients (median 5.4; 4.7 and 4.8x10^{12}/L respectively (*Online Supplementary Table S1*; visual comparisons in Figure 2D).

Uniquely, our institution has measured postural changes in the heart rate, alongside SaO_2, for more than three decades.[8] A higher pulse rate is seen in response to acute blood volume loss through hemorrhage, and in barometric responses to preserve cerebral perfusion on standing with such autonomic response stronger in younger individuals.[8,9] We, therefore, examined whether the resting pulse, a crude measure of circulation adjustment relevant to anemia, differed between the genotypes. As expected for HHT patients,[8] the pulse rate increased when patients moved from a supine to an erect position (median values 71.9 and 76.3 beats per minute [bpm], Figure 3), and a higher pulse rate on standing was seen in all three molecular genotypes. We had expected the magnitude of the increase to be marginally greater in *ENG*$^{+/-}$ patients with lower SaO_2 due to pulmonary AVM, as we have previously shown with pan-HHT genotype analyses.[8] Instead, the magnitude of pulse increase was almost twice as high in *SMAD4*$^{+/-}$ than *ENG*$^{+/}$ (Figure 3), beyond any increment predicted by their marginally younger age, lower body mass index, or pulmonary AVM status[8,9] (*Online Supplementary Table S1*).

Taken together, the study findings indicate that in the setting of iron deficiency to which *SMAD4* patients may be more susceptible through gastrointestinal bleeding and polyps,[2,10] physiological compensation mechanisms appeared to differ between HHT molecular genotypes.

The Study has strengths include a unique dataset that allowed for the comparison of multiple indices between the molecular genotypes, and a notably large sample size of genotyped patients given the rarity of the condition, particularly the *SMAD4*$^{+/-}$ genotype. As described further in the *Online Supplementary Appendix*, previous studies have predominantly compared *ENG*$^{+/-}$ and *ACVRL1*$^{+/-}$ patients, whereas this current study also assessed differences in *SMAD4*$^{+/-}$ patients. Limitations stem from its observational and retrospective nature, as the results may reflect other demographic differences or

