Mendelian randomization analysis of red cell distribution width in pulmonary arterial hypertension

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Pulmonary arterial hypertension (PAH) is a rare disease that leads to premature death from right heart failure. It is strongly associated with elevated red cell distribution width (RDW), a correlate of several iron status biomarkers. High RDW values can signal early stage iron deficiency or iron deficiency anaemia. This study investigated if elevated RDW is causally associated with PAH.

A two-sample Mendelian randomization (MR) approach was applied to investigate whether genetic predisposition to higher levels of RDW increases the odds of developing PAH. Primary and secondary MR analyses were performed using all available genome-wide significant RDW variants (n = 179) and five genome-wide significant RDW variants that act via systemic iron status, respectively.

We confirmed the observed association between RDW and PAH (OR = 1.90, 95% CI = 1.80 - 2.01) in a multi-centre case-control study (*N* cases = 642, *N* disease controls = 15,889). The primary MR analysis was adequately powered to detect a causal effect (OR) from between 1.25-1.52 or greater based on estimates reported in the RDW GWAS or from our own data. There was no evidence for a causal association between RDW and PAH in either the primary (OR_{causal} = 1.07, 95% CI = 0.92 – 1.24) or the secondary (OR_{causal} = 1.09, 95% CI = 0.77 – 1.54) MR analysis.

The results suggest that at least some of the observed association of RDW with PAH is secondary to disease progression. Results of iron therapeutic trials in PAH should be interpreted with caution as any improvements observed may not be mechanistically linked to the development of PAH.

Take home message – Mendelian randomization using genetic data from the largest-to-date pulmonary arterial hypertension (PAH) cohort do not support RDW or iron deficiency as a cause of PAH, which is important when interpreting iron replacement trials in this condition.

Introduction

Pulmonary arterial hypertension (PAH) is a rare disease with an estimated prevalence of 7-26 cases/million in the developed world [1]. It is characterized by increased pulmonary vascular resistance due to vasoconstriction and structural remodeling of pulmonary arterioles, leading to right ventricular hypertrophy and end-stage right heart failure [2]. Despite increased awareness and new therapeutic options, annual mortality remains around 10% [1]. Approximately 70–80% of heritable PAH and 10–20% of idiopathic PAH patients are known to harbour mutations in the bone morphogenetic protein type II receptor (*BMPR2*) gene [3]. A recent large study of over 1,000 PAH patients confirmed the prevalence of causal mutations in *BMPR2*, as well as in five other established genes (*TBX4, ACVRL1, ENG, SMAD9*, and *KCNK3*), and identified PAH-associated mutations in four new genes (*ATP13A3, SOX17, AQP1, and GDF2*), all together accounting for 23.5% of the cases studied [4]. The rare mutations in all these genes are inherited in an autosomal dominant manner and exhibit reduced penetrance, indicating that other genetic, epigenetic and/or environmental factors influence the development of PAH.

We and others have demonstrated that one factor strongly correlated with survival in PAH is red cell distribution width (RDW) [5, 6]. A recent hypothesis-free phenome-wide analysis indicated PAH, among several disease descriptors, as the most strongly associated with RDW (odds ratio, OR = 2.0, 95% CI = 1.75-2.4 per % increase in RDW) in a hospital population [7]. RDW - a measure of red blood cell (RBC) size variability in an individual – is part of the full blood count in standard hospital practice and readily available as a biomarker. RDW correlates with iron status biomarkers and high values can signal early stage iron deficiency or iron deficiency anemia [8]. RDW increases with decreasing iron as available body iron stores fail to meet the iron demand of RBC synthesis resulting in RBCs of varied size.

Iron deficiency is commonly observed in PAH patients and is under investigation as a therapeutic target [9-14]. An observed correlation between two traits does not necessarily imply that interventions on one trait will change the other and there are numerous examples where false positive associations have led to unsuccessful randomized controlled trials [15, 16]. Clinical trials are expensive and time-consuming, and recruitment can be challenging, especially in rare diseases. With the growing availability of genetic data in large disease-focused and population-based studies, testing causal relationships between traits of interest has become possible by harnessing the naturally occurring genetic variation in the population. The collection of methods used to test causal relationships using genetic variants is called Mendelian randomization (MR) [17, 18]. MR has been successfully used to help prioritize intervention and drug targets and to identify causal factors for several diseases [19-23]. In general, candidate drugs with genetic evidence for effectiveness are more successful in drug trials compared to those without such genetic support [24].

It remains to be investigated whether elevated RDW is largely a consequence of PAH or plays a causal role in the condition. The common genetic variation determining RDW levels has been defined in very large population studies [25], with more power than equivalent studies of iron status. This makes RDW a strong candidate for MR analysis and our primary aim was to dissect the epidemiological association between RDW and PAH by assessing causality using MR. We refined the estimate of association between RDW and PAH using biomarker data in 642 PAH cases and over 15,000 controls with common diseases from the *Imperial College PH Biobank*, the *UK PAH Cohort*

and the *Vanderbilt University Medical Centre (VUMC)* and applied MR to investigate whether being genetically predisposed to higher levels of RDW increases the odds of developing PAH.

Methods

Definition of pulmonary arterial hypertension (PAH)

PAH was defined by internationally agreed criteria [26], specifically mean pulmonary artery pressure >25mmHg, pulmonary vascular resistance >3 Woods and pulmonary capillary wedge pressure <15 mmHg. Patients with concurrent diseases known to cause pulmonary hypertension were excluded such that all were considered to have idiopathic, heritable or anorexigen-induced PAH.

Data: genetic and phenotype data in contributing studies

For our analyses we used both individual-level and summary-level data. Individual-level data including clinical laboratory RDW was available for a total of 524 PAH patients from the Imperial College PH Biobank and the multicentre *UK PAH Cohort,* a study that facilitates collaboration and the sharing of data and samples between the specialist pulmonary hypertension centres in the United Kingdom [27].

In addition, the hospital population-based *VUMC* study provided longitudinal clinical laboratory RDW measurements, detailed clinical diagnoses and genome-wide genotype array data (genotyping platform: Illumina MEGAex) for an additional 118 PAH patients and 15,889 common disease controls (*Supplementary Table 1*). *VUMC* hosts a collection of electronic medical records linked to genetic data derived from blood collected during routine clinical assessment in outpatient clinics where all patients are shown the consent form during check-in [28, 29]. The exclusion criteria (*Supplementary Figure 1*) and the imputation of genotype array data are described in the *Supplementary Methods*.

Summary-level data for both RDW and PAH susceptibility were used in the MR analyses to maximize the sample size and therefore the statistical power. Genetic instruments serving as a proxy for RDW were obtained from the largest-to-date (over 170,000 individuals) GWAS on hematological traits (hereon referred to as the 'RDW GWAS') in a population-based study by Astle et al. (25).

A large proportion of the idiopathic and familial PAH cases from the *UK PAH Cohort* were whole genome sequenced as part of the *UK National Institute for Health Research BioResource (NIHRBR) [4, 30]* study. For genetic effects on PAH susceptibility, we used a recent study published by our group and others investigating in the largest-to-date genome-wide association study the effects of common genetic variation on PAH risk (PAH GWAS) involving a total of 11,744 individuals of which 2,085 were PAH cases. The results of the PAH GWAS were combined through the meta-analysis of four independent studies, one of which is the *NIHRBR* with 847 PAH cases and 5,048 rare disease controls. The other major contributing study, the *US PAH Biobank (PAHB)* used a control population with mixed common diseases recruited from outpatient clinics (694 PAH cases and 1560 controls), whilst the two smaller studies used population-based controls, with 269/275 PAH cases and 1068/1983 controls in the PHAAR and BHFPAH studies, respectively [4].

