Hemoglobin concentration, total hemoglobin mass and plasma volume in patients: implications for anemia

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Details of authors' contributions
H.E.M., T.R., M.G., and J.O. conceived the study. J.O., J.O.M.P., E.C., S.K., and D.W. assisted with data collection, with J.O. performing all statistical analyses. H.E.M., J.O. & J.O.M.P drafted the manuscript, to which T.R., M.G., W.S. then contributed. All authors read and approved the final version of the manuscript.

Running heads
[Hb], tHb-mass and PV in anemia

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Word Count
Abstract: 254 words
Main text: 4221
Tables: 2
Figures: 6
Supplemental files: 1

Trial registration
Not applicable

Acknowledgements

Abstract

In practice, clinicians generally consider anemia (circulating hemoglobin concentration < 120 g$\cdot$l^{-1} in non-pregnant females and < 130 g$\cdot$l^{-1} in males) as due to impaired hemoglobin synthesis or increased erythrocyte loss or destruction. Rarely is a rise in plasma volume relative to circulating total hemoglobin mass considered as a cause. But does this matter? We explored this issue in patients, using the optimized carbon-monoxide rebreathing method to measure hemoglobin concentration and thereby calculate plasma volume in healthy volunteers, surgical patients, and those with inflammatory bowel disease, chronic liver disease or heart failure.

We studied 109 participants. Hemoglobin mass correlated well with its concentration in the healthy, surgical and inflammatory bowel disease groups (r= 0.687-0.871, p< 0.001). However, they were poorly related in liver disease (r= 0.410, p= 0.11) and heart failure patients (r= 0.312, p= 0.16). Here, hemoglobin mass explained little of the variance in its concentration (adjusted R^2= 0.109 and 0.052; p= 0.11 and 0.16), whilst plasma volume did (R^2 change 0.724 and 0.805 in heart and liver disease respectively, p<0.0001). Exemplar patients with identical (normal or raised) total hemoglobin masses were diagnosed as profoundly anemic (or not) depending on differences in plasma volume that had not been measured or even considered as a cause. The traditional inference that anemia generally reflects hemoglobin deficiency may be misleading, potentially resulting in inappropriate tests and therapeutic interventions to address 'hemoglobin deficiency' not 'plasma volume excess'. Measurement of total hemoglobin mass and plasma volume is now simple, cheap and safe, and its more routine use advocated.

Introduction

Anemia is defined as a reduction in the circulating concentration of hemoglobin ([Hb]) to < 120 g·l^{-1} in non-pregnant females and < 130 g·l^{-1} in males.[1] Such reductions can result from the destruction or loss of erythrocytes or a failure of their production, or from impaired hemoglobin synthesis. Given its diverse etiology, anemia is common, affecting over 1.6 billion people worldwide [2], and is associated with impaired functional capacity, reduced quality of life [3], and poorer outcome in diverse disease states. [4-9]

For all these reasons, circulating hemoglobin concentrations are routinely measured in clinical practice. Once anemia is identified, the cause of impaired hemoglobin synthesis or erythrocytosis, or of increased red cell loss or destruction, is often sought and treatment (either of the underlying cause, or through the administration of packed donor red cells) initiated.

However, it is now becoming clear that this approach may be somewhat simplistic. The concentration of circulating hemoglobin will depend not just on the total circulating quantity of hemoglobin (total hemoglobin mass, (tHb-mass)), but also on the volume of plasma (plasma volume, PV) in which it is suspended. These factors are not routinely considered separately, or measured, in clinical practice and even in experienced hands their estimation is not trivial. However, such assessment may be important if the drivers of an altered [Hb]- and the appropriate therapeutic response- are to be truly understood. Thus, the circulating hemoglobin concentration in athletes matches that in sedentary individuals, meaning that a contribution of increased red cell oxygen carriage to

elite performance was largely dismissed. Then, in 2001, Heinicke and colleagues demonstrated that tHb-mass was increased by some 35% in elite endurance athletes, [Hb] matching that in the untrained only because PV was expanded to a similar degree. [10]

Likewise, alterations in the relationship between plasma volume and tHb-mass might strongly influence [Hb] in disease states. Anemia is common in diseases such as cancer [11], inflammatory bowel disease (IBD) [12], chronic heart failure (CHF) [8], chronic kidney disease (CKD) [13] and chronic liver disease (CLD). [14] Traditionally, this has been considered the result of a reduced tHb-mass. But a low [Hb] might also be found when hemoglobin synthesis, erythrocytosis and tHb-mass are all entirely normal (or even high), if plasma volume is disproportionately expanded. This can occur through disease-related changes in global water balance or distribution of body water. Thus, patients with inflammatory bowel disease might face enteric blood loss, suppressed hemoglobin synthesis or anemia of chronic inflammation; CLD patients may likewise lose blood, have an expanded circulating plasma volume due to hyperaldosteronism, or face fluid shifts as a result of raised portal venous pressure or hypoalbuminemia; and patients with chronic heart failure may suffer an increase in circulating volume due to factors including increased renin-angiotensin-aldosterone axis activity. [15] In contrast, contractions in PV caused by pharmacotherapy may mask a fall in tHb-mass by maintaining [Hb]. [16]

The extent to which this is true has not been addressed, largely due to historical methodological limitations: circulating red cell volume (RCV, ml) or plasma

volume has generally have been determined through radiolabelling of red blood cells or albumin respectively. [17] Such techniques are costly and time consuming, and not without risk, and are thus not routinely deployed unless in special circumstances (this method is recommended for disease diagnosis by the Polycythemia Rubra Vera Study Group). [18]

Such barriers may be overcome through the use of an 'optimized carbon monoxide (CO) rebreathing' (oCOR) method [19]: inhaled CO binds avidly to circulating hemoglobin, and the concentration of the resultant COHb complex can be readily measured. Knowing the quantity of absorbed CO, tHb-mass can be measured and, knowing [Hb], PV calculated.   The method is cheap, simple and safe to use- but has only rarely been applied in the clinical setting.

Thus, the relative contributions of PV and tHb-mass to measured [Hb] across disease states have not been described. We sought to address this issue.

<u>Methods</u>

This was a prospective, observational clinical study. We studied five groups: healthy volunteers (HV), preoperative patients awaiting major surgery and those suffering inflammatory bowel disease (IBD), as well as patients in whom alterations in plasma volume might more commonly occur (those with chronic liver disease, CLD) or chronic heart failure (CHF)). Each participant was studied on one occasion between February 2015 and May 2016.