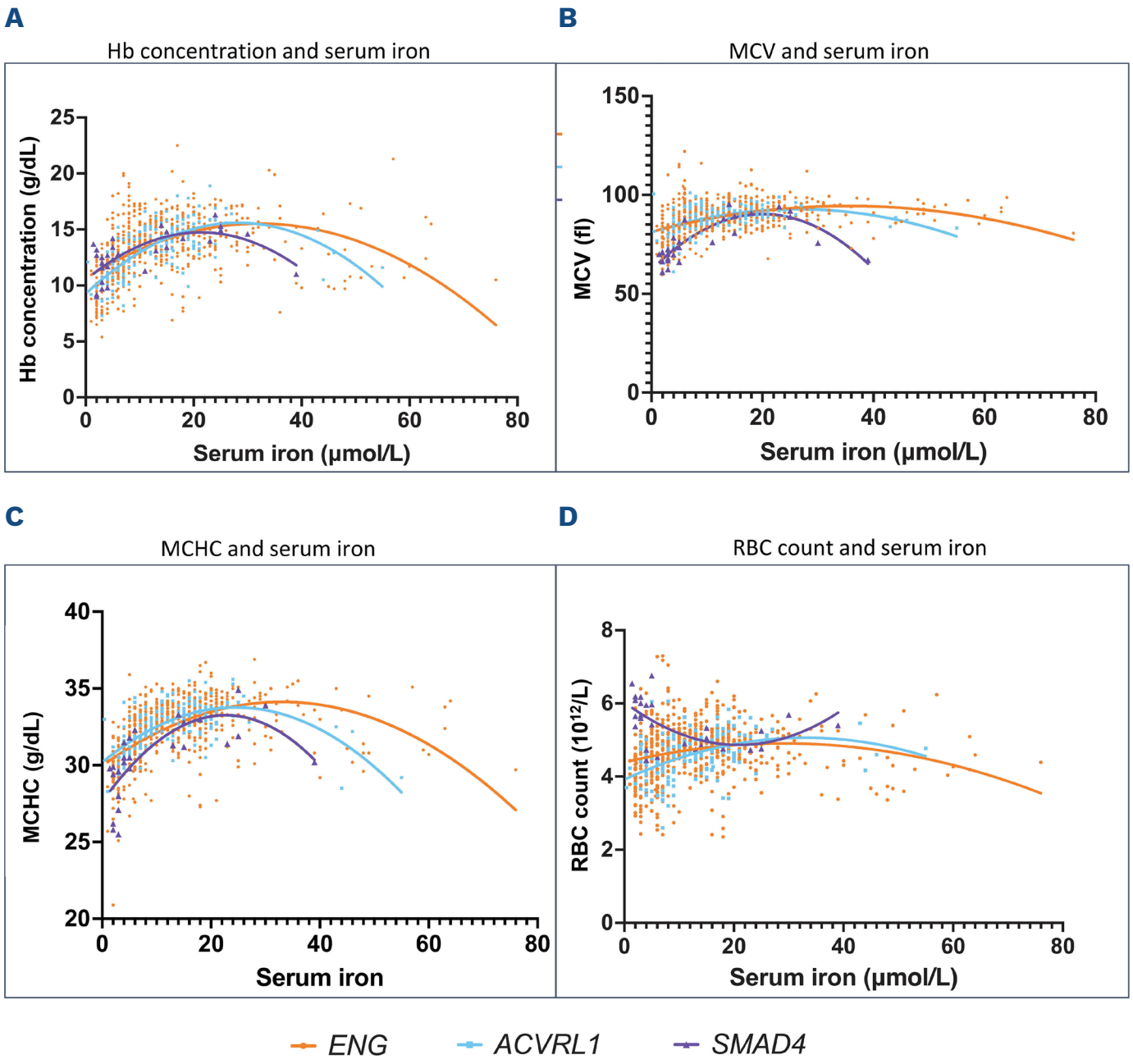


Figure 2. Comparison of the relationship between hemoglobin and red cell indices. Data in patients with confirmed hereditary hemorrhagic telangiectasia (HHT) molecular genotypes; 865 values are plotted on each graph, with quadratic regression lines for the 3 HHT genotypes illustrated for *ACVRL1* (blue), *ENG* (orange) and *SMAD4* (purple). (A) Hemoglobin concentration (Hb). (B) Mean corpuscular volume (MCV). (C) Mean corpuscular hemoglobin concentration (MCHC). (D) Red blood cell count (RBC).

confounders between the HHT molecular genotype cohorts. Furthermore, the small *SMAD4* numbers prevented two-way adjustments, thus it is not possible to assign causation to possession of the *SMAD4* variant.

That said, the evidence presented begins to point to physiologically different iron homeostasis in *SMAD4*$^{+/-}$ patients, highlighting new concepts to be considered as HHT management recommendations and clinical trials proceed. The pattern resembles functioning as though the patient is in a more iron-deficient state: despite similar serum ferritin values, *SMAD4*$^{+/-}$ patients displayed lower serum iron and T*f*SI than the other HHT molecular genotypes. Furthermore, similar hemoglobin was achieved by a greater number of smaller red cells. *SMAD4* regulates hepcidin,[11,12] the key regulator of iron homeostasis,[11] which is reduced in individuals with active bleeding (via erythroferrone[13]) and iron deficiency.[11] Our previously reported hepcidin dataset[3] included only one individual with *SMAD4*$^{+/-}$. Their hepcidin:ferritin ratio (1.2 ng/mL/µg/L) was higher than the other HHT patients (0.2-0.5 [mean 0.3] ng/mL/µg/L), and controls (0.1-1.0 [mean 0.5] ng/mL/µg/L), but the *SMAD4* patient was iron replete, limiting interpretations. Whole-genome sequencing of *ACVRL1*$^{+/-}$, *ENG*$^{+/-}$ and *SMAD4*$^{+/-}$ patients was performed through the 100,000 Genomes Project,[14] but no hepcidin (*HAMP*) DNA variants were identified in these individuals, precluding similar analyses to those we have recently performed for hemorrhage susceptibility in HHT.[15] Published murine data is of only limited help - while basic erythropoiesis was normal in *SMAD4*-deficient mice,[10] it was not examined during iron-restricted erythropoiesis, nor in the setting of secondary erythrocytosis that compensates for pulmonary AVM-induced low oxygen saturation in order to maintain arterial oxygen content,[7,8] and we speculate may be employed differently in the maintenance of hemoglobin in iron-deficient *SMAD4*$^{+/-}$ patients. Future prospective studies will be enhanced by incorporating measurements at the time of iron deficiency. Importantly, and unexpectedly, there were differing magnitudes of response to acute changes to the circulation on standing. Given the separate arterial pathologies observed in *SMAD4* patients (e.g., aortic dilatation),[1,2] possession of a *SMAD4* causal variant is a plausible differential to why the orthostatic pulse discrepancies were observed and further examination is warranted, particularly as this may highlight arterial pathology beyond the expected HHT-specific variables. In conclusion, this study contributes to the growing body of literature that indicating phenotypic differences between