Statistical analyses

To confirm and refine the estimate for the association between RDW and PAH, we combined PAH patients from the *Imperial College PH Biobank*, the *UK PAH Cohort* and the *VUMC* and compared them to common disease controls from the *VUMC* recruited in outpatient clinics (*Supplementary Methods, Supplementary Table 1*).

To test for causality between RDW and PAH, we applied a two-sample MR strategy that requires effect estimates for the genetic instrument on the risk factor (here RDW) and the outcome (here PAH) from two non-overlapping datasets (*Supplementary Methods and Supplementary Figure 2*). The genetic instrument for RDW comprised genetic variants associated with RDW levels in the RDW GWAS at a study-specific genome-wide level of significance ($P< 8.31\times10^{-9}$). In the PAH GWAS, 179 variants (inclusive of 12 proxy variants with a minimum r^2 of 0.8) out of the 212 independent ($r^2 <$ 0.01) RDW quantitative trait loci (QTL) were available after excluding 13 palindromic variants (A/T or C/G) with intermediate allele frequencies (minor allele frequency > 45%) to ensure that the effects of the variants for the two traits can be harmonized to the same allele. To obtain the causal estimate, we applied the inverse variance weighted (IVW) [31] - and weighted median [32] methods. We assessed heterogeneity between the causal estimates from each QTL using the Cochran's Q test (*Supplementary Methods*).

In the primary MR analysis, we included all available genome-wide significant RDW QTL (n = 179). The secondary analysis explicitly tested the role of iron deficiency in the RDW-PAH association using five RDW QTL mapped to genes involved in iron homeostasis (*HFE, TMPRSS6, TFRC* and *TFR2*) from the full set of genome-wide significant RDW QTL (*Supplementary Figure 2*). All of these five RDW QTL concomitantly increase serum iron, ferritin, and transferrin saturation and decrease transferrin, reflecting systemic iron status (*Supplementary Table 2*) and were first reported by an independent GWAS - the Genetics of Iron Status GWAS - as genome-wide significant signals for at least two of the above-mentioned iron status biomarkers [33]. These five RDW QTL are among the signals which explain the highest proportion of variance in the RDW GWAS, highlighting the importance of iron availability in RDW levels.

Furthermore, we validated the RDW genetic instrument as a proxy for RDW levels in our common disease controls from VUMC. To do so, we regressed the first available RDW measurement on the RDW genetic risk score (GRS) constructed for each individual (*Supplementary Methods*) and obtained the proportion of variance explained (R^2). The R^2 for the same RDW GRS in the RDW GWAS study populations (UK Biobank – UKB and INTERVAL) was calculated from the summary statistics of the RDW-QTL associations (*Supplementary Methods*).

Since genetic variants typically explain a small proportion of the variability in the associated trait, MR studies often require large sample sizes to detect a non-zero causal effect. Our power to detect a causal association in the current MR analyses was calculated [34] using the R^2 values estimated in VUMC and also those estimated in the RDW GWAS populations (*Supplementary Methods*).

Results

Defining the association of RDW and PAH

Within this observational analysis, each standard unit (1.4%) increase in RDW was associated with 90% higher odds of prevalent PAH after adjusting for the effects of age and sex (odds ratio, OR = 1.90, 95% CI = 1.80 - 2.01). There were no marked between-cohort differences in RDW levels (*Figure 1* and *Supplementary Table 1*).

Genetic risk score using RDW QTL

We estimated that the 179 RDW QTL would explain over 12% of the variability (R^2 = 12.7%, 95% CI = 12.32% -12.99%) in RDW levels in the RDW GWAS population (UK Biobank and INTERVAL) in which they were discovered. The RDW GRS constructed for the VUMC controls explained 2.6% (95% CI = 2.17% - 3.19%) of the variability in the first available RDW measurement *(Supplementary Table 3)*. The set of five variants specific to iron status explain an estimated 1.7% (95% CI = 1.62% – 1.87%) of the RDW variability in the UKB and INTERVAL populations whilst they explain 0.7% (95% CI = 0.43% - 0.92%) of the total variability in RDW in the VUMC controls *(Supplementary Table 3)*. The RDW GWAS study populations had a lower mean RDW level than our common disease controls (UKB and INTERVAL = 13.45; VUMC = 13.60) and lower standard deviation (UKB and INTERVAL = 0.82; VUMC = 1.40) which could in part explain differences between the R^2 estimates in these studies.

Statistical power to detect causal effect

With a genetic instrument that explains a relatively high proportion (R^2 = 12%) of the total variation in RDW levels (Figure 2, red curve) we have 80% power to detect a causal effect as small as 1.25 (OR). If the true variance explained lies closer to that estimated in the VUMC controls (R2 = 2.6%, Figure 2, green curve), this changes to 1.52. When the variance explained by the genetic instrument is small (R^2=1.7%, Figure 2, blue curve), we are limited, with our sample size, to an OR of 1.7 or above. However, if the effect of RDW calculated in our observational analysis (OR=1.80-2.01) was causal in nature, either of the two MR analyses, based on R^2 estimates from the RDW GWAS, would have over 80% power to detect an effect of that magnitude. Using the estimates based on the VUMC data, the analysis using all 179 RDW QTL (Figure 2, green line) would have sufficient (>80%) power in our sample, while the iron-specific model (Figure 2, purple line) would be underpowered.

RDW-PAH causal relationship

We tested for a causal effect of RDW on development of PAH in our primary MR analysis using 179 RDW QTL and found no significant relationship (IVW OR_{causal} = 1.07, 95% CI = 0.93 – 1.23, Q p-value = 0.57, Figure 3). A secondary MR analysis based on five RDW QTL provided no evidence for a causal effect of iron status on PAH (IVW OR_{causal} = 1.09, 95% CI = 0.77 – 1.54, Q p-value = 0.91, Figure 3). The weighted median method, which is more robust to violations of MR instrument assumptions, yielded similar estimates for both the primary (WM OR_{causal} = 1.11, 95% CI = 0.89 – 1.38) and the secondary (WM OR_{causal} = 1.04, 95% CI = 0.68 – 1.59) MR analyses.

If the OR estimated in the primary MR analysis was indicative of the magnitude of a real causal effect, the number of PAH cases needed to detect such a causal effect with a genetic instrument that explains 10% of the variance in RDW would be ~ 20,600 (with the same 1:4.6 ratio of cases:controls as in this study) to achieve 80% power at a false positive rate of 5% (P = 0.05). We tested for heterogeneity between causal effects estimated in the four studies contributing to the PAH GWAS separately (Supplementary Figures 1-2) to assess if differences in the nature of their control populations yielded heterogenous effect estimates for the instrumental variants. The two heterogeneity tests on the IVW estimates (main MR: Q = 0.83, df = 3, p = 0.84; secondary MR: Q = 0.55, df = 3, p = 0.91) did not detect considerable variability between the causal effects in the four contributing studies based on a random-effects model (*Supplementary Figures 3 and 4*).

Discussion

We applied a two-sample MR approach to test whether the epidemiological relationship between elevated RDW levels, which are associated with iron deficiency, and PAH is causal in nature. We estimated the effect of RDW on PAH in a large sample of cases and common disease controls. By using genetic variants as instruments for RDW, we found no evidence for a causal effect of RDW on PAH of the magnitude suggested by observational studies.

