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Early View

Original article

The ADAMTS13-VWF axis is dysregulated in chronic thromboembolic pulmonary hypertension

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Title Page

Full Title

The ADAMTS13-VWF axis is dysregulated in chronic thromboembolic pulmonary hypertension

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Take home message

The ADAMTS-13 VWF axis is dysregulated in chronic thromboembolic disease with and without pulmonary hypertension and implicated in their pathogenesis.

Abstract

Chronic thromboembolic pulmonary hypertension (CTEPH) is an important consequence of pulmonary embolism (PE) that is associated with abnormalities in haemostasis. We investigated the ADAMTS13-VWF axis in CTEPH, including its relationship to disease severity, inflammation, *ABO* groups and *ADAMTS1*3 genetic variants.

ADAMTS13 and VWF plasma antigen levels were measured in patients with CTEPH (n=208), chronic thromboembolic disease without pulmonary hypertension (CTED; n=35), resolved PE (n=28), idiopathic pulmonary arterial hypertension (n=30) and healthy controls (n=68). CTEPH genetic *ABO* associations and protein quantitative trait loci were investigated. ADAMTS-VWF axis abnormalities were assessed in CTEPH and healthy control subsets by measuring ADAMTS13 activity, D-dimers and VWF-multimeric size.

CTEPH patients had decreased ADAMTS13 (adjusted β (95% CI) = -23.4 (-30.9 to -15.1)%, $p<0.001$) and increased VWF levels (β=+75.5 (44.8 to 113)%, $p<0.001$) compared to healthy controls. ADAMTS13 levels remained low after reversal of pulmonary hypertension by pulmonary endarterectomy surgery and were equally reduced in CTED. We identify a genetic variant near the *ADAMTS13* gene associated with ADAMTS13 protein that accounted for ~8% of the variation in levels.

The ADAMTS13-VWF axis is dysregulated in CTEPH. This is unrelated to pulmonary hypertension, disease severity or markers of systemic inflammation and implicates the ADAMTS13-VWF axis in CTEPH pathobiology.

Introduction

Chronic thromboembolic pulmonary hypertension (CTEPH) results from failure of thrombus resolution in the pulmonary arteries following acute pulmonary embolism (PE) in ~3% [1]. Organisation and fibrosis of thrombotic material leads to obstruction of proximal pulmonary arteries and the subsequent development of a secondary small vessel vasculopathy, both of which contribute to pulmonary hypertension and subsequent right heart failure [2, 3].

Abnormalities in haemostasis are implicated in CTEPH pathobiology [4, 5]. This includes elevated von Willebrand factor (VWF), a multimeric plasma glycoprotein that is synthesized by vascular endothelial cells and megakaryocytes [6, 7]. VWF plays an important role in platelet recruitment by mediating adhesion of platelets to the endothelium and is also a carrier protein for the pro-coagulant blood clotting factor VIII [7]. VWF activity is normally regulated by ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13), a plasma protein that specifically cleaves the more active high molecular weight VWF multimers [8]. ADAMTS13 is predominately produced by hepatic stellate cells, in addition to vascular endothelial cells and megakaryocytes [9]. The critical role of ADAMTS13 levels in haemostasis is exemplified by thrombotic thrombocytopenic purpura (TTP), characterised by micro-angiopathic thrombosis, in which plasma levels of ADAMTS13 are severely reduced by autoantibodies or rare *ADAMTS13* mutations [10].

Plasma VWF is increased in a range of thrombotic conditions including coronary artery disease (CAD), ischaemic stroke and venous thromboembolism (VTE) [11, 12]. Conversely,

plasma ADAMTS13 is modestly reduced in CAD and ischaemic stroke [11, 13]. There are discordant findings in patients with acute PE, with increased, no difference and decreased ADAMTS13 reported [14-16]. VWF and Factor VIII are known to be elevated in CTEPH and do not change following pulmonary endarterectomy (PEA) suggesting a role in pathogenesis [6]. Whilst VWF cleaving protease has been indirectly assessed in CTEPH the direct role of ADAMTS13 has not been investigated to date [6].

A large proportion of the variation in VWF levels is genetically determined, with 30% due to *ABO* groups [17]. The *ADAMTS13* gene is situated ~200 kilobases (kb) downstream of *ABO* and is genetically regulated with 20% of its variance attributable to common variants at the *ADAMTS13* locus [18]. ADAMTS13 is not known to vary with *ABO* groups in healthy cohorts [19]. Similar to other thrombotic diseases, the non-O blood groups are over-represented in CTEPH suggesting a mechanism by which VWF levels are increased [20]. We aimed to investigate the ADAMTS13-VWF axis in CTEPH patients including its relationship to *ABO* groups and *ADAMTS1*3 genetic variants.

Methods

Study samples and participants

The study was approved by the regional ethics committee (REC no. 08/H0304/56 and 08/H0802/32) and all study participants provided written informed consent from their respective institutions.

Consecutive CTEPH patients from the national pulmonary endarterectomy (PEA) centre (Royal Papworth Hospital, United Kingdom (UK)) with available plasma samples (August 2013-December 2016) (supplementary figure S1) and genotype data were included in the study (n=208). Healthy volunteers (n=68) were used as a control group (Papworth and Hammersmith Hospital, UK). Additional patient groups were recruited as disease comparators including: chronic thromboembolic disease (CTED, n=35), idiopathic pulmonary arterial hypertension (IPAH, n=30) and pulmonary embolism (PE, n=28). CTED was characterised by persistent pulmonary arterial thromboembolic occlusions without pulmonary hypertension (mean pulmonary arterial pressure <25mmHg) in symptomatic patients, and other diagnoses were made using international criteria [21, 22].

ADAMTS13 and VWF plasma concentrations

Plasma samples were used to measure ADAMTS13 and VWF antigen (Ag) levels by enzyme-linked immunosorbent assays (ELISA). Samples for the CTEPH, CTED and IPAH groups were obtained closest to the time of diagnosis, and pre-operatively for the CTEPH and CTED patients undergoing PEA. Additionally, ADAMTS13 and VWF levels were measured in 22 paired post-PEA samples taken at a follow-up time within 1 year of surgery to assess the effect of PEA. The PE group were sampled from a specialist PE follow-up service (Hammersmith, UK) at a median of 220 (interquartile range (IQR) 218) days following an acute PE.

ADAMTS13 and VWF plasma antigen levels were quantified using polyclonal rabbit anti-ADAMTS13 and anti-VWF antibodies as previously described (supplementary material) [19, 23].

ADAMTS13 activity, D-dimer, anti-ADAMTS13 autoantibodies and VWF multimeric size

Additional experiments were performed on a subset of the CTEPH (n=21-23) and healthy control (n=14) groups to identify potential mechanisms for any dysregulation of the ADAMTS13-VWF axis. Plasma samples were used to measure ADAMTS13 activity (fluorescence resonance energy transfer (FRETS) assay), D-dimer concentrations (ELISA) and anti-ADAMTS13 autoantibodies (CTEPH: n=23) with further details in the supplementary material. An estimate of VWF multimeric size was made by measuring VWF collagen binding (VWF:CBA) and comparing this with VWF antigen levels (CTEPH: n=21).

Clinical phenotype data

Phenotype data for the CTEPH, CTED and IPAH groups were recorded closest to the time of diagnosis and pre-operatively for the CTEPH and CTED patients undergoing PEA. This included demographics, haemodynamics, WHO functional class, 6-minute walk distance (6mwd), clinical blood tests, smoking history and anticoagulation therapy usage.

Genotype data

Imputed genotype dosages were available from an ongoing international GWAS in CTEPH that will be published separately on recruitment of a validation cohort. All individuals were genotyped on commercially available Illumina assays and imputed to the Haplotype Reference Consortium build 1.1. Additional details and quality controls steps are described in the supplementary material.

Genotypes were available for 207 (185 CTEPH; 22 CTED) after GWAS quality control exclusions. These patients were included in the genetic *ABO* group and protein quantitative

trait loci (pQTL) analyses. Matched genotypes and ADAMTS / VWF antigen levels were not available for the healthy control, IPAH or PE groups.

Genetic *ABO* groups

The *ABO* groups A1, A2, B and O were reconstructed using haplotypes from phased data and a described list of tagging *ABO* SNPs (supplementary materials). This resulted in 10 groups (A1A1, A1A2, A1B, A1O, A2A2, A2B, A2O, BB, BO, OO), from which blood groups A, B, AB and O were inferred.

Protein quantitative trait loci

Associations between genetic variants in the *ADAMTS13* gene ± 40kb (n=396 variants), and ADAMTS13 protein levels were evaluated using multivariable linear regression. The model was adjusted for age, sex and ADAMTS13 plasma antigen experimental batch. Additional models were adjusted for VWF antigen levels and the first 5 ancestry informative principal components used in the GWAS analysis. The *ADAMTS13* ± 40kb region included the *ADAMTS13* cis-pQTLs that have previously been described [18, 24, 25].

Statistical analysis

Group differences in ADAMTS13 and VWF antigen levels were assessed using multivariable linear regression adjusted for age, sex, experimental batch (batch1 vs. batch2) and self-reported ethnicity (Caucasian vs. non-Caucasian). The β coefficients and confidence intervals (CI) are presented as percentage change.

Data is presented as median ± interquartile range. Spearman's rank correlation coefficients were used to describe associations between ADAMTS13 or VWF protein levels and clinical phenotypes associated with disease severity and blood markers of inflammation.

Results

Baseline group characteristics are summarised in table 1 and supplementary table S1. Age and sex differed across the groups (p<0.001 and p=0.014) with CTEPH patients being older (median ± IQR: 64 ± 19years) than healthy controls (49 ± 24years). Ethnicity also differed (p<0.001) with more non-Caucasians in the PE group. In the whole CTEPH group, 176 (87%) had a proximal distribution of pulmonary arterial obstruction deemed to be surgically accessible and 150 (72%) underwent pulmonary endarterectomy.

ADAMTS13 plasma concentrations

ADAMTS13 antigen levels were decreased in CTEPH patients (0.889 ± 0.397μg/mL; p<0.001) compared to healthy controls (1.15 ± 0.300μg/mL) (figure 1a). ADAMTS13 was also reduced in CTED (0.831 ± 0.224μg/ml, p<0.001) but levels were similar to CTEPH (p=0.205) (supplementary table S2). There was no difference in ADAMTS13 levels between IPAH (1.12 ± 0.413μg/mL; p=0.373) and healthy controls, though the PE group did exhibit slightly lower levels (0.969 ± 0.704μg/ml; p=0.049).

Multivariable linear regression confirmed that ADAMTS13 was lowest in the CTEPH (β (95% CI) (% change) = -23.4 (-30.9 to -15.1)%, p<0.001) and CTED groups (β = -25.9 (-35.1 to -15.4)%, p<0.001) (supplementary table S3). These observations should be interpreted with the additional models utilising interaction terms presented in the supplementary materials. Increasing age was also associated with lower ADAMTS13 (β = -5.06 (-2.99 to -7.08)% per 10 years, p<0.001). ADAMTS13 antigen levels were not significantly associated with the PE group (β = -12.0 (-24.0 to 1.97)%, p=0.089), nor were they associated with IPAH, sex or ethnicity.

VWF plasma concentrations

We confirmed that VWF antigen levels are increased in CTEPH (16.7 ± 15.2μg/mL; $p<0.001$) compared to healthy controls (8.45 ± 8.77μg/mL) (figure 1b). Furthermore, VWF was increased in CTED (17.0 ± 10.1μg/mL, $p<0.001$) compared to healthy controls, but was no different to CTEPH ($p=0.834$) (supplementary table S2). There was no difference in VWF antigen levels between IPAH (11.6 ± 12.3μg/mL; $p=0.071$) or PE (9.23 ± 9.82μg/mL; $p=0.433$) and healthy controls.

Multivariable linear regression was also used for VWF plasma concentrations as described for ADAMTS13. This confirmed that VWF was significantly increased in the CTEPH (β=+75.5 (44.8 to 113)%, $p<0.001$) and CTED groups (β=+89.5 (48.0 to 143)%, $p<0.001$) (supplementary table S4). VWF plasma concentrations were not significantly associated with the IPAH or PE groups, sex or ethnicity.

The combination of low ADAMTS13 and high VWF antigen levels had a synergistic effect on the odds of CTEPH (Odds ratio (OR) = 14.5 (5.33 to 47.4), $p<0.001$) compared with healthy controls (supplementary figure S2 and supplementary table S5).

ADAMTS13 and VWF: Pre- and post-pulmonary endarterectomy

In 22 CTEPH patients matched samples were taken post-PEA, after a median of 343 (IQR 216) days. There were no differences in ADAMTS13 (median of differences ± IQR: -0.0328 ± 0.250μg/mL, $p=0.777$) or VWF protein levels (-3.05 ± 10.7μg/mL, $p=0.777$) following removal of proximal organised thrombus material by pulmonary endarterectomy (figure 2).

ADAMTS13 activity, D-dimer, anti-ADAMTS13 autoantibodies and VWF multimers

Specific ADAMTS13 activity (Activity:antigen (Act:Ag) ratio) was increased in CTEPH (Act:Ag 1.57 ± 0.32) compared with healthy controls (1.05 ± 0.190; $p<0.001$) (figure 3a).

Plasmin and thrombin are able to inactivate ADAMTS13 proteolytically *in vitro* and plasmin mediated ADAMTS13 cleavage has been observed in TTP [26, 27]. Furthermore, abnormalities in the fibrinolysis pathway have been implicated in CTEPH [4]. Therefore, we used fibrinogen degradation products measured by D-dimer as a potential surrogate marker of plasmin and thrombin activity. D-dimer was increased in CTEPH (1.24 ± 1.25µg/mL) compared to healthy controls (0.538 ± 0.344µg/mL; $p=0.030$) (figure 3b). Specific ADAMTS13 activity was not correlated with D-dimer in the CTEPH (rho=0.0938, $p=0.761$) or healthy control groups (rho=-0.220, $p=0.313$) (figure 3c).

As the ADAMT13 reduction in TTP has an autoimmune mechanism, we investigated whether anti-ADAMTS13 autoantibodies are increased in CTEPH. There was no significant difference in anti-ADAMTS13 autoantibodies between CTEPH (92.3 ± 38.9%) and healthy controls (76.0 ± 16.5%; $p=0.180$) (supplementary figure S3).

We hypothesised that a decrease in ADAMTS13 antigen levels would result in reduced VWF cleavage and an increase in high multimeric VWF as occurs in TTP [28]. There was no difference in VWF multimeric size between CTEPH (VWF CBA:Ag ratio, 0.659 ± 0.537) and healthy controls (0.866 ± 0.494; $p=0.160$) (figure 3d).

Clinical phenotype associations with ADAMTS13 and VWF

In CTEPH, ADAMTS13 and VWF did not significantly correlate with markers of disease severity (6mwd, pulmonary vascular resistance or N-terminal pro b-type natriuretic peptide) (supplementary figure S4). Since inflammation has been associated with both CTEPH and abnormalities in the ADAMTS13-VWF axis we investigated if they were correlated [29, 30]. There were no correlations with blood markers of inflammation (C-reactive protein, white cell count, neutrophil and lymphocyte percentages) (supplementary figure S5).