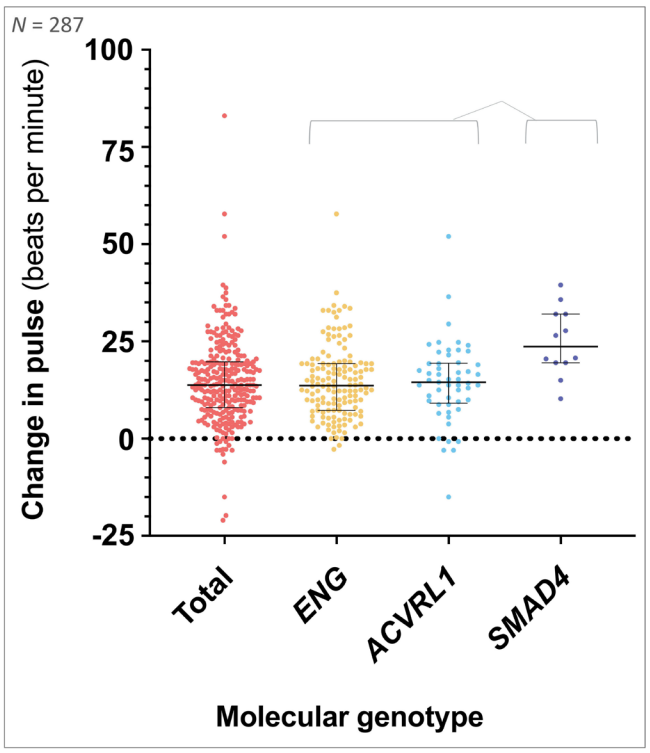


Figure 3. Comparison of orthostatic changes in pulse. Data are shown for all patients in the database (N=286), and separated by confirmed hereditary hemorrhagic telangiectasia (HHT) molecular genotypes, showing the change in pulse (in beats per minute) between a patient in a supine position and the same patient in an erect position. Data were recorded at 1-minute intervals over a 20-minute period for 10 minutes supine and 10 minutes erect, with the pulse rates recorded as the mean recorded across minutes 7, 8, 9 and 10 in each posture. Error bars indicate median and interquartile range. For the indicated comparisons using first measurements only, the *P* value was 0.04 by Mann Whitney comparing *SMAD4* to *ENG* and *ACVRL1* combined.

HHT molecular genotypes, by demonstrating differences in iron, red cell and hemodynamic indices that *SMAD4*$^{+/-}$ patients may use to in settings of iron deficiency. Future studies should aim to confirm with larger numbers, requiring introduction of routine measurements of iron indices and postural pulse into assessment, and likely multicenter analyses given the sparsity of *SMAD4*$^{+/-}$ HHT patients and importance of adjusting for confounders in multivariable analyses. Future studies should also evaluate if differences identified in *SMAD4*$^{+/-}$ patients are associated with a modified symptom burden, for instance, tolerance of standing; elucidate better mechanistic understanding of the relationship between SMAD4 and hepcidin in the setting of iron deficiency to which all HHT patients are prone, and address whether there should be changes to the type of iron deficiency treatment these patients receive.

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Contributions

LS performed the analyses, generated the figures and *Online Supplementary Appendix* and wrote the first draft. FA examined 100,000 Genomes Project data. HM, JS and HCT acquired postural pulse data. The Genomics England Research Consortium sequenced whole genomes for selected patients. CLS reviewed all patients, generated the database, conceived the study, supervised LS, wrote the manuscript and is the guarantor for the study.

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Data-sharing statement

Categorized data that do not risk breaching anonymity may be found in the *Online Supplementary Appendix*. Primary data from the 100,000 Genomes Project are held in a secure Research Environment, are available to registered users. Please see https://www.genomicsengland.co.uk/about-gecip/for-gecip-members/data-and-data-access for further information.

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