Previous work has shown that iron deficiency is common in PAH and associated with a poor prognosis, reduced exercise capacity and worsening hemodynamics [9, 11, 13]. A physiological link has been described in healthy volunteers where iron infusion attenuated the rise in pulmonary artery pressure induced by acute hypoxia [35, 36] and in rats where chronic iron deficiency results in pulmonary hypertension [37]. This relationship could be driven by the role of iron in de-stabilizing the hypoxia-inducible factor, thereby deficiency can mimic the hypoxic state [38]. Our study confirmed the association of raised RDW with PAH using controls from a hospital population and cases from multiple centres (with an effect size 1.90 for one standard unit RDW, 1.4%), supporting a recent hypothesis-free phenome-wide analysis which indicated, among several disease descriptors, PAH as the most strongly associated with RDW with a similar effect size (OR 2.0, 95% CI = 1.75-2.4 per % increase in RDW) [7]. Our MR analysis was adequately powered to detect a causal role for RDW with an effect of this magnitude. The fact that we did not detect a causal effect at this level suggests that at least some of the observed association is secondary to the disease.

The results of this study may appear to be at odds with previous clinical studies of the efficacy of iron supplementation in PAH patients, which have focused on functional capacity rather disease pathology. An open-label study of twenty patients with idiopathic PAH with iron deficiency reported improved iron status, 6-minute walk distance (6MWD) and quality of life (QoL) two months after a single infusion of 1000 mg ferric carboxymaltose [14]. Another open-label study in fifteen iron deficient idiopathic PAH patients reported improvement of iron status, QoL and exercise endurance capacity on cardiopulmonary exercise testing after receiving 1000 mg of intravenous iron [10]. Neither of the clinical studies were placebo controlled, although Viethen *et al.* compared their intervention group to a group of matched iron-replete patients who did not receive iron infusion. It remains possible that iron supplementation in PAH could have benefits through mechanisms distinct from those driving the cardiovascular pathology, for example on muscle function [39].

MR studies using data from large consortia support a causal effect of iron status in other diseases. The genetic instruments (two variants in *HFE* and one variant in *TMPRSS6*) used in these studies were also used in our secondary MR analysis. Gill and colleagues found iron to have a protective effect against coronary artery disease (IVW causal OR = 0.94 per standard deviation (SD) change in serum iron) [40] but increase the risk of cardioembolic stroke (IVW causal OR = 1.16 per SD iron) [41]. The authors suggest the opposing effects of iron status on CAD and stroke might be due to different underlying mechanisms. Pichler et al. [19] have reported a that iron protects against the risk of developing Parkinson`s disease (IVW causal OR = 0.88 per SD iron). Iron deficiency is a common risk factor and these causal effect estimates in common diseases are modest; this study was not powered to detect an effect if this is also true of PAH.

In the light of this MR analysis, alternative explanations for the association of RDW and PAH have to be considered; namely, that PAH causes raised RDW (reverse causation, for example reduced oxygen delivery and/or haemolysis related to PAH may stimulate reticulocytosis which would increase RDW) or that PAH and elevated RDW are caused by an independent common mechanism. One such mechanism is chronic inflammation, which is a common feature of PAH [42] and leads to intracellular sequestration of iron. Other mechanisms which may modify RDW such as folate or vitamin B12 could be studied for their association with PAH. To directly test whether PAH is causal for raised RDW levels a stronger genetic instrument for PAH is required than currently known common variation identified by PAH GWAS.

An important strength of our study lies in the sample size available with phenotype and genetic data achieved through extensive collaboration. Given the rarity of PAH, data had to be pooled from several centres to allow the investigation of common genetic variation in PAH and to test causal relationships. Our study also has some limitations. The control population for our observational study was not specifically selected to represent a population at risk of developing PAH. An example of a population at risk of PAH are relatives of patients with pathogenic *BMPR2* mutations (and other rare pathogenic variants). Given the reduced penetrance of familial/heritable PAH (~42% in women and ~14% in men carrying known mutations) [43], following up the families of affected individuals, especially relatives harbouring mutations associated with PAH, would be an invaluable source to identify environmental triggers of PAH.

Despite our efforts to exclude controls with conditions that likely affect RDW and to use the first available RDW measurement, we expect genetic effects of RDW levels to differ between individuals with common diseases and a cohort of healthy volunteers. The R^2 of a genetic variant describes the variance explained in the phenotype in a given population at a given time. Therefore, there can be no single population parameter that applies to multiple populations or the same population at multiple timepoints. Although the R^2 estimated in the RDW GWAS is likely upwardly biased (since it contains the discovery as well as the replication samples), it might reflect better the extent to which genetic variation influences long-term RDW levels in disease-free populations than the R^2 estimate from the VUMC disease controls, who may be expected to have more variable RDW levels due to comorbidities. This highlights a potential challenge in estimating power for MR studies; we now estimate that upwards of 20,000 PAH patients would be required to detect any likely causal effect of iron deficiency.

It is important to note that in our MR analyses, causal effects were estimated using the results of the PAH GWAS [4]. Four independent studies contributed to the overall results of the PAH GWAS comparing allele frequencies of their PAH cases to control cohorts selected according to different criteria. The NIHRBR study used a control population with a mixture of rare diseases while the other major contributing study from the United States used mixed common disease controls. The two smaller contributing studies used population-based controls; a preferable design for estimating the effects of surrogate genetic variants for common risk factors. Selection bias can affect the estimates of the instrumental genetic variants on disease susceptibility. This is especially true if the control cohort is enriched for conditions also affected by the risk factor of interest.

Conclusions

There is strong observational evidence for an association between elevated RDW, a surrogate for iron deficiency, and PAH. However, this Mendelian randomization analysis does not indicate that RDW is causally linked to disease development. Our study was powered to detect a causal effect similar in size to that observed. A more modest causal effect remains possible but would require a significantly larger study population to detect. Extending international collaborations and careful follow up of populations at risk will allow increasingly sophisticated study designs to investigate causal relationships, shared underlying mechanisms with other conditions and overall genetic susceptibility in PAH.

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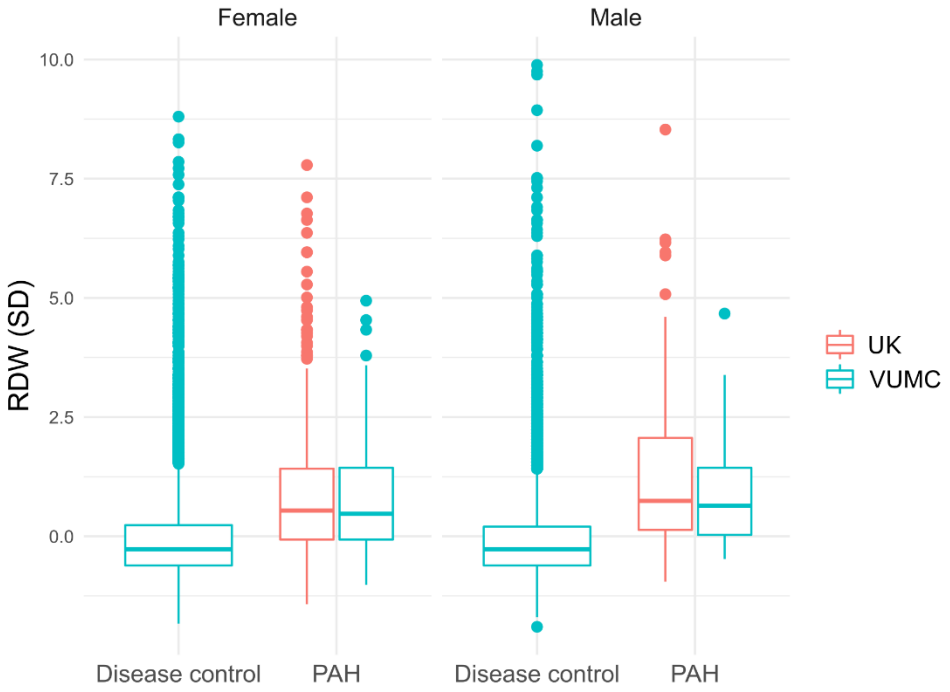


Figure 1 Boxplot of RDW levels in the merged cohort of PAH cases (N=642) and the disease control cohort (N=15,889). The bottom and the top lines of the box indicate the 25th and 75th percentiles, while the centerline indicates the median value. The whiskers extend to 1.5 times the interquartile range from both ends of the box with individual points being more extreme observations. SD = standard deviation.