ABO groups and ADAMTS13-VWF

There was no difference in ADAMTS13 antigen levels when stratified by simple genetic *ABO* groups (O, A, B, AB) (figure 4a) (p=0.443) or more comprehensive genetic *ABO* groups (supplementary figure S6a) (p=0.616).

VWF levels did not vary by *ABO* groups (figure 4b and supplementary figure S6b) however, when accounting for covariates (supplementary table S6), *ABO* group B had a higher VWF level (β=+51.3 (5.30 to 117)%, p<0.001) compared to group O. *ABO* group A also had a higher VWF level, although this was not statistically significant (β=+19.8 (-1.75 to 46.1)%, p<0.073). Patients with *ABO* group O had the lowest VWF levels within the CTEPH group (14.5 ± 13.0μg/mL), which was still significantly higher than healthy controls (8.45 ± 8.77μg/mL, p<0.001).

There was no difference in ADAMTS13 antigen levels between *ABO* groups, when accounting for covariates with multivariable linear regression.

Protein quantitative trait loci for ADAMTS13

There were 5 SNPs in the *ADAMTS13* ± 40kb region that were significantly associated with ADAMTS13 protein in a multivariable linear regression model (supplementary table S7). The most significant SNP (rs3739893, risk allele C, β=-37.1 (-48.1 to -23.8)%, p=3.78x10^{-06}) is a 5' untranslated region (UTR) variant in the *C9orf96* gene, which is ~8kb 5' of the *ADAMTS13* gene. In a model adjusted for age, sex and batch, the lead SNP (rs3739893) explained 7.7% of the variance in ADAMTS13 levels within the CTEPH group (supplementary table S8). In the whole CTEPH GWAS, the effect allele frequency for rs3739893 in CTEPH cases (0.0128) and healthy controls (0.0158) was not significantly different, which suggests that it is not associated with CTEPH disease risk.

Discussion

This is the first study demonstrating a marked reduction in plasma levels of ADAMTS13 in CTEPH. This is independent of pulmonary hypertension, disease severity or systemic inflammation. We confirm that VWF is increased in CTEPH and implicate dysregulation of the ADAMTS13-VWF axis in CTEPH pathobiology.

The magnitude of ADAMTS13 reduction and VWF increase in CTEPH is greater than observed in studies of ischaemic stroke using the same methodology [23]. Furthermore, levels are lower in CTEPH than CAD when considering the proportion of patients in the lowest ADAMTS13 quartile (65% versus 28% respectively) [13]. Additionally, the combination of decreased ADAMTS13 and increased VWF has a synergistic effect on the odds of CTEPH that is greater than observed in CAD or ischaemic stroke [23]. The more pronounced ADAMTS13-VWF dysregulation in CTEPH may reflect the larger surface area of

the vascular endothelium involved or alternatively that ADAMTS13-VWF dysregulation is more important in CTEPH pathobiology. Although ADAMTS13 is predominately produced by the liver, the contribution to plasma levels from vascular endothelial cells could be substantial given the large surface area of the lung vasculature [9]. A reciprocal relationship has previously been described between ADAMTS13 and VWF [31, 32]. The reduction in ADAMTS13 remained in our study when VWF levels were adjusted for, which is consistent with low ADAMTS13 being an independent risk factor in other thrombotic diseases [11].

Following pulmonary endarterectomy and removal of proximal thromboembolic material, the ADAMTS13-VWF axis remains dysregulated despite normalisation of haemodynamic parameters. Additionally, there is an equal perturbation of the axis in CTED, and no correlation with CTEPH disease severity, confirming the changes are not due to the presence of pulmonary hypertension or organised thrombus *per se*. Interestingly, there was no abnormality in ADAMTS13 levels in IPAH despite this group having a higher pulmonary vascular resistance, implying that distal pulmonary artery endothelial dysfunction and small vessel vasculopathy are not responsible [33]. Taken together, these observations demonstrate the dysregulation of the ADAMTS13-VWF axis in CTEPH pathogenesis.

Low ADAMTS13 could be driven by activation of fibrinolytic pathways and an increase in thrombin and/or plasmin, which have the potential to proteolytically inactivate ADAMTS13 [26]. D-dimer was raised in CTEPH though there was no correlation with ADAMTS13. High multimeric forms of VWF appear not to be increased in CTEPH. This is surprising, as increased high multimeric VWF occurs when ADAMTS13 is reduced in TTP and has been suggested to occur in ischaemic stroke and CAD [23, 28]. VWF multimeric size measured systemically may not reflect the local disease microenvironment in the pulmonary vascular endothelium. Additionally, the localised flow conditions that may be altered in CTEPH are

important in VWF structure, cleavage by ADAMTS13 and thrombus resolution [34]. The increase in specific ADAMTS13 activity in CTEPH may reflect an increased conformational activation of ADAMTS13 by its substrate VWF, due to the altered ADAMTS13:VWF ratio [35].

The *ABO* gene is located in close proximity and modest linkage disequilibrium with the ADAMTS13 gene, raising the possibility that *ABO* may influence the ADAMTS13-VWF axis. ABO blood groups are associated with CTEPH with an over-representation of the non-O groups [20]. Genetic variation in *ABO* has also been associated with ischaemic stroke, coronary artery disease and venous thromboembolism [36, 37]. The proposed mechanism of this association has been via VWF plasma levels, which are 25% higher in non-O individuals [38]. We demonstrate that VWF is increased in some non-O groups within CTEPH however, VWF is still significantly higher in the CTEPH O group compared with healthy controls. This implies that there are additional contributing causes for the increased VWF in CTEPH. Conversely, *ABO* is a pleiotropic locus and may have alternative functional effects in CTEPH including mediating pathways involved in inflammation and angiogenesis [25].

We identified a protein qualitative trait loci (rs3739893) in the *C9orf96* gene (~8kb 5' of the *ADAMTS13* gene) that is associated with ADAMTS13 protein levels and has been described in two previous studies [18, 24]. In a GWAS of ADAMTS13 antigen levels in a healthy cohort, this SNP is significantly associated with a similar effect size (β = -22.3%). Whilst this confirms that ADAMTS13 protein is genetically regulated, this SNP only accounts for a modest variance of ~8% in ADAMTS13 protein levels and is not primarily associated with CTEPH disease risk.

A strength of this study is that we investigated the ADAMTS13-VWF axis in a spectrum of thromboembolic disease from acute PE to chronic thromboembolic disease with and without pulmonary hypertension. Our study contains a large sample of well characterised CTEPH patients that have been extensively phenotyped in an experienced national CTEPH centre. ADAMTS13-VWF imbalance does not occur in PE when assessed by multivariable regression, although we were underpowered to detect smaller effect sizes. This raises an intriguing possibility, that there are differences in the ADAMTS13-VWF axis in the spectrum of thromboembolic disease. Future studies using robustly phenotyped PE cohorts to ascertain the presence and extent of residual perfusion defects, should investigate if the ADAMTS13-VWF axis varies in post-PE syndrome. Clinical prediction scores for CTEPH following acute PE do not currently incorporate biomarkers [39]. Determining if dysregulation of the ADAMTS13-VWF axis precedes the development of chronic thromboembolic pathology could inform CTEPH risk stratification.

In summary, we report that the ADAMTS13-VWF axis is dysregulated in CTEPH and this is unrelated to pulmonary hypertension, disease severity or systemic inflammation. This implicates the ADAMTS13-VWF axis in CTEPH pathogenesis.

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Tables

TABLE 1

Baseline group characteristics

	Healthy control	**CTEPH**	**CTED**	**IPAH**	**PE**
Subjects	68	208	35	30	28
Age, Years	49 ± 24	64 ±19	58 ± 27	64 ± 27	52 ± 26
Sex, Female	32 (47)	90 (43)	9 (26)	21 (70)	15 (54)
Ethnicity, Caucasian	53 (78)	180 (95)	28 (88)	26 (90)	13 (54)
WHO functional class					
1		4 (2)	6 (18)	5 (17)	
2		42 (21)	17 (50)	4 (13)	
3		151 (74)	11 (32)	21 (70)	
4		7 (3)	0 (0)	0 (0)	
6mwd, Metres		318 ± 176	366 ± 180	342 ± 244	
Pulmonary haemodynamics					
mPAP, mmHg		42 ± 18	21 ± 4	42 ± 17	
CI, L/min/m^2		2 ± 0.6	2.4 ± 0.6	1.7 ± 0.8	
PVR, dynes.s.cm^{-5}		639 ± 476	151 ± 71	808 ± 642	
Clinical blood tests					
Haemoglobin, g/L		140 ± 27	138 ± 16	142 ± 22	
Platelet count, x10^9		246 ± 82	200 ± 56	222 ± 77	
WCC, x10^9		7 ± 3	6.6 ± 2.1	6.9 ± 2.4	
Lymphocyte, %		25 ± 10	28 ± 13	18 ± 13	
Neutrophil, %		64 ± 14	59 ± 14	72 ± 14	

CRP, mg/L		5 ± 10	3 ± 3	3 ± 4	
NT-proBNP, pg/mL		592 ± 1576	113 ± 194	334 ± 695	
Smoking status					
Never		91 (47)	16 (50)	15 (52)	
Ex-smoker		87 (45)	13 (41)	11 (38)	
Current smoker		15 (8)	3 (9)	3 (10)	
Anticoagulation medication		137 (94)	15 (94)	30 (100)	

Data is presented as median ± interquartile range or number of patients (%). Percentages were calculated using the number of patients that data was available for as the denominator. 6mwd (6-minute walk distance), CI (cardiac index), mPAP (mean pulmonary arterial pressure), NT-proBNP (N-terminal pro b-type natriuretic peptide), PVR (pulmonary vascular resistance), WCC (white cell count).

Figures

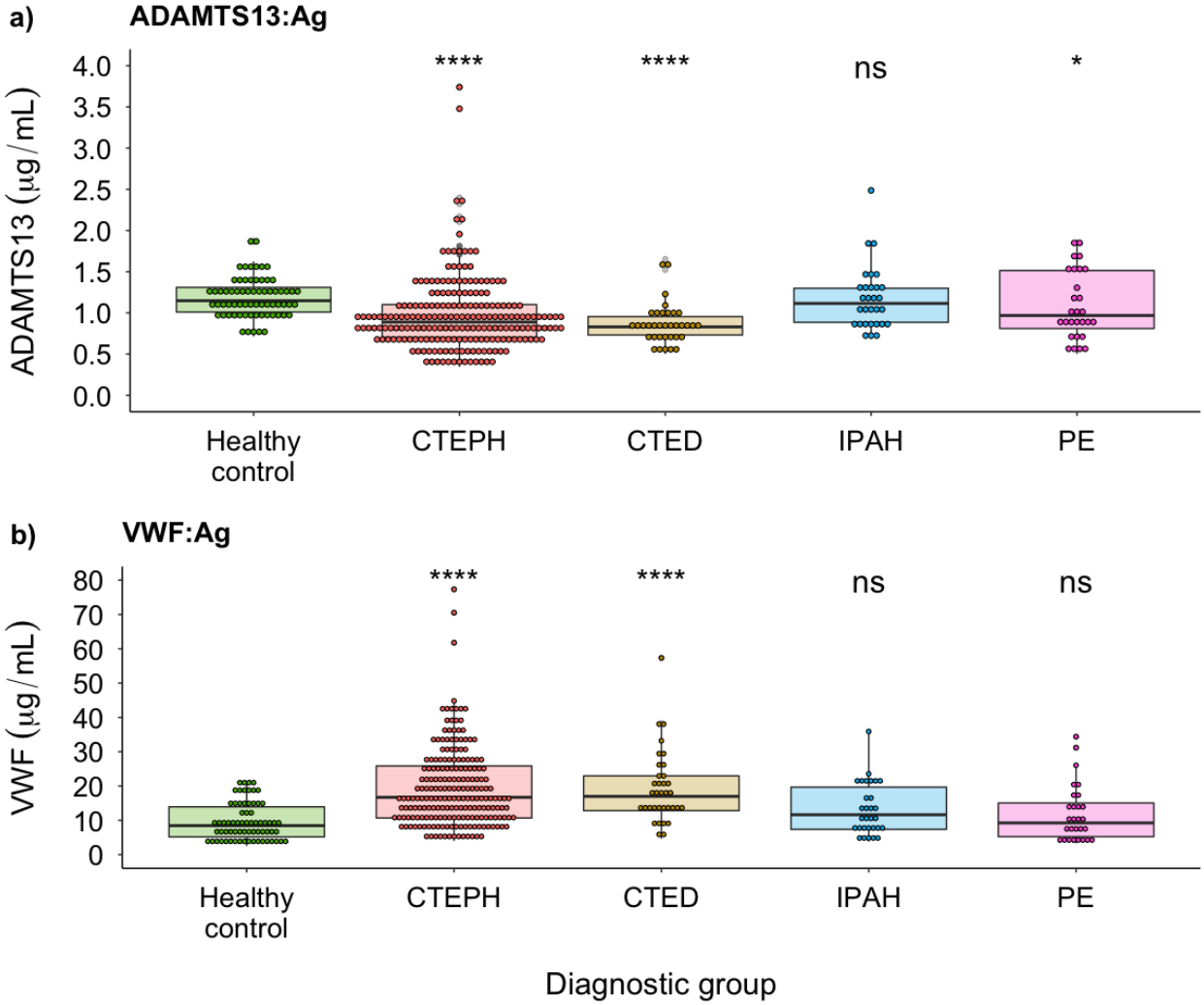

FIGURE 1

a) ADAMTS13 and b) VWF antigen (Ag) levels by diagnostic groups. Dunn's test with false discovery rate (FDR) adjustment was used to calculate *p*-values. ****: *p* <=0.0001, ***: *p*<=0.001, **: *p*<=0.01, *: *p*<=0.05, ns (not significant): *p*>0.05. Healthy control (n=68), CTEPH (chronic thromboembolic pulmonary hypertension, n=208), CTED (chronic thromboembolic disease, n=35), IPAH (idiopathic pulmonary arterial hypertension, n=28), PE (pulmonary embolism, n=28).