Optimized Carbon Monoxide Rebreathing Method (oCOR)

The use of CO to determine tHb-mass was first proposed in the late 1800s, with refined techniques being published 100 years later. [20] In 2005, Schmidt and Prommer reported a simpler and faster technique (described in detail below) which also required less blood sampling. [19] It was applied almost exclusively, however, in the fields of athletic physiology, and thus failed to come to the attention of the bulk of the broader clinical/medical community.

tHb-mass was determined using the validated oCOR method described in detail by Schmidt and Prommer. [19] In brief, COHb concentration in blood was measured before and after 2-min rebreathing a known CO volume (0.5 to 1.0 $ml^.kg^{-1}$ in this study depending on gender). Each participant was seated for 15 minutes to allow stabilization of plasma volume, after which a mouthpiece connected them via a container of 'soda lime'~10g (carbon dioxide scrubber) to a spirometer (Spico-CO Respirations-Applikator, Blood Tec, Germany) and a 3 liter anaesthetic bag pre-filled with 100% oxygen. The patient exhaled to residual volume, breathed in the CO dose via the spirometer, held their breath for 10 s,

then continued normal breathing into the closed circuit via the spirometer for 1 min 50s. The participant then exhaled to residual volume, this exhaled volume being collected and analyzed to quantify the CO not absorbed into the bloodstream. Disconnected from the mouthpiece, participants finally fully exhaled to residual volume into a CO gas analyzer (Dräger Pac 7000, Dräegerwerk AG & Co. KGaA, Germany) before and at minutes 4 after CO rebreathing, to determine the CO concentration exhaled after disconnecting the patient from the spirometer that will also have not been adsorbed into the blood.

Statistical Analysis

Statistical analysis was performed using SPSS Statistics (Version 23.0 for Apple Macintosh, Chicago, IL, USA). Values are presented as mean ± standard deviation (SD), unless otherwise stated. Median and interquartile range (IQR) are reported when variables were not normally distributed. Categorical variables are presented as frequency (%). Pearson's correlation coefficient assessed the relationship between [Hb] and tHb-mass, allowing adjustment for PV (ml). Linear regression assessed the proportion of variance in tHb-mass explained by [Hb], allowing adjustment for PV (ml). In both correlation and regression analyses [Hb] and tHb-mass are expressed in $g \cdot l^{-1}$ and grams, respectively and PV in ml. This is also the case elsewhere, unless otherwise stated.

Differences across sub-groups were assessed by one-way analysis of variance (ANOVA), prior to which the assumption of normality was tested by the

Levene's test for homogeneity of variances. Where homogeneity of variance was verified, being the case for [Hb], Hct, tHb-mass (g), PV (%) and weight, a one-way ANOVA was performed, with *post hoc* comparisons by Gabriel's test due to the slightly different sample sizes across sub-groups. When homogeneity of variance was violated, as was the case for PV (ml & ml·kg^{-1}), age and tHb-mass (g·kg^{-1}), a Welch ANOVA [21] was used with *post hoc* comparisons made by the Games-Howell test, as this does not rely on the assumption of equal variances. [22] All tests were two-sided with statistical significance being accepted as a p-value of < 0.05.

Power calculation

The power calculation was based on the study by Hinrichs and colleagues [23] and was performed using G*3 Power version 3.1.9.2. [24] According to Hinrichs and colleagues, the relationship (expressed as Pearson's correlation coefficient) between [Hb] and tHb-mass was r= 0.59, p< 0.05. Based on this, using a two-tailed correlation: bivariate normal model, we calculated that 21 patients would provide 80% power at the 5% significance level to detect a correlation of at least r= 0.59 between [Hb] and tHb-mass. Given that five groups were studied, a total of 105 participants were required.

Ethical approval

Ethical approval was granted by the London- Camden and Kings Cross Research Ethics Committee (REC reference: 13/LO/1902). Written informed consent was obtained from all participants.

Results

One hundred and nine patients (61% male: mean (IQR) age 52 (36-64) years) consented to take part in the study. Supplementary Figure 1 shows a Consolidated Standards of Reporting Trials (CONSORT) flow diagram indicating included and excluded patients and sub-groups. Sixteen patients were tested at Southampton General Hospital, ninety at UCLH (including HV) and three at the Royal Free Hospital (RFH). Surgical specialties are shown in Supplementary Table S1 online with patient characteristics and etiology of disease for IBD patients in Supplementary Table S2. Patient characteristics, medications and etiology of CLD and CHF are shown in Supplementary Table S3 and Table S4 online, respectively. There were no differences in weight between sub-groups. Healthy volunteers (n= 21) were younger compared to all patient groups (p< 0.0001) with CHF patients (n= 22) being older than IBD (n= 21, p= 0.001), surgical (n= 28, p= 0.008) and CLD patients (n= 16, p= 0.002). Figure 1 A-D shows hematological variables and Figure 2 A-B plasma volumes across different sub-groups.

Hemoglobin concentration

Hemoglobin concentration was 128.4 ± 18.1 g·l^{-1} in the subjects overall. Across sub-groups, CLD patients had lower [Hb] when compared to HV and other disease groups (p< 0.0001, Figure 1B). Anemia prevalence (n=34 [39%]) varied across disease sub-groups (CLD 15 [94%], CHF 12 [55%], IBD 2 [9%], surgical 5 [18%]). Amongst healthy volunteers, 5 (24%, all female) were anemic.

Total hemoglobin mass

Mean ± SD tHb-mass was 669 ± 181 grams (8.5 ± 1.9 $g \cdot kg^{-1}$ body mass) in subjects overall, with no statistically significant differences across sub-groups (Figure 1, C, D). tHb-mass was higher in males than females: (758 ± 152 vs. 533 ± 132 g, p< 0.0001; and 8.9 ± 1.8 vs. 7.9 ± 2.0 $g \cdot kg^{-1}$, p= 0.006, respectively).

Plasma volume

Mean ± SD plasma volume was 3667 ± 1020 ml (47.1 ± 11.3 $ml \cdot kg^{-1}$) in subjects overall. PV was higher in males than females (4040 ± 1038 vs. 3093 ± 672 ml respectively, p< 0.0001), but not when weight adjusted (47.6 ± 11.3 vs. 46.2 ± 11.5 $ml \cdot kg^{-1}$, p= 0.556). It also differed across disease groups, being expanded and more varied in CLD (4965 ± 1447 ml) compared to HV (3429 ± 538 ml, p= 0.006), IBD (3202 ± 653 ml, p= 0.002) and surgical patients (3297 ± *590* ml, p= 0.003), but not CHF (3883 ± 953 ml, p= 0.100). Adjusted for body weight, PV was similarly expanded in CLD (59.1 ± 16.0 $ml \cdot kg^{-1}$) when compared to IBD (42.5 ± 8.3 $ml \cdot kg^{-1}$, p= 0.008) and surgical patients (41.3 ± 7.8 $ml \cdot kg^{-1}$, p= 0.004), but again was similar to that in CHF patients (48.6 ± 9.2 $ml \cdot kg^{-1}$, p= 0.172) or HV (49.1 ± 7.9 $ml \cdot kg^{-1}$, p= 0.191).