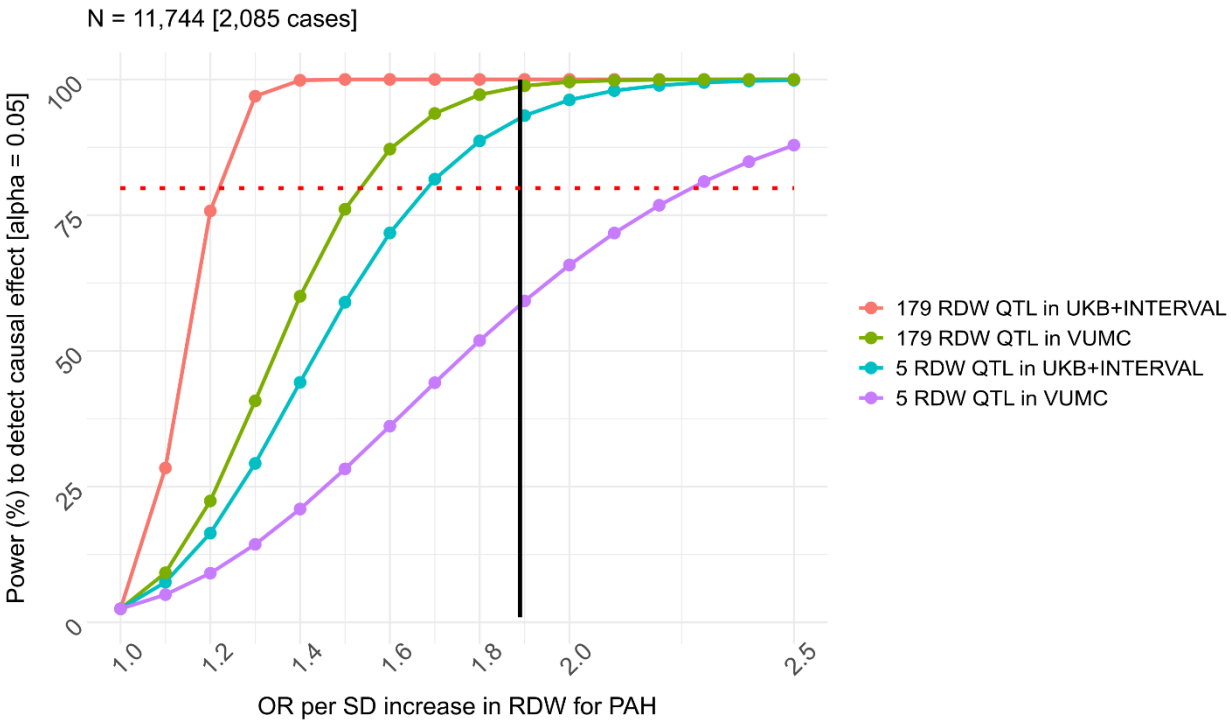


Figure 2 Power (%) to detect a causal association (y-axis) given the size of the true underlying causal effect of one standard unit increase in RDW on PAH risk (x-axis). The effect estimate obtained from the observational study is indicated with the vertical black line at OR = 1.90 whilst the red dotted line marks the desired power of 80%. Red curve: MR using all overlapping genome-wide significant variants from the RDW GWAS, given the true R^2 = 12% as per estimated in the UKB and INTERVAL cohorts. Green curve: MR using all genome-wide significant QTL from the RDW GWAS, given the true R^2 = 2.6% as per estimated in our VUMC control cohort. Blue line: MR using five genome-wide significant variants from the RDW GWAS reflecting systemic iron status, given the true R^2 = 1.7% as per estimated in the UKB and INTERVAL cohorts. Purple line: MR using five genome-wide significant QTL from the RDW GWAS reflecting systemic iron status, given the true R^2 = 0.7% as per estimated in our VUMC control cohort.

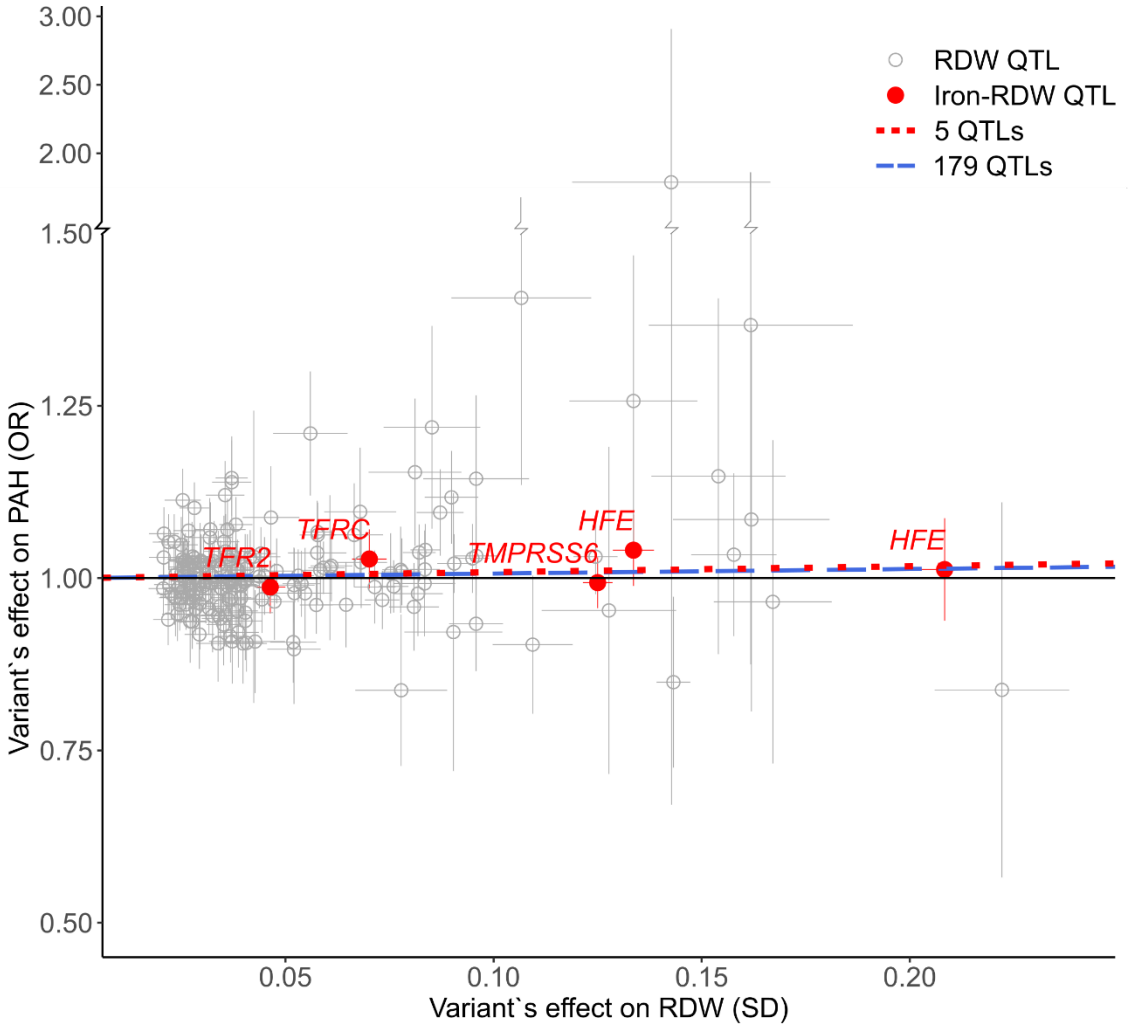


Figure 3 Scatterplot of variant – RDW associations (x-axis) plotted against variant – PAH associations (y-axis) where each dot represents a single RDW QTL. The effect estimates and their standard errors (grey bars) are given in standard units for RDW and in odds ratios for PAH. The solid black line denotes an OR of 1 (no effect) whilst the dashed blue line is the overall causal effect from the IVW regression using all 179 RDW QTL. The five iron-specific RDW QTL (red dots) used as instruments in the secondary MR analysis are labelled with their corresponding gene names and the red dotted line denotes the corresponding causal effect.