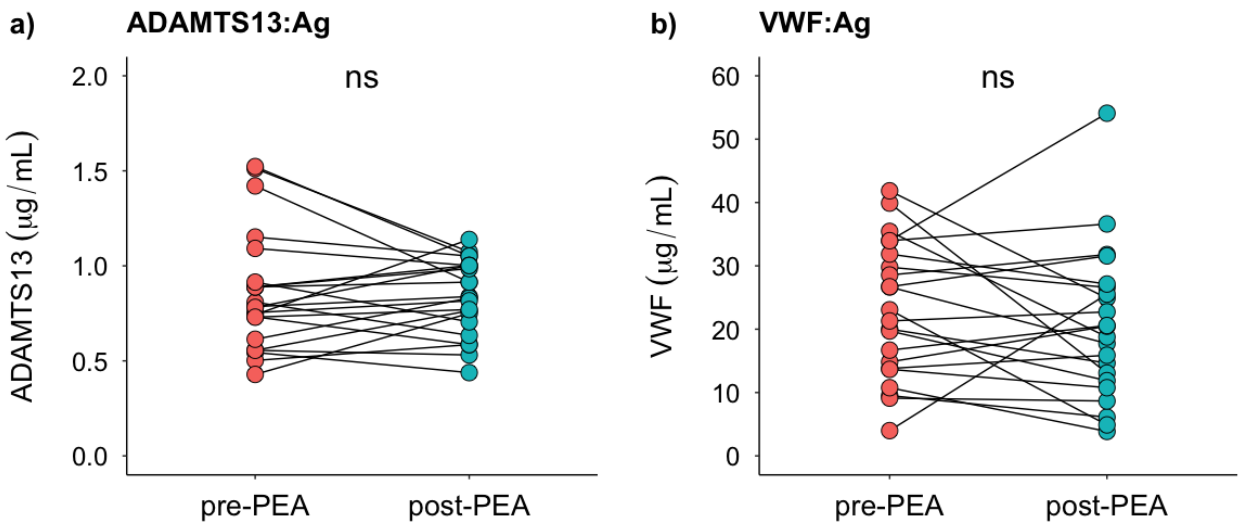

FIGURE 2

a) ADAMTS13 and b) VWF antigen levels pre- and post-pulmonary endarterectomy (n=22). Wilcoxon signed-rank test was used to calculate *p*-values.

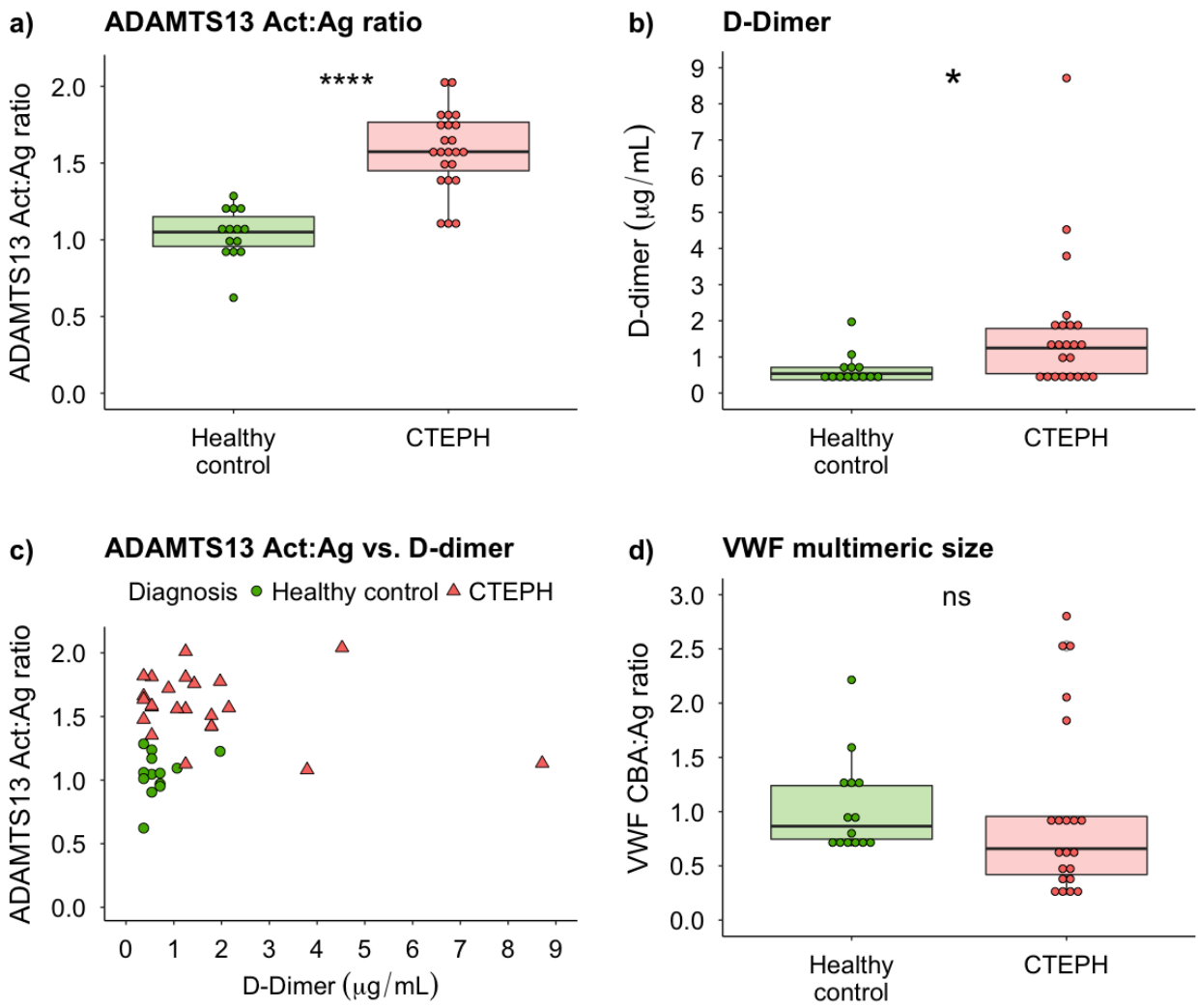

FIGURE 3

ADAMTS13 activity, D-dimer and VWF multimeric size in CTEPH and healthy controls. A subset of CTEPH patients (n=23) with the lowest ADAMTS13 antigen levels (below the first quartile of the CTEPH group) and healthy controls (n=14) were used for a-c). VWF multimeric size was measured in CTEPH (n=21) samples with the highest VWF antigen concentrations (above the third quartile of the CTEPH group) using the same healthy control subset and displayed in d). The Mann-Whitney *U* test was used to calculate group differences (a, b, d) and correlation was assessed with Spearman's rank correlation coefficients (c). A. Specific ADAMTS13 activity (Act:Ag ratio). b) D-dimer antigen levels. c) Specific ADAMTS13 activity and D-dimer antigen correlation. Healthy control correlation: rho=0.0938, *p*=0.761;

CTEPH correlation: rho=-0.220, *p*=0.313. d) VWF multimeric size (VWF Collagen binding assay:Antigen ratio).

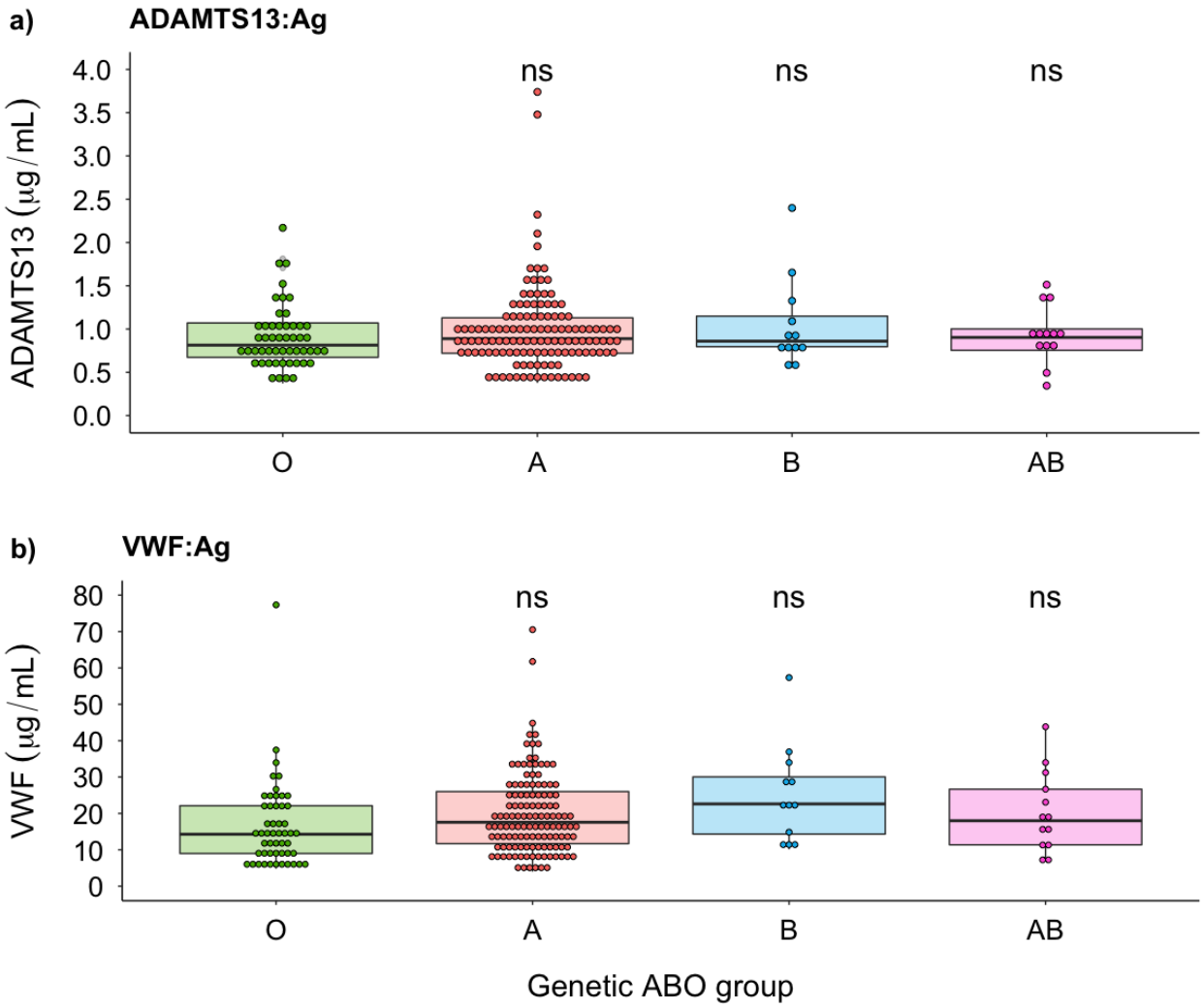

FIGURE 4

ADAMTS13 and VWF antigen levels by *ABO* genetic groups. CTEPH (n=182) and CTED (n=22) patients with genotypes and protein levels available (in n=3 a genetic ABO group could not be inferred) were included. Dunn's test with FDR adjustment was used to calculate *p*-values. Numbers in each group: O = 51, A = 128, B = 12, AB = 13.

SUPPLEMENTAL MATERIAL

Supplementary Methods

Sample size calculations

The effect size for a difference in ADAMTS13 between other thrombotic diseases and healthy controls was calculated from a study of myocardial infarction and stroke (Andersson). An estimated 142 individuals would be required for a small-medium effect size at a power of 80% (supplementary figure S7a). However, to establish what effect size is appropriate for CTEPH a pilot study was performed in 2016 to refine the sample size estimates. ADAMTS13 plasma antigen levels were measured in 94 CTEPH patients and 29 healthy controls. This confirmed a larger effect size between CTEPH and healthy controls than had previously been reported for other thrombotic diseases. Consequently, an estimated 30 individuals (per group) would be required for a large effect size in ADAMTS13 with 80% power (supplementary figure S7b). Furthermore, as CTEPH, CTED and IPAH are rare diseases with limited sample numbers available, the study was powered for larger effect sizes.

Study samples and participants

The 208 CTEPH patients represented 40% (208/514) of all the patients diagnosed with CTEPH during the same period at Royal Papworth Hospital, UK. The healthy controls (n=68) were volunteers bio-banked from Royal Papworth Hospital and Hammersmith Hospital, UK. Propensity matching for age and sex was explored for CTEPH, healthy controls and disease comparators (CTED, IPAH and PE). However, given the known demographic differences across these disease groups and limited sample availability for rare diseases, it was not possible to fully propensity match. Therefore, healthy

controls and disease comparators were selected for the closest possible age- and sex- matching to the CTEPH group. The closest matched (age and sex) CTED patients (n=35) from Royal Papworth Hospital with available bio-banked samples were selected. The definition used for CTED patients has been previously described [1]. IPAH patients (n=30) from Royal Papworth Hospital were also selected for the closest age/sex matching and additionally all IPAH patients were matched for anticoagulation therapy usage but had not had a venous thromboembolism. The PE group were sampled from a specialist PE follow-up service (Hammersmith Hospital) at a median of 220 (interquartile range (IQR) 218) days following an acute PE. All consecutive patients (n=28) with bio-banked samples available (November 2013 to October 2014) were included in the study.

As the CTEPH, healthy control and disease comparator groups could not be fully matched for age and sex, these variables were included in multivariable linear models described in the statistical analysis section.

ADAMTS13 and VWF plasma concentrations

Plasma ethylenediaminetetraacetic acid (EDTA) samples were used to measure ADAMTS13 and VWF antigen (Ag) levels by enzyme-linked immunosorbent assays (ELISA). Normal human control plasma (Technoclone, Vienna, Austria) with known concentrations of ADAMTS13 and VWF was used as the reference [2].

ADAMTS13

ADAMTS13 plasma antigen levels were quantified using a polyclonal rabbit anti-ADAMTS13 antibody (5μg/mL, anti-TSP2–4 depleted) as previously described [2, 3]. The antibody was immobilised in 96-

well microplates (Nunc, Rochester, USA) in 50mM carbonate buffer, pH 9.6 at 4°C overnight. Washes were performed with phosphate buffered saline (PBS) + 0.1% Tween-20 (PBST), and this was repeated between each step. Wells were blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour. Plasma samples were diluted 1:20 using 1% BSA in PBS and added to the wells in duplicate for 2 hours. A standard curve of 0-126 ng/mL was made with normal human control plasma (NHP) (Technoclone, Vienna, Austria). Bound ADAMTS13 was detected with biotinylated anti-TSP2–4 polyclonal antibody for 2 hours followed by incubation of wells with streptavidin-horseradish peroxidase (HRP) (GE Healthcare, UK) for 1 hour. Plates were developed with a peroxidase substrate (o-phenylenediamine dihydrochloride (OPD); Sigma-Aldrich, Darmstadt, Germany) for 5 minutes and the reaction was stopped with 65µL/well of 2.5M H_2SO. Absorbance was read at 492nm (FLUOstar Omega plate reader, BMG Labtech). ADAMTS13 concentrations were obtained by interpolating from the standards fitted with a four-parameter logistic curve. The intra- and inter-assay coefficients of variation were 8 and 12% respectively.

To enable a comparison with other published thrombotic diseases, each ADAMTS13 plasma antigen level was divided by the median of the healthy control group and expressed as a percentage. The CTEPH group was then divided into quartiles of the ADAMTS13 distribution of the healthy control group. The odds ratios for a combination of ADAMTS13 and VWF antigen levels. The quartile thresholds were used to stratify CTEPH patients and healthy controls into groups using a combination of ADAMTS13 and VWF levels. Odds ratios for the different groups were then assessed using logistic regression adjusted for age, sex, ethnicity and experimental batch.