Hemoglobin concentration was influenced by the degree of PV expansion (Figure 4A), being lower in patients with severe PV expansion (n= 46, 119.0 ± 17.7 $g \cdot l^{-1}$) than mild to moderate PV expansion (n= 36, 131.1 ± 13.8 $g \cdot l^{-1}$, p= 0.005), and normal PV (n= 24, 140.7 ± 15.5 $g \cdot l^{-1}$, p< 0.0001), but not PV contraction (n= 3, 143.8 ± 11.8 $g \cdot l^{-1}$, p= 0.139)

Relationships between hemoglobin concentration and total hemoglobin mass

In the study cohort as a whole, [Hb] ($g \cdot l^{-1}$) correlated with tHb-mass (g) (r= 0.500, p< 0.0001, n= 109), this being true in both males (r= 0.452, p< 0.0001) and females (r= 0.462, p< 0.0001). Whilst true in HV and in IBD and surgical patients (r= 0.871, p< 0.0001; r= 0.687, p< 0.0001; and r= 0.763, p< 0.0001 respectively; Figure 3 A-E) this was *not* the case in patients with CLD or CHF (r= 0.410, p= 0.114 and r= 0.312, p= 0.157, respectively).

Whilst consistently statistically significant, the strength of the relationship between [Hb] and tHb-mass weakened as plasma volume rose, r-values falling from 0.94 to 0.91, 0.86 and 0.57 for those with low (n=3), normal (n=24), mild-moderately expanded (n=36) and severely expanded (n= 46) plasma volumes respectively (p= 0.216 for the 3 with low PV, and p<0.0001 for the others). Sub-group analysis is hampered by small numbers, but data are presented in Supplementary Table S5 online for completeness.

Total hemoglobin mass, plasma volume and hemoglobin concentration on an individual level

The data presented thus suggest that, at an individual level, [Hb] was not a good guide to tHb-mass- being strongly influenced by PV. This is illustrated in Figure 5 A-E, which shows data from individual participants ranked by weight-adjusted tHb-mass from smallest to largest with corresponding PV ($ml \cdot kg^{-1}$) and [Hb] ($g \cdot l^{-1}$) in HV (A), IBD (B), surgical (C), CLD (D) and CHF (E) patients, respectively. These show that patients who share a very similar tHb-mass may exhibit

markedly different [Hb] due to differences in PV. For example, in patients with IBD (Figure 5B), patient numbers 17 and 18 have a very similar tHb-mass (9.2 $g^.kg^{-1}$ and 9.3 $g^.kg^{-1}$, respectively), yet one is defined as having a high normal [Hb] (161 $g^.l^{-1}$) and the other as being anemic ([Hb] 107 $g^.l^{-1}$), due to substantial differences in PV (37.2 $ml^.kg^{-1}$vs 65.7 $ml^.kg^{-1}$). Similarly, in CLD patients (Figure 5E), tHb-mass in patient numbers 2 and 3 are the same (5.2 $g^.kg^{-1}$) but the first is considered to have a normal [Hb] (110 $g^.l^{-1}$), but the second to be markedly anemic ([Hb] 69 $g^.l^{-1}$) due to a relatively raised PV in the latter (36.5 vs. 67.8 $ml^.kg^{-1}$ respectively).

Linear regression models

In the whole population, tHb-mass explained 25.0% of the variance in [Hb] (adjusted R^2 = 0.250, p< 0.0001). However, tHb-mass explained different amounts of the variance in [Hb] across patient groups, adjusted R^2 for HV, surgical and IBD patients being 0.746, 0.565 and 0.446 respectively (p< 0.0001 in all cases). Of particular note, tHb-mass did *not* significantly explain variance in [Hb] in the two patient groups most likely to suffer expanded plasma volume and shifts in fluid- CLD (adjusted R^2= 0.109, p= 0.114) or CHF patients (adjusted R^2= 0.052, p= 0.157). In keeping, PV independently accounted for a greater proportion of the variance in [Hb] in these groups (R^2 change 0.724 in CHF and 0.805 in CLD) than in HV (0.192), surgical patients (0.374) or IBD patients (0.479) (p<0.0001 in all cases).

Hematological variables by anemia status

In the 39 anemic subjects (mean ± SD [Hb] 109.6 ± 12.5 g·l^{-1}), when compared to the 70 non-anemic ([Hb] 138.9 ± 10.6 g·l^{-1}), tHb-mass (g & g·kg^{-1}) was significantly lower (594 ± 192 vs. 711 ± 162 g, p= 0.001; 7.7 ± 1.9 vs. 9.0 ± 1.8 g·kg^{-1}, p= 0.001). However, PV (ml, and ml·kg^{-1}) was also significantly higher in anemic subjects (4083 ± 1351 vs. 3434 ± 686 ml, p= 0.007; 52.6 ± 12.2 vs. 44.0 ± 9.6 ml·kg^{-1}, p< 0.0001: (see Table 1).

We have studied the contribution of changes in circulating hemoglobin and plasma volume to differences in the concentration of circulating hemoglobin. We have done so in healthy volunteers and across a variety of disease states, selected to be more or less likely to influence intravascular fluid status. By doing so, we have shown that (i) variation in plasma volume contributes significantly to variation in hemoglobin concentration, (ii) this contribution is greater in cases of chronic liver disease of heart failure- diseases in which changes in total body water and in its distribution are more likely to occur, and (iii) perhaps most importantly, this may lead to some individuals being diagnosed as anemic whilst having a tHb-mass which is normal or even supra-normal. These findings are of direct clinical relevance. A basic medical education teaches that plasma volume may be expanded in some disease states. But it is rarely if ever mentioned that this might lead to hemodilution of such degree as to cause anemia. Thus, a diagnosis of anemia generally leads to investigation of a cause for reduced hemoglobin synthesis or increase erythrocyte destruction or loss. When such features are not identified, a diagnosis of 'anemia of chronic disease' is likely made. Rarely if ever is hemodilution considered as a cause, and measurement of tHb-mass or plasma volume performed. This is exemplified by the fact that no such measurements had been performed electively in the patients we studied. Such deficits in consideration or action may in part relate to the difficulty and expense of measuring such variables through traditional methods (radiolabelling of red blood cells, for instance).

Thus, tHb-mass was generally strongly related to [Hb] (r $\geq$0.687 and p <0.0001 in all cases); tHb-mass explained a good deal of the variance in [Hb] (adjusted R^2 in HV, surgical and IBD patients being 0.746, 0.565 and 0.446 respectively, p< 0.0001 in all cases); and PV independently accounted for only a small proportion of the variance in [Hb] over that due to tHb-mass (R^2 change 0.192, 0.374) or 0.479 in HV, surgical and IBD patients respectively). By contrast, in the two patient groups most likely to suffer expanded plasma volume (CLD and CHF), tHb-mass did not correlate with [Hb] (r= 0.410, p= 0.114; r= 0.312, p= 0.157, respectively). Likewise, tHb-mass did not significantly explain variance in [Hb] (adjusted R^2= 0.109, p= 0.114; adjusted R^2= 0.052, p= 0.157, respectively), whilst PV independently accounted for a greater proportion of the variance in [Hb] over and above tHb-mass in these groups (R^2 change 0.724 in CHF and 0.805 in CLD). Thus, [Hb] is strongly influenced by disease-related changes in PV. The relationship between [Hb] and tHb-mass weakened as plasma volume rose (r-values falling from 0.94 and 0.91 in those with low or normal PV, to 0.57 amongst those in whom PV was severely expanded.