Supplementary Appendix
for

Mendelian randomization analysis of red cell distribution width in pulmonary arterial hypertension

Anna Ulrich *et al.*

Table of Contents

Supplementary Methods

RDW and PAH association

RDW values were natural log-transformed and for ease of interpretation z-score normalized to have a mean of zero and a standard deviation of one. The association between RDW levels and PAH was tested in a logistic regression framework adjusted for age and sex (Supplementary Table 1). For PAH cases we used closest to diagnosis RDW measurements. For controls we retrieved the first available RDW measurement.

We excluded individuals from non-white ethnic backgrounds to avoid potential bias from ethnicity effects. Since RDW is known to be elevated in a number of diseases, we excluded all individuals with either of the following in their medical history: polycystic kidney disease, chronic kidney disease, liver disease and transfusion therapy received. Furthermore, children and adolescents under the age of 18 as well as individuals with extreme RDW values (below 10%, N=20 or above 30%, N=15) were not included in the analysis. Out of the 35 excluded for extreme RDW, 14 were cases from VUMC in the range of 43-55 and 20 were all below 10 from one of the NIHRBR centers. Both centers confirmed reporting errors for these samples (*Supplementary Figure 1*).

Mendelian randomization; causal effect estimation using MR

Alleles were aligned to correspond to an increase in RDW followed by the harmonization of the effects. The causal effect was estimated with the inverse variance weighted (IVW) and weighted median estimator (WM) methods as implemented in the MR-Base software (1).

Inverse-variance weighted method

We used the conventional inverse variance weighted (IVW) method for estimating the causal effect. The IVW method is efficient when all variants in the genetic instrument are valid instruments. Briefly, each variant in the genetic instrument provided a causal estimate calculated by simply dividing the variant's effect on PAH by the variant's effect on RDW (ratio of coefficients or Wald ratio). These individual causal estimates were then meta-analyzed in a fixed-effects model weighted by the reciprocal of the standard error of the variant association with PAH.

This is equivalent to regressing the variant-RDW estimates on the variant-PAH estimates with the above-mentioned weighting whilst forcing the regression line to pass through the origin.

The causal estimate from the IVW method (β_{IVW}) is:

$$\beta_{IVW} = \frac{\sum_{k=1}^{K} X_k Y_k \sigma_{Yk}^{-2}}{\sum_{k=1}^{K} X_k^2 \sigma_{Yk}^{-2}}$$

Where:
k is an index for each of the variants used in the two-sample MR analysis
X is the effect estimate on the exposure (RDW) as reported in the RDW GWAS summary statistics
Y is the effect estimate on the outcome (PAH) as reported in the PAH GWAS summary statistics
The standard error of the causal estimate is:

$$se(\beta_{IVW}) = \sqrt{\frac{1}{\sum_{k=1}^{K} X_k^2 \sigma_{Yk}^{-2}}}$$

Weighted median estimator

We used the WM estimator to allow for up to (but not including 50%) of the variants in our genetic instrument to be invalid instruments.

Analogously to the IVW method, a Wald ratio (see above) is calculated for each variant. These Wald ratios are then ordered and weighted by the same weights used in the IVW method (see above). Let wj be the weight of the jth ordered Wald ratio estimate.

$$s_j = \sum_{k=1}^{j} w_k$$

Where:

k is an index for each of the variants used in the two-sample MR analysis

w is the weight of the variant

s_j is the sum of weights up to and including the jth Wald ratio estimate

The weights are standardized, so that the sum of weights is 1. The WM estimator is the median of the empirical distribution of weighted Wald ratios. Each Wald ratio is the $100(s_j - \frac{w_j}{2})$th percentile of this distribution.

Quality control and imputation procedures of VUMC genotype data

VUMC participants were genotyped in 6 batches (30,886 in total) using the Infinium Expanded Multi-Ethnic Global Array-8 (MEGA-ex) array (Illumina, San Diego, California, US).

Variant QC (pre-imputation): Variants were excluded if they had a low call rate (< 95%), deviated from the Hardy-Weinberg equilibrium ($P \leq 0.00005$), were rare (minor allele frequency ≤ 1%) or had more than two alleles.

Sample QC: Individuals with high proportions (> 5%) of missing genotype data, unresolved sex discrepancies (discordant phenotype-genotype sex information), heterozygosity outliers, self-reported and/or principal component-based ethnic outliers, intentional duplicates and related individuals (PI-HAT > 0.2) were excluded.

Imputation of non-genotyped variants: The filtered genotype array data was imputed to the Haplotype Reference Consortium panel using the free Sanger Imputation Service provided by the Wellcome Sanger Institute (2).

Imputed variants were further filtered for deviations from the Hardy-Weinberg equilibrium ($P \leq 0.00005$), rare variants (minor allele frequency ≤ 1%) which are often poorly imputed and other low-quality variants with an INFO score lower than 0.9.

Genetic risk score (GRS) derivation and calculation of variance explained (R^2) from individual-level data

Weighted genetic risk scores (GRS) comprising the single nucleotide polymorphisms (SNPs) from the RDW genetic instrument were regressed onto the first RDW values which provided the coefficient of determination (R^2) as an estimate for the correlation between RDW and the RDW GRS in our population. The GRSs can be derived by summing the effect alleles multiplied by the effect size at each of the variants (3).

GRS were calculated using the software PRSice-2 (3). Genotypes for the 179 SNPs in the RDW GRS were extracted from the imputed VUMC controls dataset. The same inclusion criteria as for our observational study were applied. Out of the 15,889 VUMC controls included in the observational study, 14,964 had genetic data that passed standard variant and sample quality control.

The GRS for an individual is the summation of the effect (trait-increasing) alleles (0, 1 or 2) weighted by the effect size of the variant taken from the genome-wide significant summary statistics of the RDW GWAS (4). We used an additive model meaning that homozygotes for the effect allele had twice the increase in RDW levels as the heterozygotes. This was in line with the model used in the RDW GWAS.

The following models were used to assess the validity of the RDW GRS as a proxy for RDW levels:

Full model: *RDW ~ GRS + sex + age + principal components (1st and 2nd) + batch (study specific)*

Null model: *sex + age + principal components (1st and 2nd) + batch (study specific)*

The R^2 for the GRS alone is calculated by subtracting the R^2 of the model not containing the GRS (null model) from the R^2 of the full model.

The confidence intervals for the GRS R^2 were computed using the adjusted bootstrap percentile method as implemented in the R software package 'boot' (5) (number of replicates = 20,000).