VWF

VWF plasma antigen levels were quantified in a similar well-described manner using a polyclonal rabbit anti-VWF antibody (3.1 µg/mL; Dako, Santa Clara, USA) [3]. After overnight antibody immobilisation and washes, wells were blocked with 1% BSA in PBST for 1 hour. Plasma samples were diluted 1:400 in PBST 1% BSA and a standard curve of 0-125 ng/L was made with NHP. VWF was detected with 1.1µg/mL Polyclonal Rabbit Anti-Human VWF/HRP (Dako) followed by plate development with OPD for 3 minutes. The intra- and inter-assay coefficients of variation were 5 and 8% respectively.

The ADAMTS13 and VWF ELISAs were performed for all groups in 2016 (batch1) and additional CTEPH samples (n=115) were included in 2017 (batch2). Batch variation in ADAMTS13 and VWF was adjusted using replicate samples (ADAMTS13: n=24, VWF: n=12) and additionally batch was included as a covariate in multivariable linear regression models.

ADAMTS13 activity, D-dimer, anti-ADAMTS13 autoantibodies and VWF multimeric size

Additional experiments were performed on a subset of the CTEPH (n=23) and healthy control (n=14) groups to identify potential mechanisms for any dysregulation of the ADAMTS13-VWF axis. Plasma lithium heparin samples were used to measure ADAMTS13 activity, D-dimer concentrations and anti-ADAMTS13 autoantibodies. The CTEPH sample subset were those with the lowest ADAMTS13 antigen levels (below the first quartile of the CTEPH group) and the healthy controls were those with ADAMTS13 antigen levels closest to the median of the control group. An estimate of VWF multimeric size was made by measuring VWF collagen binding (VWF:CBA) and comparing this with VWF antigen levels in the CTEPH (n=21) samples with the highest VWF antigen concentrations (above the third quartile of the CTEPH group) and the same healthy control subset.

ADAMTS13 Activity

ADAMTS13 activity was measured with a fluorescence resonance energy transfer (FRETS) assay using a short synthetic VWF peptide (VWF73: PeptaNova, Sandhausen, Germany) containing the ADAMTS13 cleavage site for VWF [4]. Plasma samples and NHP were diluted to 1:10 in reaction buffer (5 mM Bis-Tris, 25 mM $CaCl_2$ and 0.005% Tween-20 at pH 6.0) in 96-well plates (Nunc, Rochester, USA). FRETS-VWF73 substrate (an equal volume of 4μM) was added and fluorescence was recorded at 1-minute intervals for 1 hour (FLUOstar Omega plate reader) to monitor substrate proteolysis. Assays were repeated 3 times to obtain the mean fluorescence and ADAMTS13 activity was normalised to NHP, which was defined as 100%.

D-Dimer plasma levels

Plasma D-Dimer levels were quantified using an ELISA kit (ab196269, abcam, Cambridge, USA) according to the manufacturer's instructions. Plasma lithium heparin samples from CTEPH patients and healthy controls were used at a dilution of 1:1000.

Anti-ADAMTS13 autoantibodies

ADAMTS13 was immobilised directly on Maxisorp plates (Nunc) in carbonate buffer overnight at 4°C. After blocking with 1% BSA, patient plasma samples (1:10 dilution in 1% BSA) were applied to the plates for 1 hour. Bound antibodies were detected and quantified using a rabbit anti-human IgG polyclonal antibody conjugated with HRP. A standard curve of normal human plasma, containing known concentrations of the purified, patient derived anti-ADAMTS13 monoclonal antibody (II-1),

was used as a positive control for the binding and detection of autoantibody [5]. Anti-ADAMTS13 autoantibodies were normalised to NHP, which was defined as 100%.

VWF multimeric size

VWF multimeric size was evaluated with a collagen binding assay (CBA) which utilises the increased collagen binding of higher multimeric VWF. Human type III placental collagen (5µg/mL) was immobilised in 96-well microplates plates (Nunc) in 50mM carbonate buffer, pH 9.6 at 4°C overnight. After washes with PBST, wells were blocked with 2% BSA in PBST for 1 hour. Plasma lithium heparin samples were diluted 1:100 in PBST 1% BSA and a standard curve of 0-1000ng/mL was made with NHP. VWF was detected with 1.1µg/mL polyclonal rabbit anti-human VWF/HRP (Dako) followed by plate development with OPD for 3 minutes. Collagen binding is reported as a ratio over the total plasma VWF antigen.

Clinical phenotype data

Phenotype data for the CTEPH, CTED and IPAH groups were recorded closest to the time of diagnosis and pre-operatively for the CTEPH and CTED patients undergoing PEA. This included demographics, haemodynamics, WHO functional class, 6-minute walk distance (6mwd), clinical blood tests, smoking history and anticoagulation therapy usage. Additionally, post-operative haemodynamics were recorded within 1 year of surgery for the CTEPH and CTED patients that underwent PEA, as part of routine care. Haemodynamics were evaluated by right heart catheterisation according to international guidelines and PEA was performed as previously described [6, 7]. The PE group had phenotype data recorded at a follow-up visit (median 220 days) after their acute PE, which also included a ventilation perfusion (VQ) scan to assess residual perfusion defects.

The relationship between the ADAMTS13-VWF axis and the amount of chronic thromboembolism was not evaluated as a validated radiological classification system does not exist.

Genotype data and genome-wide association study

To date, 1457 Caucasian CTEPH patients have been recruited from 8 European and US specialist pulmonary hypertension centres. The participating centres were:

- Royal Papworth Hospital, Cambridge, UK
- Hammersmith Hospital, London, UK
- University of California, San Diego, USA
- Hospital Clínic - IDIBAPS-CIBER Enfermedades Respiratorias, University of Barcelona, Spain
- KU Leuven – University of Leuven
- Kerckhoff Heart and Lung Centre, Bad Nauheim, Germany
- VU University Medical Centre, Amsterdam, Netherlands
- Medical University, Vienna, Austria

They have been compared to 1536 healthy Caucasian controls from the Wellcome Trust Case Control Consortium (WTCCC) [8].

Genomic DNA was extracted and quantified from whole blood or buffy coat fractions (LGC, Hoddesdon, Herts, UK). Genotyping was performed using the Illumina HumanOmniExpressExome-8 v1.2 BeadChip containing 964,193 single-nucleotide polymorphism (SNP) markers (Kings College,

London, UK). The Genome Reference Consortium human genome (build 37) (GRCh37) was used for genomic positions. Three batches have been genotyped from 2014-16 (batch1: 2014, batch2: 2015, batch3: 2016), with all WTCCC controls genotyped in batch1.

Each batch of micro-array intensity data was normalised, clustered and called independently using Illumina GenomeStudio [9]. Samples containing more than 1% of missing genotypes were removed and SNPs were then re-clustered to remove confounding from differential SNP exclusions between batches. SNPs with a low GenTrain score (<0.7) or clustering separation score (<0.5) were excluded [9, 10].

Sample/SNP QC

Divergent ancestry was assessed using principal component analysis (PCA). Each batch was merged with the 1000 Genomes data using an intersecting set of SNPs [11]. A robust set of independent SNPs (n=30,609) was used for PCA, selected by the following criteria: genotype missingness < 5%, SNPs in Hardy-Weinberg equilibrium (HWE) (p>1x10^{-5}), minor allele frequency (MAF) > 5%, non-independent SNPs (pairwise R^2 < 0.2). Furthermore, SNPs in several regions with long-range linkage disequilibrium (LD) were excluded [12]. PCA against all populations in the 1000 genomes data was conducted and samples not clustering with European population were excluded. A second PCA was then performed with the remaining samples against European samples only, and outlying samples were also excluded. Thresholds were set by visual inspection of plots.

Additionally, individual samples were removed due to: outlying heterozygosity (3 standard deviations from the mean), duplicates or relatedness (Identity by descent > 0.1875) and missing

genotypes (>1%; as described). SNP markers were removed due to: missing genotypes (>1%), deviation from HWE ($p<1x10^{-6}$), differential missingness rate between cases and controls ($p<1x10^{-5}$). and multi-allelic SNPs.

The 3 batches were then merged using an intersecting set of SNPs and further PCA was performed using the robust SNP set previously used for divergent ancestry quality control, to check for batch and recruiting centre effects.

Phasing/imputation

After quality control exclusions there were 1250 CTEPH cases, 1492 healthy controls and 915,999 SNPs. Phasing and imputation were performed using EAGLE 2 (v2.0.5) and positional Burrows–Wheeler transform (PBWT) software via the Sanger imputation service (https://imputation.sanger.ac.uk/) [13, 14]. The reference panel was the Haplotype Reference Consortium (release 1.1), containing 39 million biallelic SNPs from 32,470 individuals [15]. Following imputation, SNPs were excluded if they had a low minor allele frequency (<1%) or if they were poorly imputed (info score < 0.5), with 7,675,738 SNPs remaining for association testing.

208 CTEPH patients with ADAMTS/VWF antigen levels and 28 patients with CTED were also included in the CTEPH GWAS. Genotypes were available for 207 (187 CTEPH; 23 CTED) after GWAS quality control exclusions. Matched genotypes and ADAMTS / VWF antigen levels were not available for the healthy control, IPAH or PE groups.

Genetic *ABO* groups

The tagging SNPs used to reconstruct the genetic *ABO* groups A1, A2, B and O from phased haplotypes were: rs8176746, rs8176704, rs687289 and rs507666 [16]. The genetic *ABO* groups were compared to the available ABO antigen groups measured by serology (n=1490 Healthy control group) to confirm the accuracy of this method (98% concordance; n=29 were unable to be classified). The 10 genetic *ABO* groups were converted into A, B, AB and O groups using the following criteria: A = A1A1, A1A2, A2A2, A1O, A2O; B = BB, BO; AB = A1B, A2B; O = OO.

Protein quantitative trait loci

The post-imputation allelic dosages (0-2) of individual SNPs in the *ADAMTS13* gene ± 40kb flanking region (n=396 variants) were tested against log transformed ADAMTS13 protein levels (dependent variable) and adjusted for age, sex and ADAMTS13 plasma antigen experimental batch. This region included the ADAMTS13 cis-pQTLs that have previously been described [17-19]. A Bonferroni *p*-value threshold $<1.26 \times 10^{-4}$ (0.05/396 variants) was used to denote statistical significance. Partitioning of the variance explained by each variable within the models was performed by averaging over orders using the R package 'relaimpo' [20].

Linkage disequilibrium was quantified from 1000 Genomes project data using LDlink (https://analysistools.nci.nih.gov/LDlink/, accessed 22/01/2018) in all European populations [21].

Statistical analysis

The differences in categorical variables between groups were assessed using Chi-squared or Fisher's exact test. The differences in continuous variables were assessed using the Mann-Whitney *U* test and the Kruskal-Wallis test.

Post-hoc pairwise diagnostic group comparisons were performed using Dunn's test with false discovery rate (FDR) adjustment for multiple testing. For matched values pre- and post-PEA Wilcoxon signed-rank test was used. *P*-values are reported to 3 decimal places and experimental data are reported to 3 significant figures. Data averages are described as median ± interquartile range unless specified.

Multivariable linear regression was performed using log-transformed ADAMTS13 or VWF protein levels as the dependent (outcome) variables after assessing log-likelihoods using the Box-Cox power transformation. Log-transformed ADAMTS13 and VWF were used in all multivariable linear regression models (supplementary tables S3, S4, S6, S7 and S8). The models were adjusted for age, sex, experimental batch (supplementary tables S7 and S8) and additionally ethnicity (supplementary tables S3, S4 and S6), VWF (supplementary tables S3 and S8) and 5 ancestry informative principal components (supplementary tables S7 and S8). The β coefficients and confidence intervals are presented as percentage change $((\exp^{\beta}-1) \times 100)$ to enable clinical interpretation of the log-transformed values. Models were checked for normality of residuals, homoscedasticity and multicollinearity (variance inflation factor), with additional checks performed using the R package 'gvlma' [22]. We investigated interacting effects between the variables that were used in supplementary tables S4 and S5. The significant ($p<0.05$) and informative interactions were included in additional multivariable linear regression models (supplementary table S9).

Spearman's rank correlation coefficients were used to describe associations between ADAMTS13 or VWF protein levels and clinical phenotypes associated with disease severity (pulmonary vascular resistance (PVR), 6mwd and N-terminal pro b-type natriuretic peptide (NT-proBNP)) and blood markers of inflammation (white cell count (WCC), C-reactive protein (CRP), neutrophil and lymphocyte percentages). *P*-values from correlation testing were corrected for multiple testing using false discovery rate (FDR) adjustment.

Software

The analysis was performed using the following software: Illumina Genomestudio [9], PLINK (v1.90beta) [23], bcftools (v1.4.1) [24], LDLink [21], R (3.4.3) [25] and RStudio (1.1.414) [26]. The R packages used included: MASS [27], coin [28], gvlma [22], PMCMR [29], SNPRelate [30], relaimpo [20], jtools [31], forestmodel [32] and the tidyverse suite [33].

Supplementary Results

Data missingness

All individuals with CTEPH, CTED, PE, IPAH and healthy controls included in the analyses had ADAMTS13 and VWF antigen levels available (missingness: n=0/369, 0%). The core variables used in multivariable linear models had the following missingness: there was no missing data for age and sex (n=0/369, 0% missing) and ethnicity was missing in 7% (n=26/369). Data missingness for other variables are displayed in the figure and table legends. Analyses were performed using complete data. As missingness was low in core variables, imputation was not performed and is unlikely to have altered the results.

ADAMTS13 plasma concentrations

We were 95% powered to detect the difference in ADAMTS13 levels between CTEPH and healthy controls (healthy controls(n1)=68, CTEPH(n2)=208, effect size=0.5, two-sided t-test of means) and 99% powered to detect the difference between the CTED group and healthy controls (healthy controls(n1)=68, CTED (n2)=35, effect size=1.12, two-sided t-test of means).

When the analysis is confined to batch 1 samples with a closer 1:1 matching between CTEPH (n=94) and healthy controls (n=68), the markedly reduced ADAMTS13 in the CTEPH group remains in a multivariable linear regression model (β (95% CI) (% change) = -24.8 (-32.5, -16.2)%, p<0.001).

We investigated interaction effects for the variables used in supplementary tables S3 and S4. For ADAMTS13 antigen levels, there was a significant interaction between age and CTEPH (p=0.007) and additionally between age and sex (p=0.019) (supplementary table S9 and supplementary figure S8). This suggests that the reduction in ADAMTS13 levels with increasing age is of more relevance within the CTEPH group. Consideration of the interaction terms is most relevant for the extreme values. For example, there is less difference between a 30-year-old Caucasian female with CTEPH and a 30-year-old Caucasian male healthy control (predicted ADAMTS13: 1.07 vs. 1.28 μg/mL, 16% reduction) than an 80-year-old Caucasian male with CTEPH and an 80-year-old Caucasian female healthy control (0.688 vs. 1.18 μg/mL, 42% reduction). There were no significant interaction effects for a separate model of VWF antigen levels using the variables in supplementary table 4.

Converting ADAMTS13 antigen levels to a percentage of the median value of the healthy control group (set at 100%) allowed comparisons with thrombotic diseases in other studies. The majority of the CTEPH group (n=136, 65%) were in the lowest quartile (Q1<88% ADAMTS13) (supplementary table S10).