As a consequence, even amongst those in our small sample, we identified patients with identical and normal tHb-mass, in some of whom severe anemia would be diagnosed, likely triggering investigations focused upon failed erythrogenesis or increased red cell destruction. As specific exemplars of the phenomenon, two IBD patients had similar tHb-masses (9.2 and 9.3 g·kg^{-1}), one having a high normal [Hb] (161 g·l^{-1}) and the other being anemic ([Hb] 107 g·l^{-1}), due to substantial differences in PV (37.2 ml·kg^{-1} vs. 65.7 ml·kg^{-1}). Similarly, two CLD

patients had the same tHb-mass (5.2 g·kg^{-1}), one having a normal [Hb] (110 g·l^{-1}), but the second being markedly anemic ([Hb] 69 g·l^{-1}) due to a relatively raised PV in the latter (36.5 vs. 67.8 ml·kg^{-1} respectively).

Our findings are thus of real clinical importance, as a significantly low [Hb] can trigger a raft of (unwarranted) investigations (such as the assay of circulating hematinic factors) or treatments (such as the administration of packed red blood cells), whilst denying the administration of agents to reduce plasma volume, which might sometimes be required. Blood transfusion itself carries risks [25] as well as a price in terms of healthcare costs. Meanwhile, in other circumstances, contraction in plasma volume might offer false reassurance by maintaining [Hb], when tHb-mass is low.

Whilst tHb-mass is generally used as an index of oxygen carrying capacity and of circulating red cell mass, others have previously reported total circulating red blood cell volume (RCV) in this regard. In hematologically normal control subjects, hematocrit (Hct) in the 20-50% range reportedly correlated well with RCV (determined by ^{51}Cr labeling of RBCs: r= 0.880, p< 0.001). [26] However, this relationship was disturbed when Hct fell outside this range, owing to wider variability in PV. Such data support those from other radiolabelling studies in suggesting that direct measurement of RCV (rather than the use of [Hb] or Hct) is required for the accurate diagnosis of polycythemia. [27] Likewise, data derived from the same technique which we applied (oCOR) show PV to be expanded (variably, but along with increased RCV) in polycythemia rubra vera. [28]

The focus of such studies differed from ours: namely, they sought to address the degree to which variation in PV altered the accuracy of the diagnosis of polycythemia, whilst we assessed the influence of variation in tHb-mass and PV on [Hb] *per se* and on the diagnosis of anemia. Nor have any studies in this field been comprehensive across disease states, or assessed tHb-mass (rather than RCV). Nonetheless RCV (by ^{51}Cr labeling) has been shown to be similar in anemic and non-anemic CHF patients, suggesting that PV expansion accounted for this diagnosis. [15] Indeed, this has been shown to occur (using I^{131}-labeled albumin) in CHF due to systolic dysfunction, with poor correlation between [Hb] and RCV. [29] Likewise, and using the same technique, Miller showed 19 of 32 patients hospitalized with decompensated CHF to be anemic, with only 4 of these having a true reduction in RCV. [30] Using the carbon monoxide rebreathing method, we extend such observations (see Table 2). Overall, 39 of the 109 participants were anemic, in only 2 (13.3%) of whom was this due to a reduced tHb-mass [352 g and 449 g] in the context of a normal PV. In the remaining 86.7%, reduced [Hb] was accounted for by PV expansion (n= 4 mild to moderate, tHb-mass 494 ± 76 g, and n= 8 severe, tHb-mass 539 ± 105 g). Interestingly, 14 of the 39 anemic patients (93%) had a relatively *raised* tHb-mass, with PV elevated to a greater degree (n= 1 mild to moderate PV expansion (tHb-mass 610 g), n= 13 severe PV expansion (tHb-mass 758 ± 220 g).

The overall prevalence of anemia for our study participants (36%) is similar to that reported in previous studies in non-cardiac surgical patients (30.4%). [9] Nine per cent of IBD patients suffered anemia, which is less than has been reported across European Countries (24%). [12] Some 54% of CHF patients suffered anemia

in the current study, somewhat more than has been previously reported in the Study of Anemia in a Heart Failure Population (STAMINA-HFP) Registry (34%), [31] or in patients with advanced HF (30%), [32] but in keeping with the data of others (55.6% [33] to 61%). [34] Of CLD patients, 94% suffered anemia- a figure somewhat higher that that previously reported by some (50-75%), [35,36] but in keeping with data in decompensated CLD (86%) [37] or hepatitis C infection (75%). [38]

Amongst healthy volunteers, five (24% of healthy volunteers) were anemic- a figure in keeping with global data relating to non-pregnant females (30%), [2] and only slightly higher than the 16% reported in non-pregnant women aged 15-49 years from high income regions and 22% in menstruating women from central and eastern Europe. [39] This may be related to volunteering bias in the current study whereby those who thought they might be anemic preferentially applied to participate.

This study utilized the optimized carbon monoxide rebreathing method, validated against [51]Chromuim radiolabelling methodologies. [40] An advantage of our study was its assessment of diverse patient groups. Sample sizes, whilst small, were appropriately powered to explore the relationship between tHb-mass and [Hb]-albeit that, for administrative reasons, the sample size for patients with CLD (n= 16) was below the 21 we originally sought, based on the study by Hinrichs and colleagues. [23] Nonetheless, we were yet able to demonstrate that PV changes in this group do influence assessed [Hb] and anemia diagnoses.

Differing blood sample methods (capillary and venous) yield identical Δ%COHb (and thus tHb-mass) values. [41] However, capillary [Hb] can be marginally higher than that in venous blood. [42-45] The use of differing sampling techniques across testing sites may thus have contributed a little to variation in measured [Hb], although capillary blood [Hb] values were all corrected to venous conditions, and all blood samples were collected from the same anatomical site, and with patients in the same posture (seated). This factor does not therefore weaken the significance of our findings. Whilst the use of different blood gas machines and testing staff may have introduced error in the measurement of tHb-mass, we found a typical error (TE) of repeat tHb-mass measurements of 1.93% (95% CI 1.3-3.4%: unpublished data), values in keeping with other institutions using the oCOR method. [19, 46]

Finally, the oCOR method is quick and simple, avoids the technical difficulties of working with radiolabelled compounds, [40] offers a minimal burden for patients, is minimally invasive and is safe even in patients with serious medical conditions and comorbidities such as stable coronary artery disease. [47] This greatly widens the applicability of the oCOR test to measure tHb-mass and plasma volume in the clinical setting. To date, its clinical experimental application has been sparse- (e.g. to demonstrate that low tHb-mass may account for impaired exertional performance in otherwise healthy diabetics. [48] Whilst (rarely yet) applied to the measurement of red cell mass in patients with polycythemia rubra vera, [28] it has yet to be utilized in routine clinical practice but might find great value, for example in the estimation of red cell mass and plasma volume in cases of presumed excessive erythrocytosis. Our data might support wider use.