R^2 calculation from GWAS summary-level data

R^2 was calculated for each independent variant based from the publicly available summary statistics of the RDW GWAS in the discovery and replication populations (4). These individual estimates were then summed to give the overall variance explained by the RDW instrument.

$$R^2 = \sum_{k=1}^{K} \frac{Nsample_k + 1}{Nsample_k} \times \frac{Z_k^2}{Z_k^2 + Nsample_k} - \frac{1}{Nsample_k}$$

Where:

k is an index for each of the variants used in the two-sample MR analysis

Z is the Z-statistic as reported in the RDW GWAS summary statistics

$Nsample$ is the sample size as reported in the RDW GWAS summary statistics

The standard error for the R2 estimate was calculated as shown below:

$$SE_{R^2} = \sum_{k=1}^{K} \sqrt{\left(\frac{2}{Nsample_k}\right) \times \left(2 \times R_k^2 + \frac{1}{Nsample_k}\right)}$$

Supplementary Table and Figure Legends

Supplementary Table 1 Characteristics of the study population used for estimating/assessing the association between RDW and PAH, stratified by sex. PAH – Pulmonary Arterial Hypertension, VUMC - Vanderbilt Institute for Clinical and Translational Research, NIHRBR - UK National Institute for Health Research BioResource, RDW – red cell distribution width.

Supplementary Table 2 Logistic regression model predicting PAH disease status. We report the results of the adjusted model in the paper.

Supplementary Table 3 Five variants selected from the RDW GWAS based on their effects on systemic iron status. This table presents the effect estimates of these variants on RDW as reported by Astle et al. on RDW (Effect estimate per RDW SD; Effect estimate p-value). *The effects of these variants for the same allele go in the opposite direction on serum iron as reported by the Genetics of Iron Status GWAS (6). Elevated RDW can reflect iron deficiency which presents with decreased serum iron levels. In the Genetics of Iron Status GWAS, the two HFE variants reached genome-wide significance ($P< 5\times10^{-8}$) for all four (serum iron, transferrin, transferrin saturation, ferritin) iron status biomarkers, TMPRSS6 reached it for all but transferrin, TFRC reached it for transferrin and transferrin saturation, while TFR2 reached it for iron and transferrin saturation.

Supplementary Table 4 Summary of the RDW GRS models. The null model corresponds to the linear regression model specified above without the GRS. The R2 of the null models are identical since the sample and the covariates are the same. P.value is the significance value of the model fit (F-test). Empirical p-values that account for multiple testing and overfitting were obtained through permutation tests (n=20,000).

Supplementary Figure 1 Flow diagram of the exclusion steps in the UK centers and the Vanderbilt University Medical Centre (VUMC) of the two cohorts (UK PAH Cohort and VUMC) participating in the RDW and PAH association analysis as described in the Supplementary methods.

Supplementary Figure 2 Mendelian Randomization (MR) analyses of red cell distribution width (RDW) and pulmonary arterial hypertension (PAH). A two-sample design was used where effect estimates for the instrumental genetic variants were taken from two non-overlapping populations. RDW QTL and their effect estimates were taken from the largest-to-date population based RDW genetic association study (GWAS) (4). Effect estimates for the RDW QTL on PAH susceptibility were obtained from the largest-to-date PAH GWAS (6). The primary MR analysis included all 179 RDW QTL while the secondary MR analysis was restricted to five out of the 179 RDW QTL acting via iron status (Supplementary Table 3).

Supplementary Figure 3 Individual MR causal estimates (IVW) for the main MR analysis of association between RDW and development of PAH - using all available RDW SNPs - from the four contributing studies in PAH GWAS. PAH ORs per one standard unit increase in RDW (dot) with the corresponding lower and upper 95% confidence intervals (horizontal line). The result of the BHFPAH (IVW causal OR = 1.54, 95% CI = 1.06 – 2.23) did not survive the correction for multiple testing and was driven by the causal estimate of one variant (rs6883412). PHAAR: Pulmonary Hypertension Allele-Associated Risk (269 PAH cases, 1068 controls). PAHB: US National Biological Sample and Data Repository for Pulmonary Arterial Hypertension (694 PAH cases, 1560 controls). NIHRBR: UK National Institute for Health Research BioResource (847 PAH cases, 5048 controls). BHFPAH: British Heart Foundation Pulmonary Arterial Hypertension (275 PAH cases, 1983 controls). Meta-analyzed: overall results of PAH GWAS including all four studies.

Supplementary Figure 4 Individual MR causal estimates (IVW) for the secondary MR analysis of association of RDW to development of PAH – using 5 SNPs related to systemic iron status - from the four contributing studies in PAH GWAS. PAH ORs per one standard unit increase in RDW (dot) with the corresponding lower and upper 95% Cis (horizontal line). PHAAR: Pulmonary Hypertension Allele-Associated Risk (269 PAH cases, 1068 controls). PAHB: US National Biological ample and Data Repository for Pulmonary Arterial Hypertension (694 PAH cases, 1560 controls). NIHRBR: UK National Institute for Health Research BioResource (847 PAH cases, 5048 controls). BHFPAH: British Heart Foundation Pulmonary Arterial Hypertension (275 PAH cases, 1983 controls). Meta-analyzed: overall results of PAH GWAS including all four studies.

Supplementary Tables and Figures

	PAH (118 VUMC; 524 NIHRBR)		Controls (15,889 VUMC)	
	Female (%)	Male (%)	Female (%)	Male (%)
N	445 (69)	197 (31)	8,539 (54)	7,350 (46)
RDW (mean/SD)	15.1/2.20	15.5/2.33	13.6/1.41	13.6/1.39
Age (mean/SD)	52.4/17.6	58.2/16.2	54.3/16.1	58.1/14.7

Supplementary Table 2 Characteristics of the study population used for estimating/assessing the association between RDW and PAH, stratified by sex. PAH – Pulmonary Arterial Hypertension, VUMC - Vanderbilt University Medical Centre, NIHRBR - UK National Institute for Health Research BioResource, RDW – red cell distribution width.

Variable	Unadjusted		Adjusted	
	OR	95% CI	OR	95% CI
RDW (SD)	1.85	1.75 – 1.94	1.90	1.80 – 2.01
Age	-	-	0.98	0.98 – 0.99
Sex *(base=female)*	-	-	0.54	0.45 – 0.64

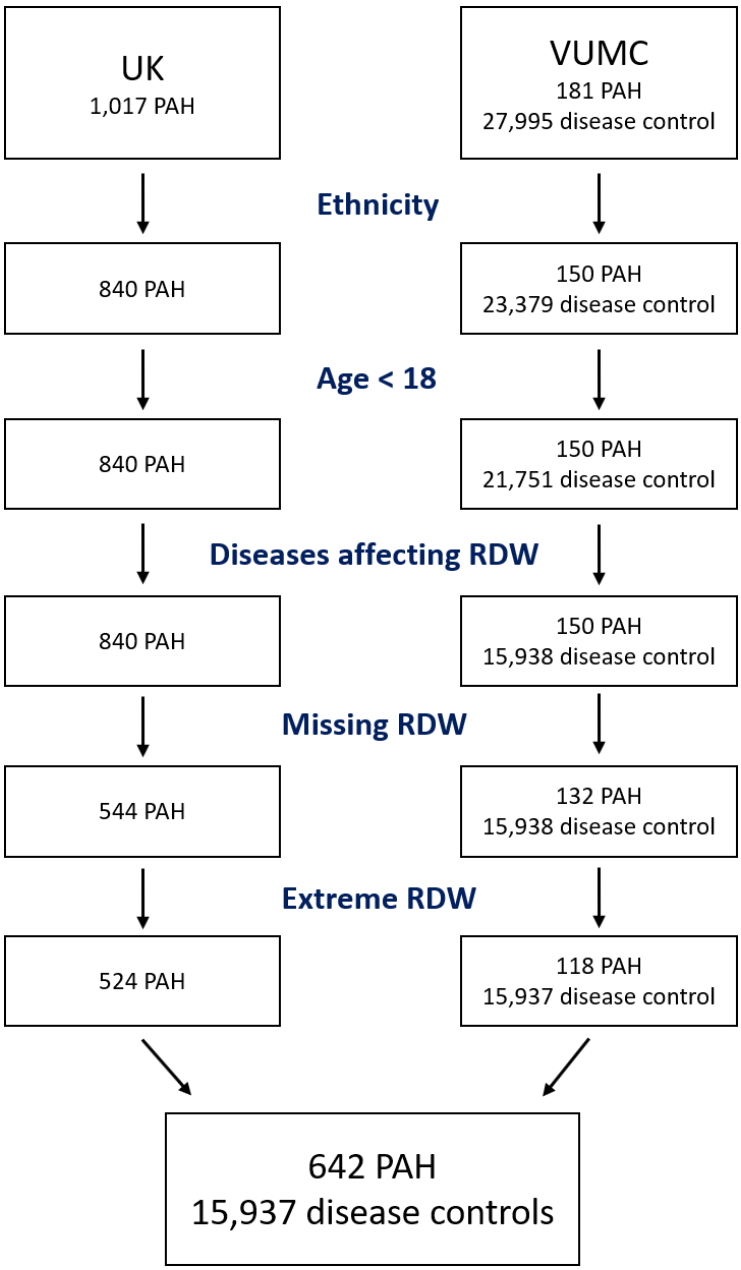
Supplementary Table 2 Logistic regression model predicting PAH disease status. We report the results of the adjusted model in the paper.