There was a modest negative correlation between ADAMTS13 and VWF plasma levels in CTEPH (rho = -0.164, p=0.018) but they were not correlated in healthy controls (rho = -0.0622, p=0.614) (supplementary figure S9). Furthermore, adjusting the multivariable linear regression model of ADAMTS13 antigen levels (supplementary table S3) by VWF had minimal effect, suggesting that the associations are not mediated by VWF antigen levels.

ADAMTS13 and VWF: Pre- and post-pulmonary endarterectomy

ADAMTS13 and VWF levels did not change pre- and post-PEA and this also applied when limited to patients with normal post-operative haemodynamics (mPAP <25mmHg) (n=7, ADAMTS13: *p*=0.742, VWF: *p*=0.195).

ADAMTS13 activity, D-dimer, anti-ADAMTS13 autoantibodies and VWF multimeric size measurements

ADAMTS13 activity and D-dimer concentrations were measured in a subset of patients with CTEPH (n=23) with the lowest ADAMTS13 protein concentrations (below the 1st Quartile of the CTEPH group) (0.556 ± 0.130 μg/mL) and compared to a subset of healthy controls (n=14, ADAMTS13: 1.03 ± 0.284 μg/mL).

ADAMTS13 activity

ADAMTS13 activity that is not adjusted for ADAMTS13 antigen levels is reduced (supplementary figure S10a). The increased specific ADAMTS13 activity (Act:Ag ratio) reflects the greater decrease in ADAMTS13:Ag compared to ADAMTS13:Act. Specific ADAMTS13 activity (Act:Ag) is not correlated with VWF:Ag in either CTEPH or healthy controls (supplementary figure S10b).

VWF multimeric size

The CTEPH (n=21) and healthy control subset (n=14) that were used to assess VWF multimeric size had a VWF plasma antigen level of 32.5 ± 6.80 μg/mL and 9.97 ± 4.99 μg/mL respectively. VWF:CBA was not correlated with ADAMTS13:Ag in CTEPH or healthy controls (supplementary figure S10c).

VWF:CBA was correlated with VWF:Ag in healthy controls but not in CTEPH (supplementary figure S10d).

Clinical phenotype associations with ADAMTS13 and VWF

There were no correlations with blood markers of inflammation (CRP, WCC, neutrophil and lymphocyte %) (supplementary figure S5). including when confining the analysis to samples that were taken on the same day as ADAMTS13 and VWF sampling (n=81, for WCC, neutrophil and lymphocyte %; n= 77 for CRP).

As proximal operable CTEPH has different risk associations to distal inoperable CTEPH and thus potentially different pathophysiological mechanisms we investigated the disease sub-types [34]. There was no difference in ADAMTS13 (p=0.070) or VWF (p=0.253) between the different sub-diagnostic categories of CTEPH (supplementary figures S11a and S11b). Furthermore, there was no difference in ADAMTS13 (p=0.366) or VWF (p=0.078) in those with and without post-operative residual pulmonary hypertension (mPAP $\geq$ 25mmHg) (n=83, 63%), which is a potential marker of distal vasculopathy (supplementary figures S11c and S11d) [35].

CTEPH is a potential severe consequence of acute PE, however there are a spectrum of changes following PE (post-PE syndrome) that may have differing pathobiology [36]. We evaluated whether there was a difference in ADAMTS13 or VWF antigen levels depending on the degree of post-PE perfusion defects on available VQ scans (n=20). There was no difference in ADAMTS13 (p=0.812) or VWF (p=0.678) levels in those that had residual perfusion defects post-PE (n=12) compared with those with no perfusion defects (n=8) (supplementary figures S12a and S12b). Furthermore, there

was no difference in ADAMTS13 (p=0.938) or VWF (p=0.427) levels when the PE group was stratified into provoked PE (n=8) and idiopathic PE (n=12) (supplementary figures S12c and S12d).

Dysregulation of the ADAMTS13-VWF axis is associated with stroke and myocardial infarction. Where co-morbidities were available for the CTEPH group, 5/74 (6%) had cerebrovascular disease and 20/124 (14%) had coronary artery disease. This is unlikely to have been a major confounder in the CTEPH group.

***ABO* groups and ADAMTS13-VWF**

Reconstructing genetic *ABO* groups allowed us to explore more complex associations within the *ABO* subgroups. Whilst the A1 and A2 groups would be classified as non-O on serological testing, they have been associated with different effects on VWF levels and VTE risk [37].

The small sample size of some non-O A*BO* groups (n: B=12, AB=13) limits the power to detect associations of smaller effect.

Protein quantitative trait loci for ADAMTS13

In a model adjusted for age, sex and batch, the lead SNP (rs3739893) explained 7.7% of the variance in ADAMTS13 levels within the CTEPH group (supplementary table S8). However, as only 10 CTEPH patients had the rs3739893 effect allele, this accounts for a small proportion of the ADAMTS13 antigen level reduction observed in CTEPH.

The 4 other significantly associated SNPs were highly correlated with the lead SNP (R^2=0.91-1.00, p<0.001). Additional analysis correcting for the first 5 ancestry informative principal components and VWF antigen levels did not alter the results.

In the linear regression model of ADAMTS13 antigen levels and rs3739893, the most variance in ADAMTS13 antigen levels was attributable to age (16%) (supplementary table S7), which is higher than reported in healthy cohorts [18]. In the CTEPH GWAS the effect allele frequency for rs3739893 was 0.0128 in CTEPH cases and 0.0158 in healthy controls and this was similar to a European (non-Finnish) reference population in gnomAD (0.0160) (http://gnomad.broadinstitute.org/, accessed Feb 2018). The results were unchanged when the analysis was confined to the CTEPH group.

Supplementary Figures

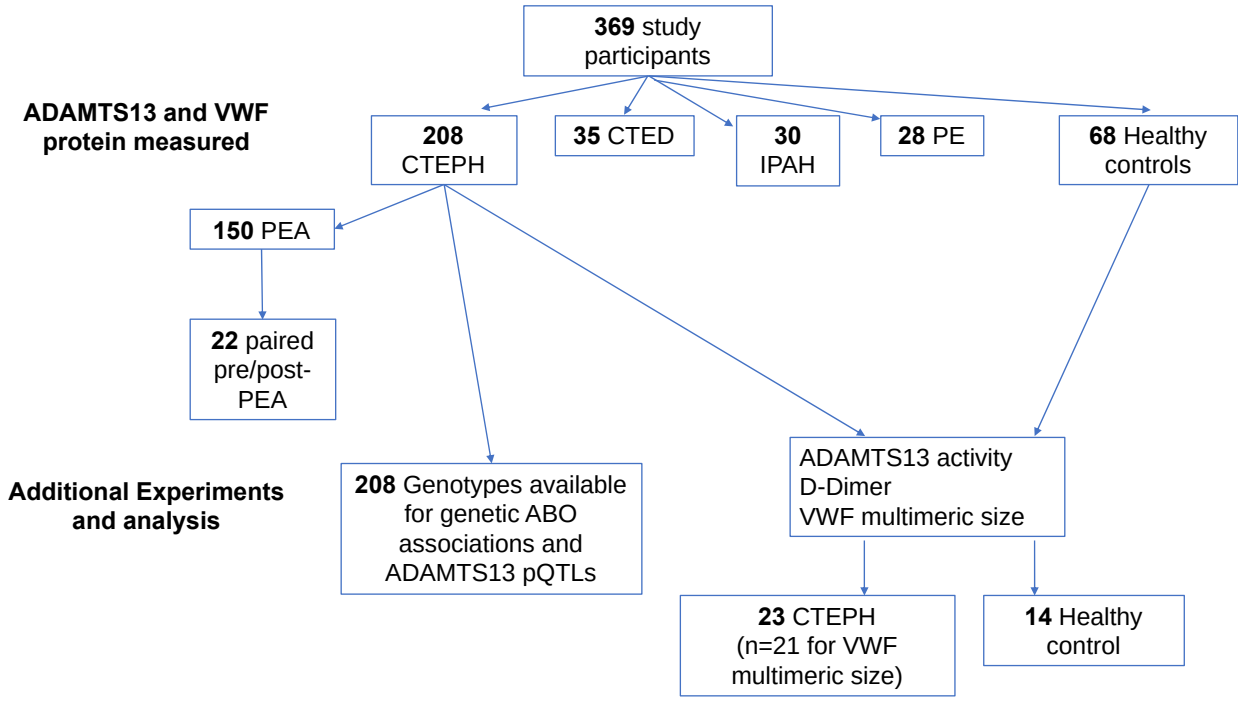

FIGURE S1

Flow chart of study design and study participant numbers. PEA (pulmonary endarterectomy), pQTL (protein quantitative trait loci). The number in each group are shown in bold.

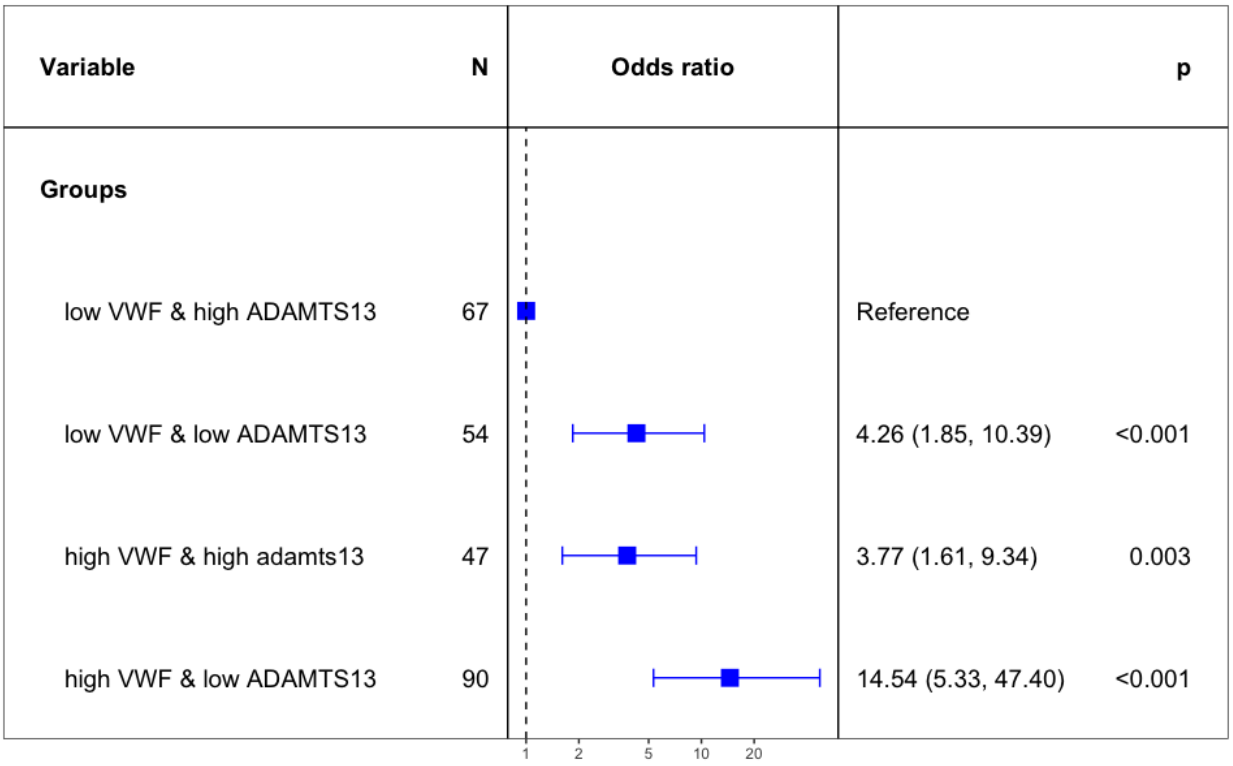

FIGURE S2

The odds ratios of CTEPH in relation to healthy controls for combined ADAMTS13 and VWF groups. The ADAMTS13 and VWF group ORs are adjusted for age, sex, ethnicity and batch in a logistic regression model. N represents the total for both the CTEPH and healthy control groups. Threshold criteria and n (%) within the CTEPH and healthy control groups are shown in supplementary table S5. Forest plot generated with the R package 'forestmodel' [32].

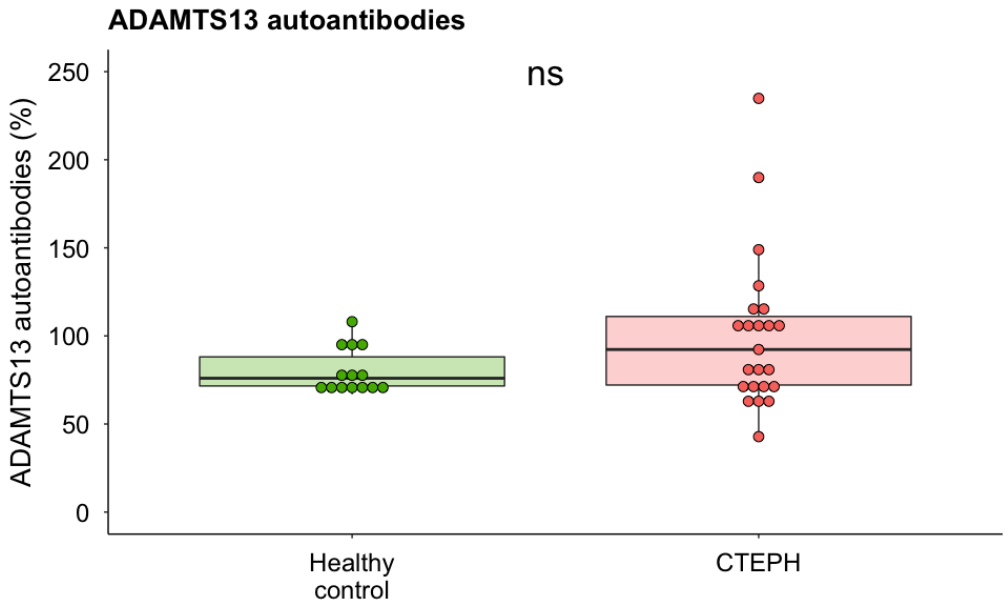

FIGURE S3

Anti-ADAMTS13 autoantibody percentage in CTEPH and healthy controls. Anti-ADAMTS13 autoantibodies were normalised to normal human control plasma, which was defined as 100%