In conclusion, measured [Hb], and the diagnosis of anemia, can be strongly influenced by (or can largely depend upon) changes in plasma volume. The scale of this impact may be greater in some diseases than others. Constraining investigation of anemia to the identification of causes of reduced Hb synthesis or of erythrocyte loss or destruction may be inappropriate for many. The concept of 'anemia' may thus need refining in clinical practice, and the oCOR method may support better and more appropriate assessments of the factors influencing circulating [Hb].

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Tables

Table 1. Hematological variables in anemic and non-anemic participants.

Variable	Anemic (n= 39)	Non-anemic (n= 70)	P-value
[Hb] (g$\cdot$l^{-1})	109.6 ± 12.5	138.9 ± 10.6	< 0.0001
Hct (%)	35.0 ± 5.9	42.4 ± 3.0	< 0.0001
tHb-mass (g)	594 ± 192	711 ± 162	0.001
tHb-mass (g$\cdot$kg^{-1})	7.7 ± 1.9	9.0 ± 1.8	0.001
BV (ml)	5978 ± 1837	5606 ± 1146	0.257
BV (ml$\cdot$kg^{-1})	77.3 ± 16.7	71.6 ± 14.8	0.066
PV (ml)	4083 ± 1351	3434 ± 686	0.007
PV (ml$\cdot$kg^{-1})	52.6 ± 12.1	44.0 ± 9.6	< 0.0001
PV (%)	68%	61%	< 0.0001
RCV (ml)	1894 ± 692	2171 ± 501	0.018
RCV (ml$\cdot$kg^{-1})	24.7 ± 7.1	27.5 ± 5.7	0.023
MCV (fl)	88.0 ± 6.2	91.0 ± 6.6	0.038
MCH (pg)	29.3 ± 2.9	30.1 ± 2.2	0.218
MCHC (g$\cdot$l^{-1})	333.1 ± 18.0	330.5 ± 12.2	0.477
RDW (%)	14.5 ± 1.7	13.7 ± 1.1	0.024
Creatinine (μmol$\cdot$l^{-1})	114.9 ± 117.4	84.3 ± 58.2	0.166
Albumin (g$\cdot$l^{-1})	37.4 ± 8.7	43.7 ± 4.9	0.001

[Hb], Hemoglobin concentration; Hct, hematocrit; tHb-mass, total hemoglobin mass; BV, blood volume; PV, plasma volume; RCV, red cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width. Anemia defined according to World Health Organization criteria ([Hb] <130 g$\cdot$l^{-1} in men and <120 g$\cdot$l^{-1} in women. Data are presented as mean ± SD, or frequency (%).

Table 2. Anemia classification based on hemoglobin concentration, red cell volume and plasma volume in all patients (n= 109).

Anemia status		Normal PV	Mild to moderate PV expansion	Severe PV expansion	PV contraction	Total
Non anemic	Normal RCV	12	8	1	1	22
	RCV deficit	4	1	0	2	7
	RCV excess	5	17	19	0	41
	Total	21	26	20	3	70
Anemic	Normal RCV	1	5	5	0	11
	RCV deficit	2	4	8	0	15
	RCV excess	0	1	13	0	14
	Total	3	10	26	0	39

Normal BV, PV and RCV were classified as derived volumes from measured tHb-mass within ± 8% of the expected normal volumes on an individual level. Mild to moderate volume expansion was considered >8% to <25% deviation from expected norms, and severe as >25% of the expected normal volume. BV, blood volume, PV, plasma volume, RCV, red cell volume. Anemia defined according to World Health Organization criteria ([Hb] <130 g^{-1} in men and <120 g^{-1} in women. Values are expressed as counts

Figure legends

Figure 1. Hematological variables in healthy volunteers and patient sub-groups. Hct (%), hematocrit percentage (A); [Hb], hemoglobin concentration (B); tHb-mass, total hemoglobin mass (g & g·kg^{-1}) (C & D). HV, healthy volunteers; IBD, inflammatory bowel disease; HF, heart failure; LD, liver disease. *p< 0.0001 for Hct and [Hb] in LD patients compared to all other groups. No differences in tHb-mass (g or g·kg^{-1}) between groups. Data expressed as mean (± standard deviation error bars).

Figure 2. Plasma volume in healthy volunteers and patient sub-groups. Plasma volume (ml & ml·kg^{-1}) (A & B). HV, healthy volunteers; IBD, inflammatory bowel disease; HF, heart failure; LD, liver disease; PV, plasma volume. (A) PV (ml) between LD and HV, *p= 0.006, PV (ml) between LD and IBD, **p= 0.002 and PV (ml) between LD and surgical patients, †p= 0.003; (B) PV (ml kg^{-1}) between LD and IBD, *p= 0.008, PV between LD and surgical patients, **p= 0.004. Data expressed as mean (± standard deviation error bars).

Figure 3. Unadjusted relationship between hemoglobin concentration and total hemoglobin mass. Healthy controls (A, n= 21), patients with IBD (B, n= 22), surgical patients (C, n= 28), liver disease (D, n= 16) and HF (E, n= 22). tHb-mass (g), total hemoglobin mass; [Hb] (g·l^{-1}), hemoglobin concentration; IBD, inflammatory bowel disease; HF, heart failure.

Figure 4. Hemoglobin concentration and hematocrit in all patients categorized by plasma volume status. Plasma volume contraction (n= 3) was classified as > minus 8% from expected norms. Normal PV (n= 24) was classified as derived PV within ± 8% of the expected normal volume on an individual level. Mild to moderate volume expansion (n= 36) was considered >8% to <25% deviation from expected norms, and severe PV expansion (n= 46) as >25% of the expected normal volume. *p= 0.005 for [Hb] in severe PV expansion vs. mild to moderate, †p< 0.0001 for [Hb] in severe PV expansion vs. normal PV. PV, plasma volume; [Hb], hemoglobin concentration (g·l^{-1}); Hct (%), hematocrit percentage. Data expressed as mean (± standard deviation error bars).