Variant information				RDW GWAS Astle et al.		Genetics of Iron Status GWAS Benyamin et al.								
Gene	Lead variant ID	Effect Allele	Allele Frequency in UKB and INTERVAL	beta RDW	*P* - value	Proxy variant ID*	beta iron	*P* – value iron	beta ferritin	*P* – value ferritin	beta TSAT	*P* – value TSAT	beta TF	P – value TF
HFE	rs144861591	C	0.92	0.21	6.50×10^{-216}	rs1800562	-0.37	4.0×10^{-77}	-0.21	1.4×10^{-29}	-0.58	1.5×10^{-178}	0.55	1.3×10^{-153}
TMPRSS6	rs855791	A	0.44	0.13	9.79×10^{-271}	-	-0.19	4.3×10^{-77}	-0.05	5.8×10^{-8}	-0.19	3.5×10^{-80}	0.04	1.3×10^{-4}
HFE	rs198851	G	0.85	0.13	2.55×10^{-161}	-	-0.19	1.6×10^{-40}	-0.06	3.6×10^{-6}	-0.23	4.7×10^{-59}	0.12	3.0×10^{-17}
TFRC	rs7619708	C	0.24	0.07	4.35×10^{-64}	rs6583288	0.03	1.2×10^{-2}	0.004	7.3×10^{-1}	0.05	3.8×10^{-6}	-0.06	3.8×10^{-8}
TFR2	rs9801017	G	0.37	0.05	9.40×10^{-37}	rs7385804	-0.06	7.2×10^{-8}	-0.02	2.5×10^{-2}	-0.05	1.8×10^{-7}	0.01	4.0×10^{-1}

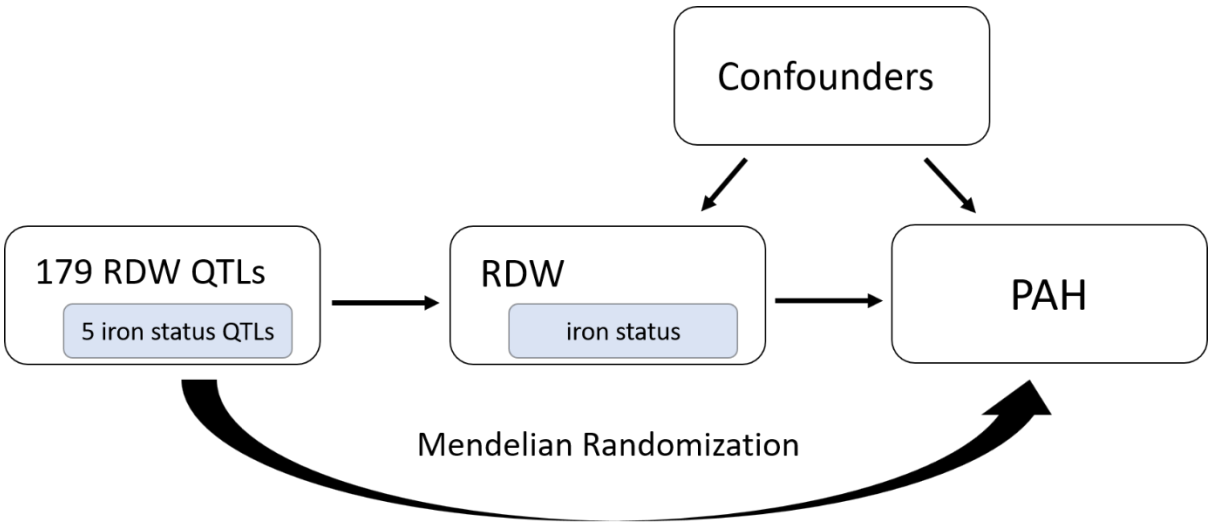
Supplementary Table 3 Five variants selected from the RDW GWAS based on their effects on systemic iron status. This table presents the effect estimates of these variants on RDW as reported by Astle et al. on RDW (Effect estimate per RDW SD; Effect estimate p-value). Elevated RDW can reflect iron deficiency which presents with decreased serum iron levels. In the Genetics of Iron Status GWAS, the two HFE variants reached genome-wide significance ($P< 5\times10^{-8}$) for all four (serum iron, transferrin, transferrin saturation, ferritin) iron status biomarkers, TMPRSS6 reached it for all but transferrin, TFRC reached it for transferrin and transferrin saturation, while TFR2 reached it for iron and transferrin saturation. The betas for RDW and the iron biomarkers from Benyamin et al. are reported in standard units. RDW = red cell distribution width; TSAT = transferrin saturation; TF = transferrin. *Where the lead RDW variant for the locus was not available in the Genetics of Iron Status GWAS we listed the results of a suitable proxy variant in strong linkage disequilibrium ($r^2 \geq 0.8$) with the RDW lead variant.

RDW GRS	GRS R^2	Null R^2	P value	Empirical P value
179 RDW QTLs	0.0264	0.054	2.4×10^{-94}	5.0×10^{-5}
5 RDW QTLs	0.0065	0.054	3.3×10^{-24}	5.0×10^{-5}