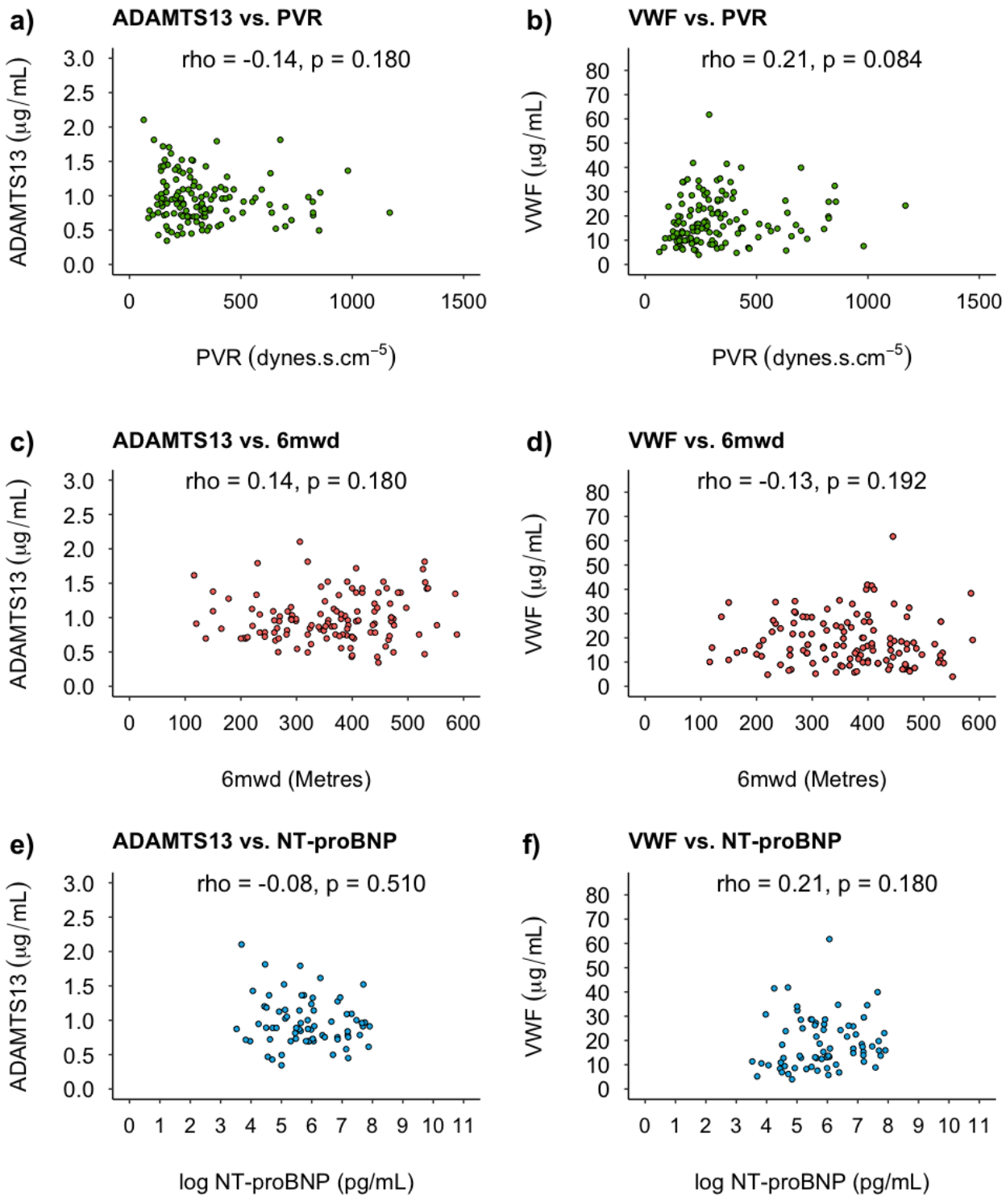

FIGURE S4

Correlation of ADAMTS13 and VWF antigen levels with markers of disease severity in CTEPH. Correlation was assessed by Spearman's rank test. *P*-values adjusted for the number of statistical tests performed using FDR correction. 6mwd (6-minute walk distance), NT-proBNP (N-terminal pro

b-type natriuretic peptide), PVR (pulmonary vascular resistance). NT-proBNP log-transformed to improve visualisation. Numbers in each group: PVR = 169, 6mwd = 165, NT-proBNP = 144.

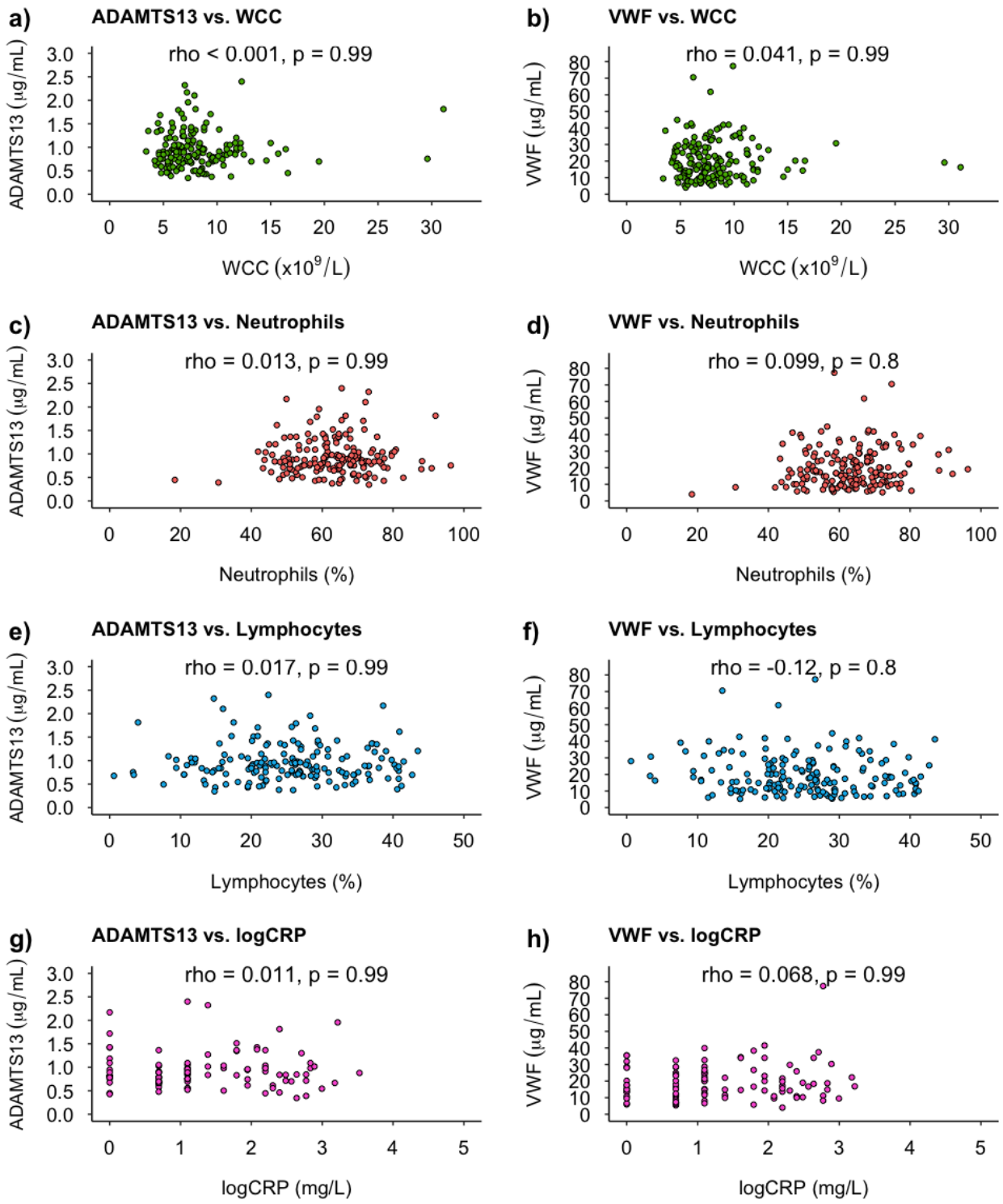

FIGURE S5

Correlation of ADAMTS13 and VWF antigen levels with blood markers of inflammation. Correlation was assessed by Spearman's rank test. *P*-values adjusted for the number of statistical tests performed using FDR correction. CRP log-transformed to improve visualisation. CRP (C-reactive

protein), WCC (white cell count). Numbers in each group: WCC = 169, Neutrophils = 169, Lymphocytes = 168, CRP = 95.

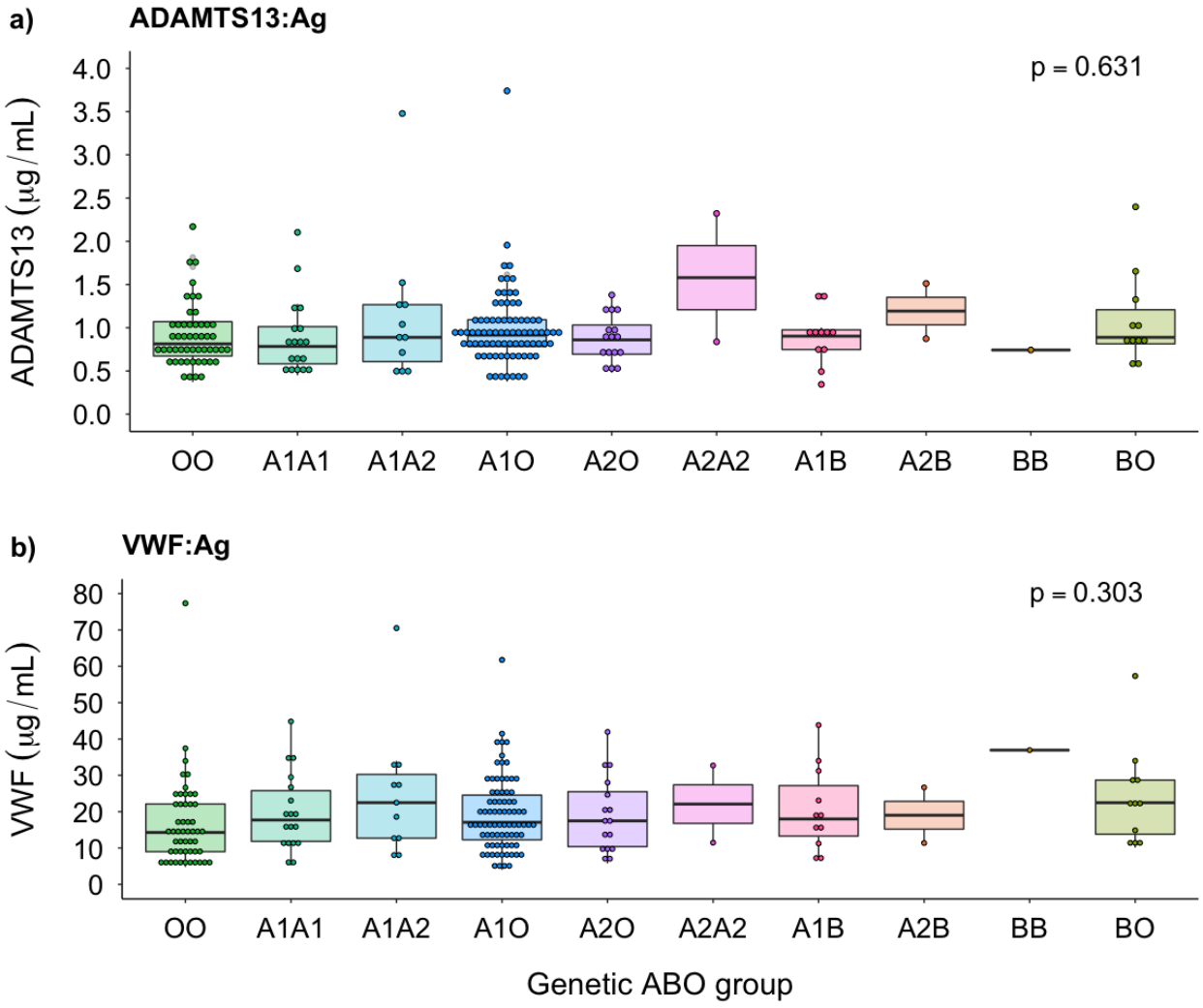

FIGURE S6

ADAMT13 and VWF antigen levels by comprehensive *ABO* genetic groups. The group differences were assessed using the Kruskal-Wallis test. Numbers in each group: OO = 51, A1A1 = 18, A1A2 = 11, A1B = 11, A1O = 81, A2A2 = 2, A2B = 2, A2O = 16, BB = 1, BO = 11.

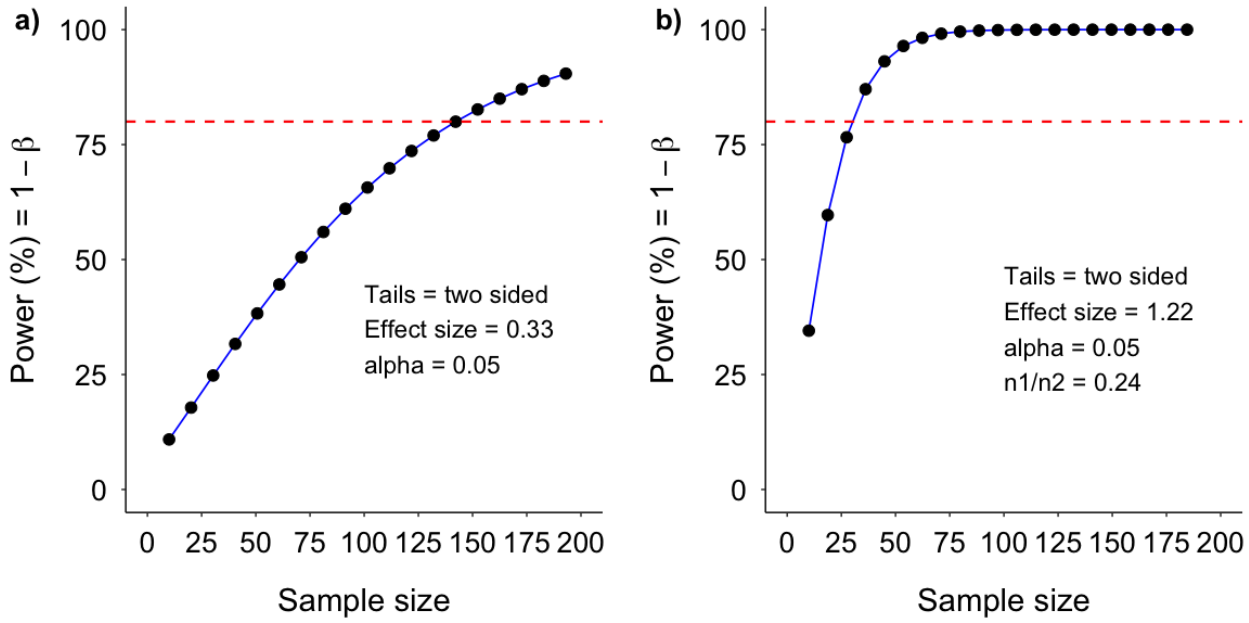

FIGURE S7

Sample size calculations for ADAMTS13 plasma concentration. Sample sizes calculated from two samples using t-test of mean (R package `pwr`).

a) Sample size estimation for a small-medium effect size. An estimated 142 individuals (per group) would be required for a small-medium effect size at a power of 80%.

b) Sample size estimation for large effect size (determined by pilot data) with unequal sample size (n: healthy controls 29, CTEPH 94).

n1/n2 (ratio of healthy controls to CTEPH), alpha (type 1 error probability), β (type 2 error probability).