Figure 5. Individual participant data for total hemoglobin mass, plasma volume and hemoglobin concentration. Participants ranked by tHb-mass (g·kg^{-1}) (dark grey bars) from smallest to largest with corresponding PV (ml·kg^{-1}) (light grey bars) and [Hb] (g·l^{-1}) (black circles) in healthy volunteers (A), inflammatory bowel disease (B), surgical (C), chronic heart failure (D), and chronic liver disease (E). tHb-mass, total hemoglobin mass; PV, plasma volume; [Hb], hemoglobin concentration.
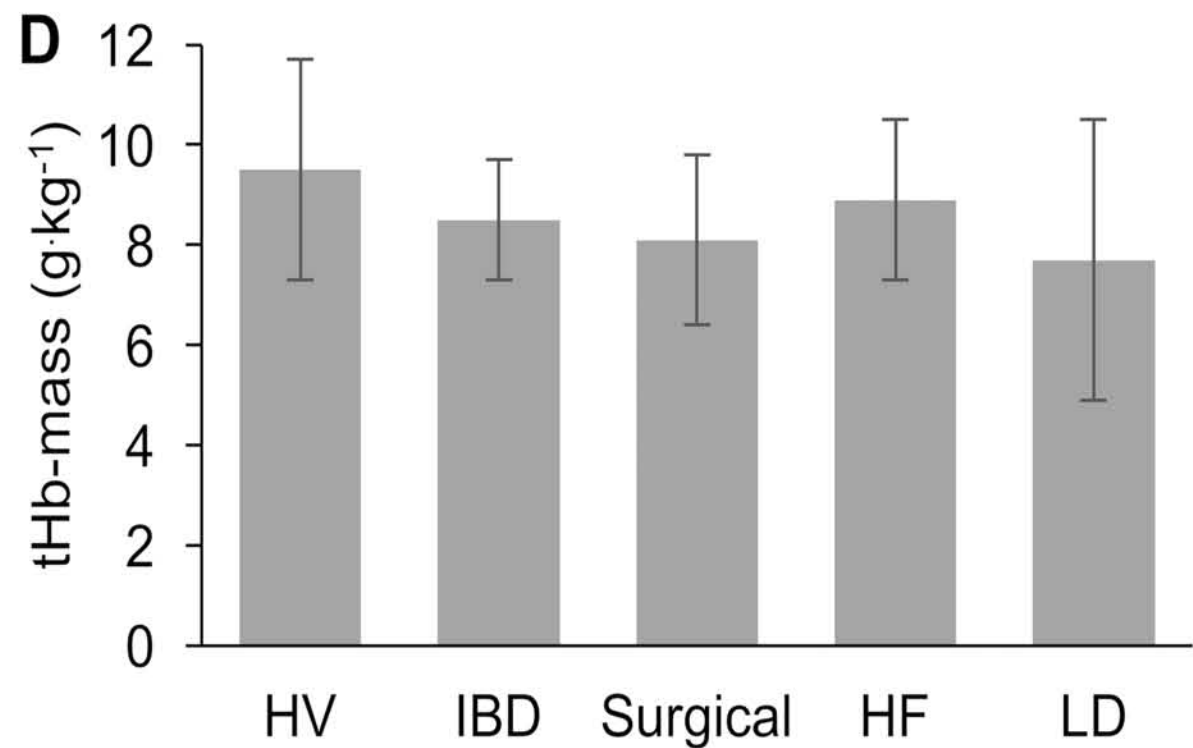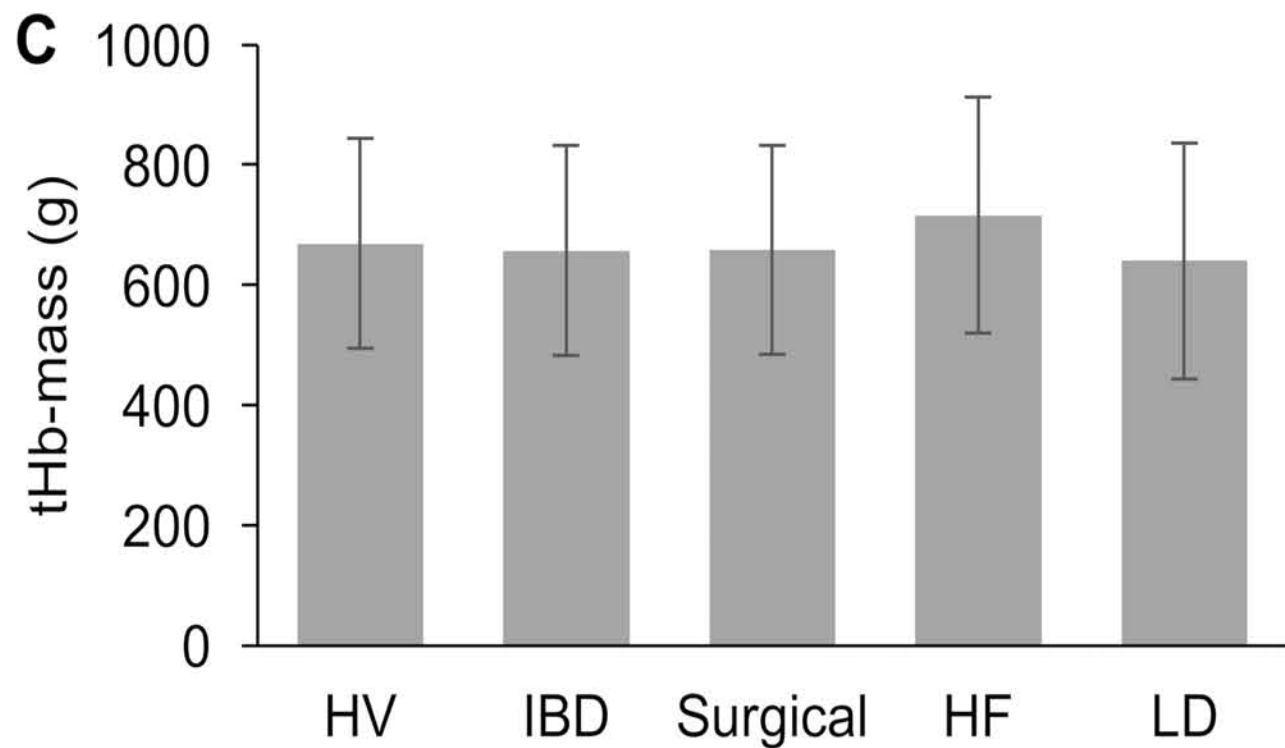