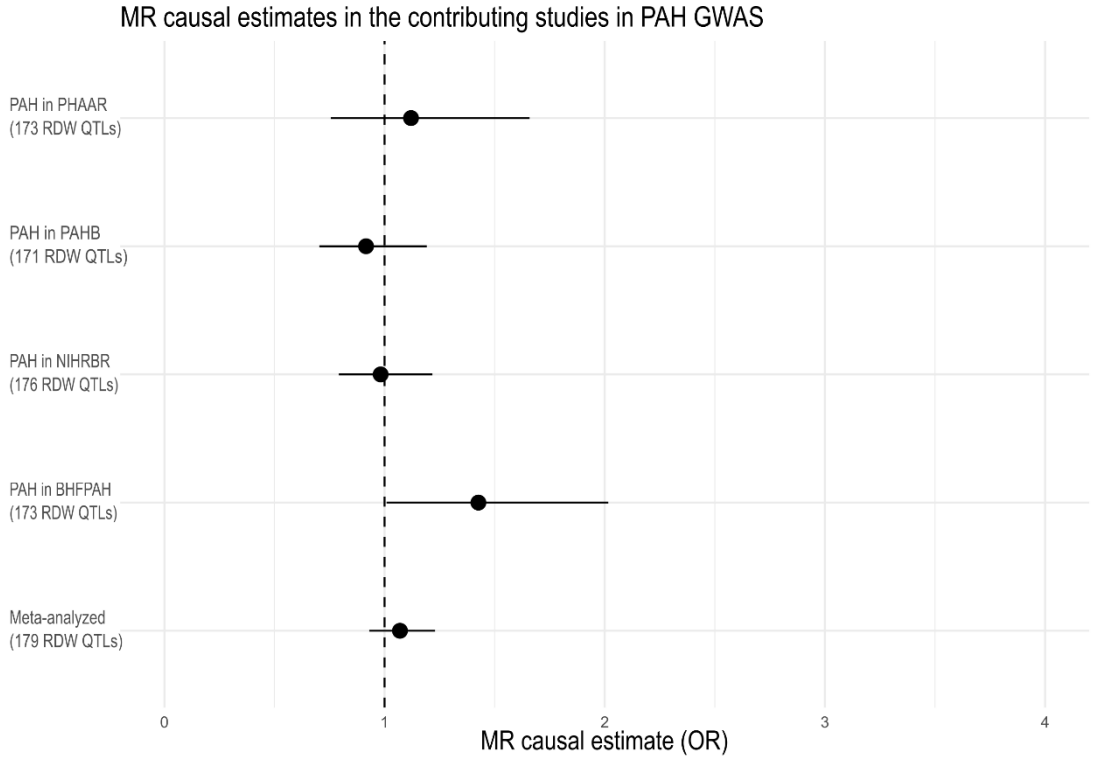
Supplementary Table 4 Summary of the RDW GRS models. The null model corresponds to the linear regression model without the GRS. The R^2 of the null models are identical since the sample and the covariates are the same. P value is the significance value of the model fit (F-test). Empirical p-values that account for multiple testing and overfitting were obtained through permutation tests (n=20,000).



Supplementary Figure 1 Flow diagram of the exclusion steps in the UK centers and the Vanderbilt University Medical Centre (VUMC) participating in the RDW and PAH association analysis as described in the Supplementary methods.

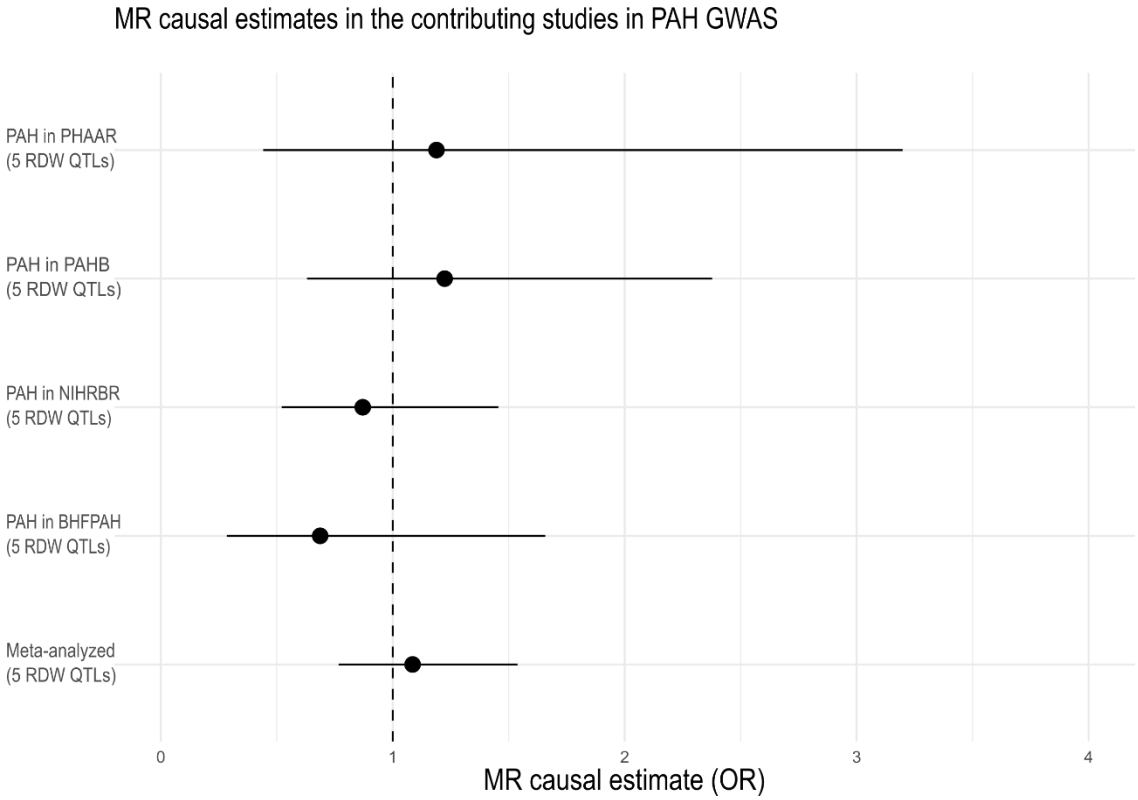


Supplementary Figure 2 Mendelian Randomization (MR) analyses of red cell distribution width (RDW) and pulmonary arterial hypertension (PAH). A two-sample design was used where effect estimates for the instrumental genetic variants were taken from two non-overlapping populations. RDW QTL and their effect estimates were taken from the largest-to-date population based RDW genetic association study (GWAS) (4). Effect estimates for the RDW QTL on PAH susceptibility were obtained from the largest-to-date PAH GWAS (7). The primary MR analysis included all 179 RDW QTL while the secondary MR analysis was restricted to five out of the 179 RDW QTL acting via iron status (Supplementary Table 3).



Supplementary Figure 3 Individual inverse variance weighted MR causal estimates for the main MR analysis of association between RDW and development of PAH - using all available RDW QTLs including suitable proxy variants with a minimum r^2 of 0.8 in each study - from the four contributing

studies in PAH GWAS. PAH ORs per one standard unit increase in RDW (dot) with the corresponding lower and upper 95% confidence intervals (horizontal line). The result of the BHFPAH (OR causal = 1.43, 95% CI = 1.01 – 2.02) did not survive the correction for multiple testing and was driven by the causal estimate of one variant (rs6883412). PHAAR: Pulmonary Hypertension Allele-Associated Risk (269 PAH cases, 1068 controls). PAHB: US National Biological Sample and Data Repository for Pulmonary Arterial Hypertension (694 PAH cases, 1560 controls). NIHRBR: UK National Institute for Health Research BioResource (847 PAH cases, 5048 controls). BHFPAH: British Heart Foundation Pulmonary Arterial Hypertension (275 PAH cases, 1983 controls). Meta-analyzed: overall results of PAH GWAS including all four studies.



Supplementary Figure 4 Individual MR causal estimates (IVW) for the secondary MR analysis of association of RDW to development of PAH – using 5 RDW QTLs related to systemic iron status - from the four contributing studies in PAH GWAS. PAH ORs per one standard unit increase in RDW (dot) with the corresponding lower and upper 95% confidence intervals (horizontal line). PHAAR: Pulmonary Hypertension Allele-Associated Risk (269 PAH cases, 1068 controls). PAHB: US National Biological Sample and Data Repository for Pulmonary Arterial Hypertension (694 PAH cases, 1560 controls). NIHRBR: UK National Institute for Health Research BioResource (847 PAH cases, 5048 controls). BHFPAH: British Heart Foundation Pulmonary Arterial Hypertension (275 PAH cases, 1983 controls). Meta-analyzed: overall results of PAH GWAS including all four studies.

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