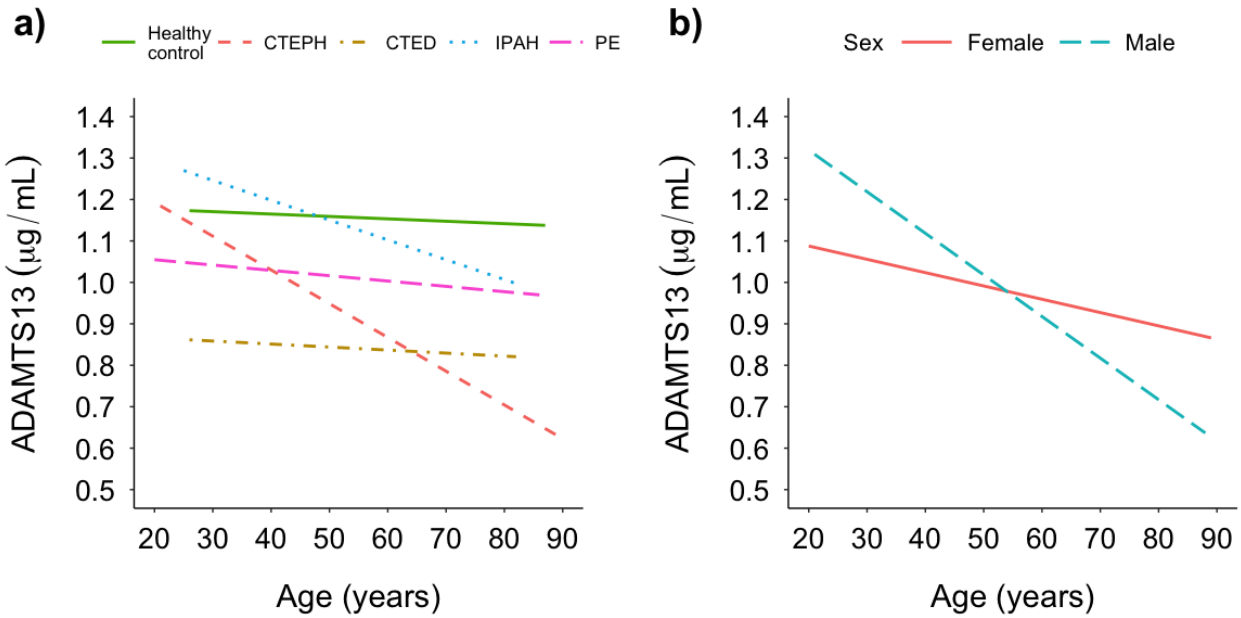

FIGURE S8

Interaction effects for the multivariable linear model shown in supplementary table S9. The predicted ADAMTS13 values are plotted with the interaction terms:

a) Age:Diagnostic group. The ADAMTS13 difference between CTEPH patients and healthy controls is more pronounced for older patients than for younger patients. ADAMTS13 levels remain lower in CTEPH patients across all ages compared with healthy controls. The rate of ADAMTS13 reduction in the CTED groups is similar to healthy controls and is also lower than healthy controls across all ages.

b) Sex:Age. The rate of ADAMTS13 reduction with age is more pronounced for males than for females.

n=343 individuals included in the models.

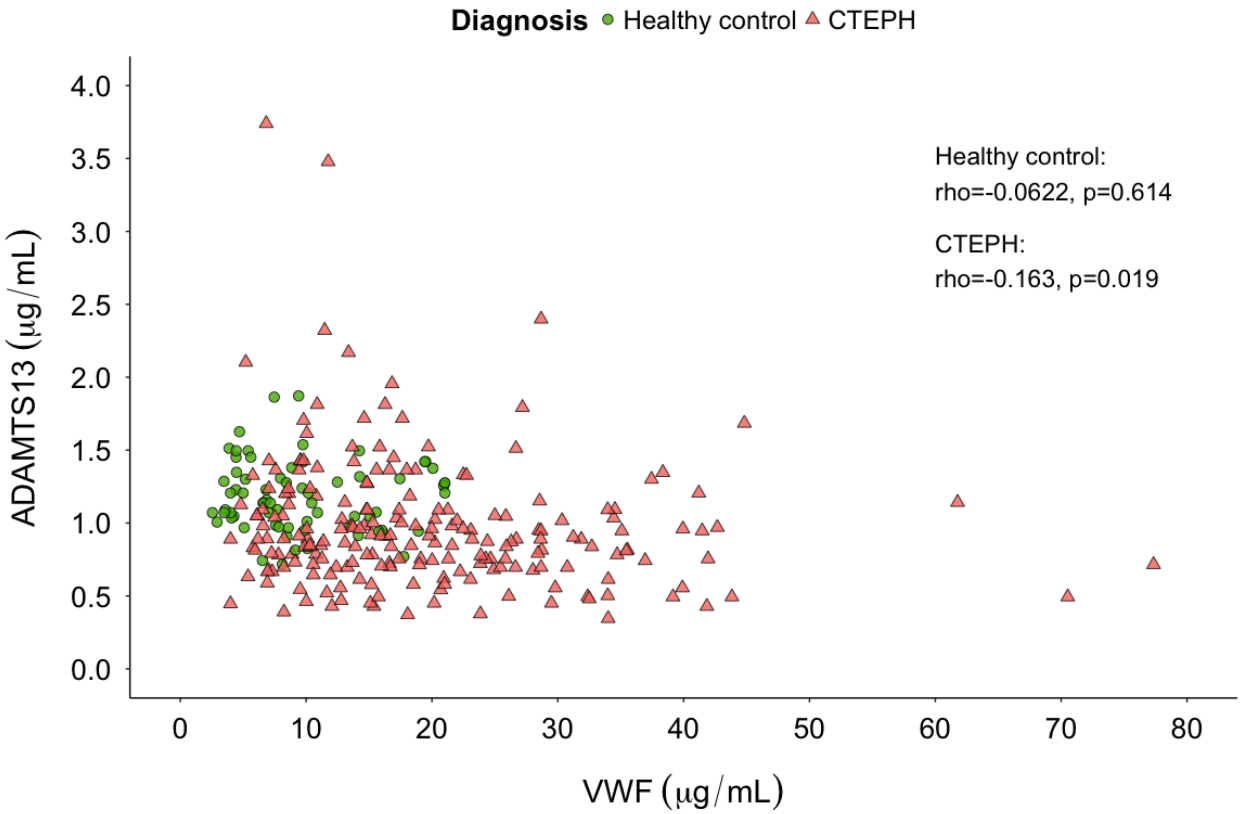

FIGURE S9

Correlation of ADAMTS13 with VWF antigen levels in CTEPH (n=208) and healthy controls (n=68). Spearman's rank correlation in other diagnostic groups: CTED (rho = -0.161, *p* = 0.354), IPAH (rho = 0.329, *p* = 0.076), PE (rho = -0.0504, *p* = 0.799).

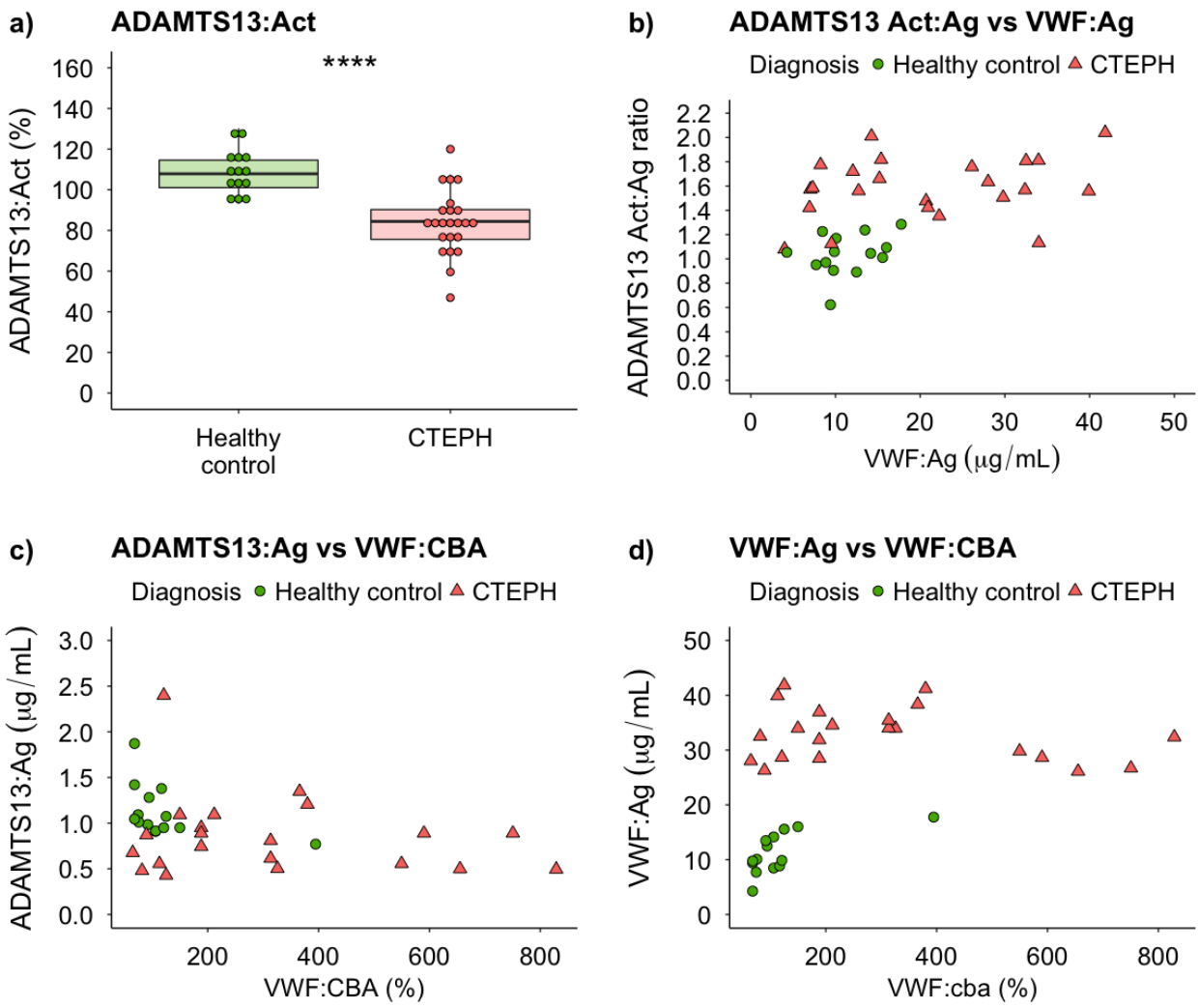

FIGURE S10

ADAMTS13 activity, D-dimer and VWF multimeric size in CTEPH and healthy controls. Additional analysis using the data presented in figure 3 (See figure 3 for group details).

a) Unadjusted ADAMTS13:Act (%) for CTEPH (84.4 ± 14.6 %) and healthy control (107 ± 13.5 %) groups. The Mann-Whitney *U* test was used to calculate group differences.

b) ADAMTS13 Act:Ag ratio vs. VWF:Ag. Healthy control correlation: rho=0.36, *p*=0.210; CTEPH correlation: rho=0.24, *p*=0.270.

c) ADAMTS13:Ag ratio vs. VWF:CBA. Healthy control correlation: rho=-0.61, *p*=0.022; CTEPH correlation: rho=-0.02, *p*=0.930.

d) VWF:Ag vs. VWF:CBA. Healthy control correlation: rho=0.670, *p*=0.008; CTEPH correlation: rho=-0.088, *p*=0.700.

Correlation was assessed with Spearman's rank correlation coefficients for both the healthy control (green circles) and the CTEPH (red triangles) groups (b, c, d).

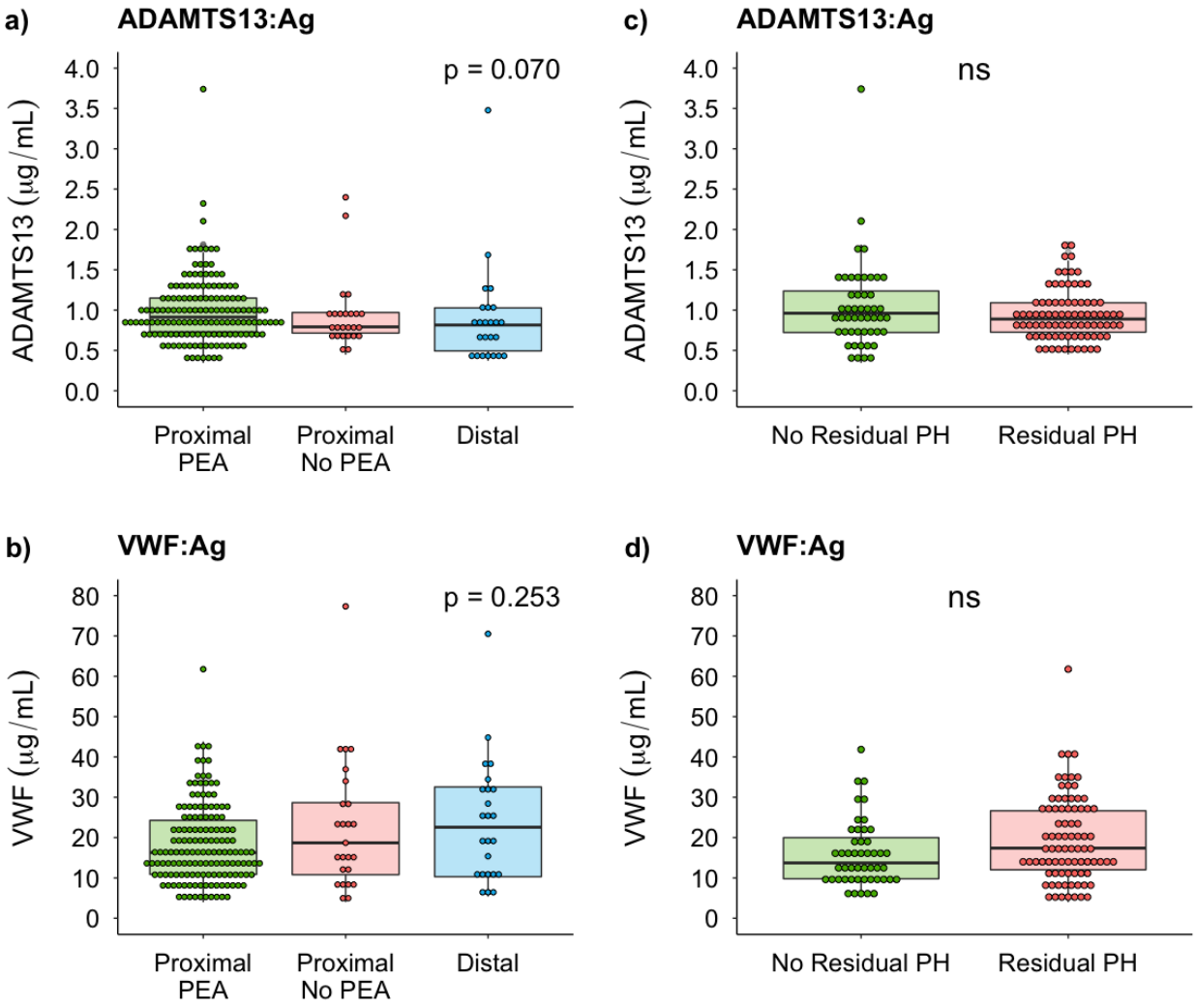

FIGURE S11

ADAMTS13 and VWF antigen levels in CTEPH sub-diagnostic and post-PEA residual pulmonary hypertension groups. a) and b) ADAMTS13 and VWF antigen levels in CTEPH diagnostic sub-groups. Numbers in each group: Proximal PEA = 150, Proximal no PEA = 25, Distal (surgically inaccessible) = 24, insufficient clinical data in 9 patients to classify them. c) and d) ADAMTS13 and VWF antigen levels in CTEPH post-PEA residual pulmonary hypertension (mPAP ≥ 25mmHg) groups. Numbers in each group: No residual PH = 49, Residual PH = 83, insufficient clinical data in 18 patients to classify them. The group differences were assessed using the Kruskal-Wallis test (a, b) and the Mann-Whitney *U* test (c, d).

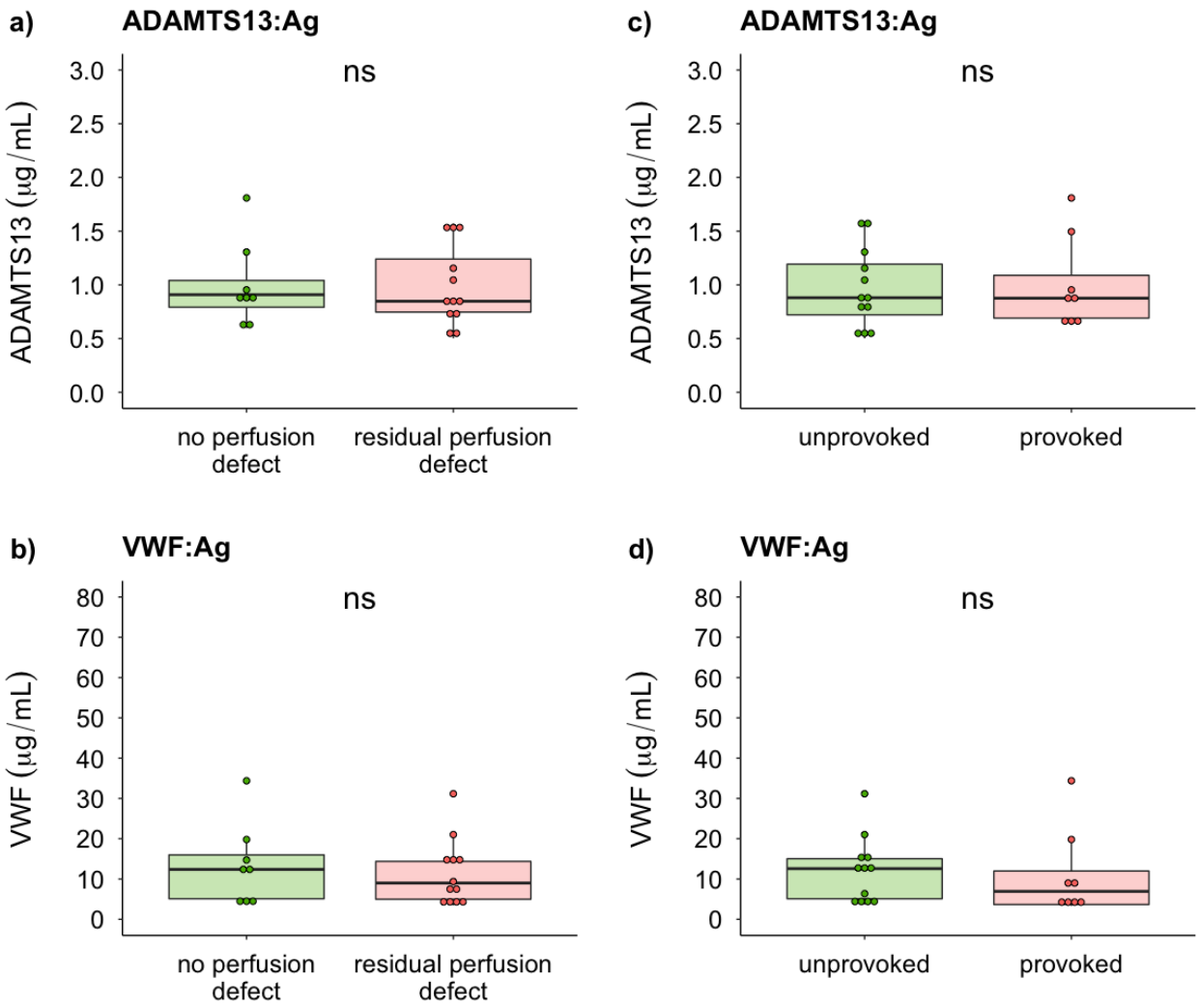

FIGURE S12

ADAMTS13 and VWF antigen levels in PE stratified by residual perfusion defects and provoked PE. The group differences were assessed using the Mann-Whitney *U* test. Numbers in each group: no perfusion defect on VQ scan = 8, residual perfusion defect on VQ scan = 12, unprovoked (no VTE risk factors) = 12, provoked (VTE risk factors) = 8. Of those with residual perfusion defects, the majority were minor (n=10).

Supplementary tables

	n (%)
CTEPH	
Disease distribution	
Proximal	176 (87)
Distal	25 (13)
PEA	150 (72)
Residual PH (>25mmHg)	83 (63)
Co-morbidities	
IHD	20 (14)
DM	19 (13)
Malignancy	19 (13)
Thrombophilia	9 (6)
Splenectomy	9 (6)
Systemic hypertension	48 (34)
Atrial fibrillation / flutter	14 (10)

COPD	8 (6)
PE	
VQ defects post PE	
None	8 (40)
Minor	10 (50)
Major	2 (10)
Idiopathic PE	8 (40)

TABLE S1

Additional clinical phenotype data for the CTEPH and PE groups. COPD (chronic obstructive pulmonary disease), IHD (ischaemic heart disease), DM (diabetes mellitus), PH (pulmonary hypertension), VQ (ventilation-perfusion).

ADAMTS13				
	Healthy control	**CTEPH**	**CTED**	**IPAH**
CTEPH	$3.00x10^{-08}$	-	-	-
CTED	$9.70x10^{-07}$	0.205	-	-
IPAH	0.373	0.003	0.001	-
PE	0.049	0.131	0.038	0.294
VWF				
	Healthy control	**CTEPH**	**CTED**	**IPAH**
CTEPH	$4.00x10^{-12}$	-	-	-
CTED	$2.20x10^{-06}$	0.834	-	-
IPAH	0.071	0.006	0.021	-
PE	0.433	$1.90x10^{-04}$	0.002	0.433

TABLE S2

ADAMTS13 and VWF antigen level pairwise diagnostic group comparisons from figure 1. Dunn's test with FDR adjustment was used to calculate *p*-values.

	β (%)	95% CI (%)	*p*	β* (%)	95% CI* (%)	*p**
Healthy Control	Reference					
CTEPH	-23.4	-30.9, -15.1	$5.91x10^{-07}$	-17.6	-27.9, -5.78	0.005
CTED	-25.9	-35.1, -15.4	$1.18x10^{-05}$	-23.1	-35.1, -8.75	0.003
IPAH	-2.18	-14.7, 12.2	0.752	1.17	-14.7, 20.0	0.894
PE	-12.0	-24.0, 1.97	0.089	-7.84	-23.3, 10.7	0.381
Female	Reference					
Male	-1.07	-7.57, 5.89	0.756	-1.98	-9.92, 6.65	0.641
Age	-0.518	-0.732, -0.303	$3.30x10^{-06}$	-0.541	-0.810, -0.271	$9.99x10^{-5}$
Batch1	Reference					
Batch2	-2.16	-10.5, 6.95	0.630	13.5	1.71, 26.8	0.024
Caucasian	Reference					
Non-Caucasian	-5.58	-15.2, 5.10	0.293	-6.7	-18.4, 6.59	0.306

TABLE S3

Multivariable linear regression model of ADAMTS13 antigen levels. Beta (β) coefficients and 95% confidence intervals are presented as percentage change with respect to healthy controls. The reference diagnostic group is healthy control, the reference sex is female, the reference batch is

batch1, the reference ethnicity is Caucasian and the β coefficient for age is per year. n=343 individuals included in the models.

* Model additionally adjusted for VWF antigen levels.

	β (%)	95% CI (%)	*p*
CTEPH	75.5	44.8, 113	$2.00x10^{-8}$
CTED	89.5	48.0, 143	$6.19x10^{-7}$
IPAH	26.7	-1.93, 63.7	0.070
PE	19.4	-9.26, 57.2	0.205
Male	7.11	-5.65, 21.6	0.288
Age	0.584	0.180, 0.990	0.005
Batch	7.33	-9.10, 26.7	0.403
Non-Caucasian	-14.5	-30.0, 4.42	0.124

TABLE S4

Multivariable linear regression model of VWF antigen levels. Reference groups are the same as described in supplementary table S3. n=343 individuals included in the model.

Groups	Thresholds	Healthy Control	CTEPH
low VWF & high ADAMTS13	VWF: < 165% (Q1-Q3) ADAMTS13 > 88% (Q2-Q4)	38 (56)	33 (16)
low VWF & low ADAMTS13	VWF: < 165% (Q1-Q3) ADAMTS13: <= 88% (Q1)	13 (19)	45 (22)
high VWF & high adamts13	VWF: >= 165% (Q4) ADAMTS13: > 88% (Q2-4)	12 (18)	39 (19)
high VWF & low ADAMTS13	VWF: >= 165% (Q4) ADAMTS13: <=88% (Q1)	5 (7)	91 (44)

TABLE S5

Summary table for combined ADAMTS13 and VWF groups. Threshold criteria in described in the table with the n (%) for CTEPH and healthy controls. ADAMTS13 and VWF antigen levels were converted to a percentage (the median of the healthy control group) and the quartile thresholds

were then determined (healthy control group). The Odds ratios for each group are shown in supplementary figure S2.

	***β* (%)**	**95% CI (%)**	***p***
***ABO* group - O**	Reference		
***ABO* group - A**	19.8	-1.75, 46.1	0.074
***ABO* group - B**	51.3	5.30, 117	0.025
***ABO* group - AB**	4.41	-26.9, 49.1	0.811
CTEPH	Reference		
CTED	7.43	-18.5, 41.6	0.609
Male	4.40	-11.9, 23.7	0.617
Age	0.921	0.341, 1.50	0.002
Batch	11.8	-5.88, 32.8	0.203
Non-Caucasian	-1.71	-51.7, 100	0.962

TABLE S6

Multivariable linear regression model of VWF antigen levels and genetic *ABO* groups in CTEPH / CTED. The reference ABO group is O and the reference diagnostic group is CTEPH. Otherwise, reference groups are the same as described in supplementary table S3. n=196 included in the model.

rsID	Chr	Position	β (%)	95% CI (%)	*p*
rs3739893	9	136243324	-37.1	-48.1, -23.8	$3.78x10^{-6}$
rs28407036	9	136252654	-39.0	-51.3, -23.5	$2.42x10^{-5}$
rs8181039	9	136253927	-37.4	-49.5, -22.5	$2.42x10^{-5}$
rs78883179	9	136241818	-41.0	-54.1, -24.1	$5.05x10^{-5}$
rs77533110	9	136286789	-43.0	-56.4, -25.5	$5.20x10^{-5}$

TABLE S7

Protein quantitative trait loci for ADAMTS13 antigen levels in CTEPH. Associations were assessed using multivariable linear regression and the SNPs included were those in the ADAMTS13 gene ± 40Kilobases (n=396). The model was adjusted for age, sex and batch. A Bonferroni *p*-value threshold $<1.26x10^{-4}$ (0.05/396 variants) was used to denote statistical significance. rsID (reference SNP identification), Chr (chromosome), position (base position). GRCh37 was used for the genomic positions of the SNPs. n=207 individuals included in the model.

	β (%)	95% CI (%)	*p*	Variance (%)
rs3739893	-37.1	-48.1, -23.8	$3.78x10^{-6}$	7.70
Age	-0.935	-1.21, -0.660	$2.23x10^{-10}$	16.3
Male	-4.55	-11.7, 3.34	0.253	0.651
Batch	-2.40	-9.67, 5.47	0.537	0.0748

TABLE S8

Multivariable linear regression with the percentage of variance of ADAMTS13 antigen levels explained by SNPs and other characteristics. Reference groups are the same as described in supplementary table S3. Partitioning of the variance explained by each variable within the models was performed by averaging over orders using the R package 'relaimpo' [20]. n=207 individuals included in the model.

	β	95% CI	*p*
(Intercept)	0.827	-0.194, 0.359	0.557
CTEPH	0.192	-0.135, 0.519	0.249
CTED	-0.325	-0.785, 0.136	0.167
IPAH	0.218	-0.236, 0.672	0.345
PE	-0.0728	-0.499, 0.353	0.737
Male	0.286	0.0305, 0.541	0.028
Age	0.00101	-0.00386, 0.00587	0.684
Batch	-0.0237	-0.111, 0.0638	0.594
Non-Caucasian	-0.0535	-0.16, 0.0527	0.322
CTEPH:Age	-0.00768	-0.0133, -0.00207	0.007
CTED:Age	0.000386	-0.00766, 0.00844	0.925
IPAH:Age	-0.00420	-0.012, 0.00358	0.289

PE:Age	-0.000410	-0.00825, 0.00743	0.918
Age:Sex	-0.00500	-0.00917, -0.000817	0.019

TABLE S9

Multivariable linear regression model of log transformed ADAMTS13 antigen levels (dependent variable) with interaction terms. The reference groups for diagnostic group, batch and ethnicity are the same as described in supplementary table S3. The interaction terms included in the model are those that were significant (p<0.05) and informative from the combination of variables in supplementary table S3. The beta coefficients should be interpreted with consideration of the interaction effects. For example, the predicted ADAMTS13 antigen level in an 80-year-old male Caucasian from experimental batch1 would be: exp(0.0827 + 0.192 + (80 x 0.00100) + (0.286) + (80 x -0.00768) + (80 x -0.00500)) = 0.688 µg/mL. This is 34% lower than an 80-year-old male Caucasian healthy control from experimental batch1: exp(0.827 + (80 x 0.00100) + 0.286 + (80 x -0.00500)) = 1.04 µg/mL. n=343 included in the model.

	CTEPH (n=208)	Healthy control (n=68)
Q1 (<88%)	136 (65)	16 (24)
Q2 (88-100%)	24 (12)	18 (26)
Q3 (100-114%)	12 (6)	17 (25)
Q4 (>114%)	36 (17)	17 (25)

TABLE S10

ADAMTS13 antigen level quartiles for CTEPH and healthy controls. ADAMTS13 levels were divided by the median of the healthy control group and expressed as a percentage. The CTEPH group was then divided into quartiles (Q1-Q4) of the ADAMTS13 distribution of the healthy control group.

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