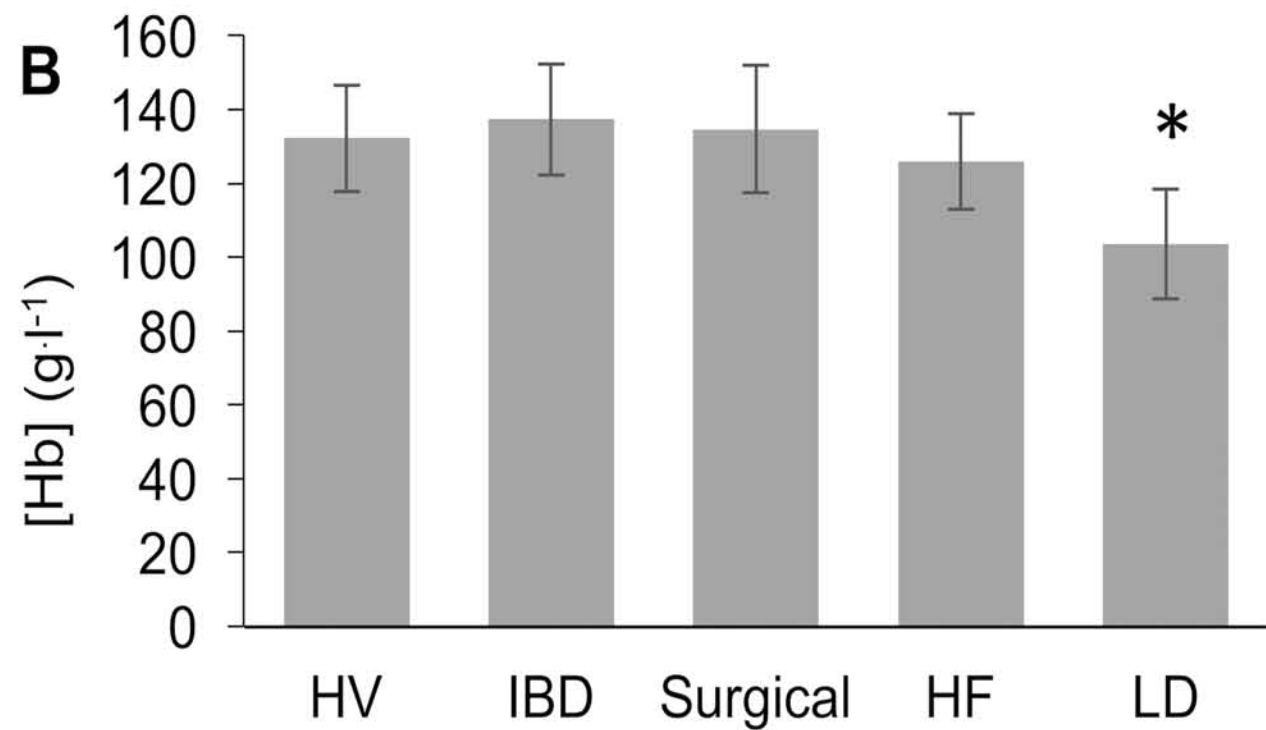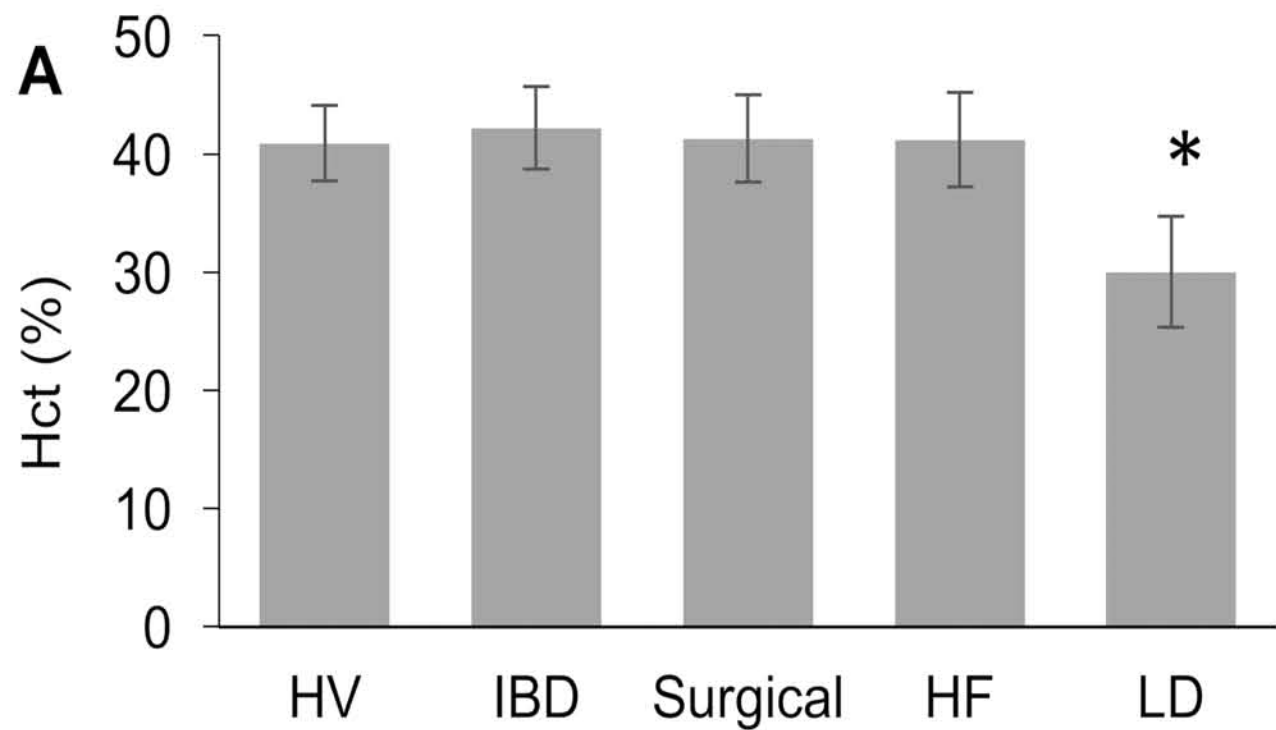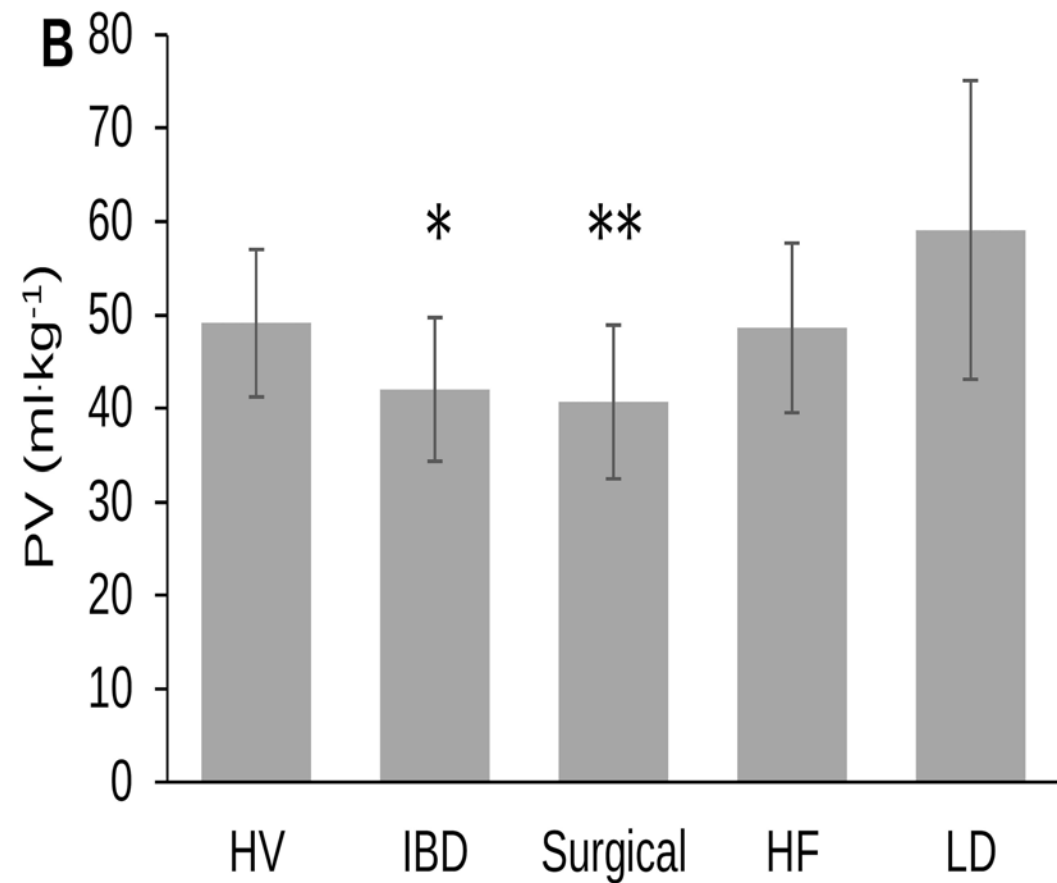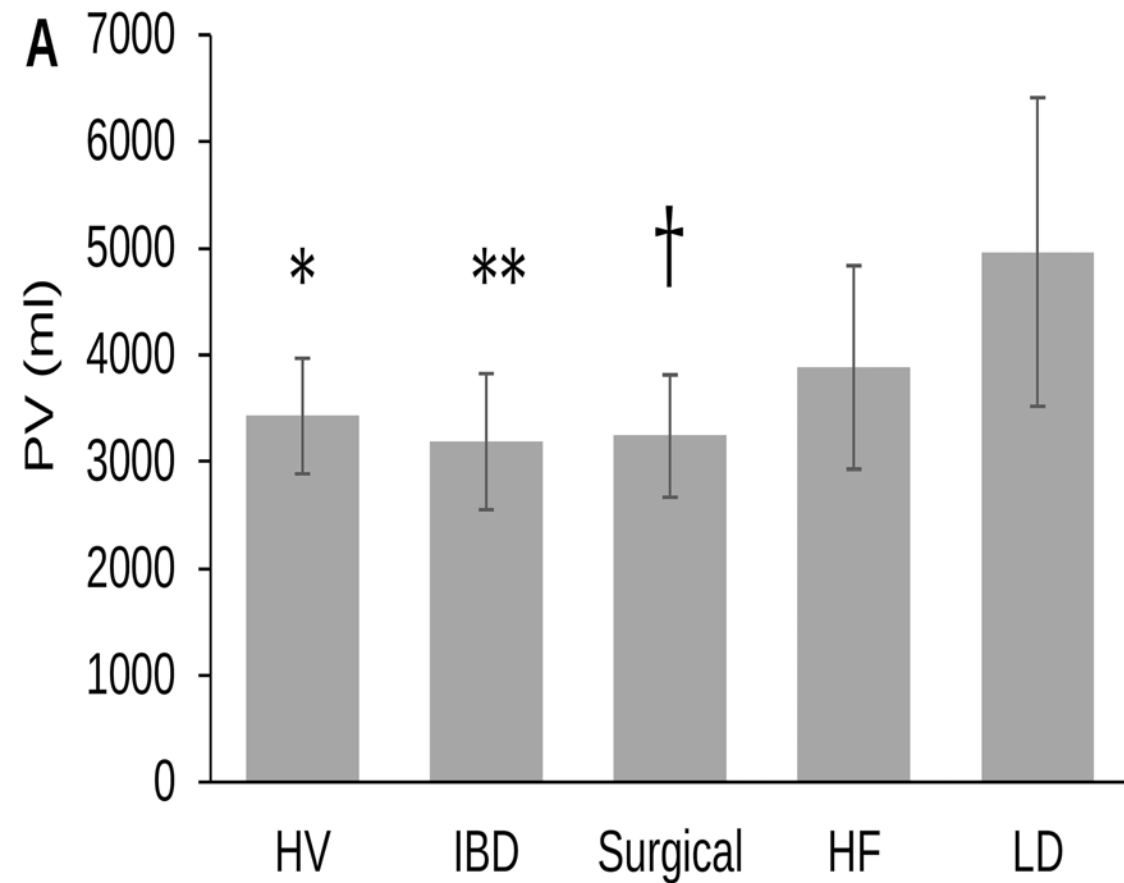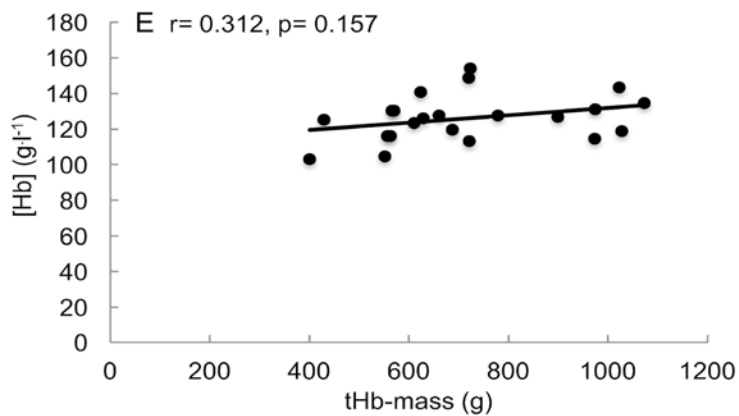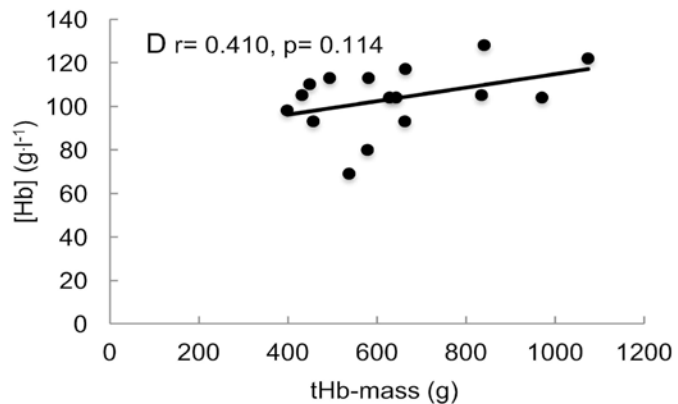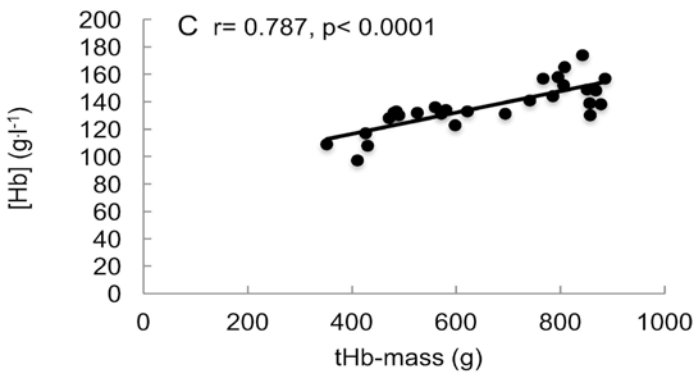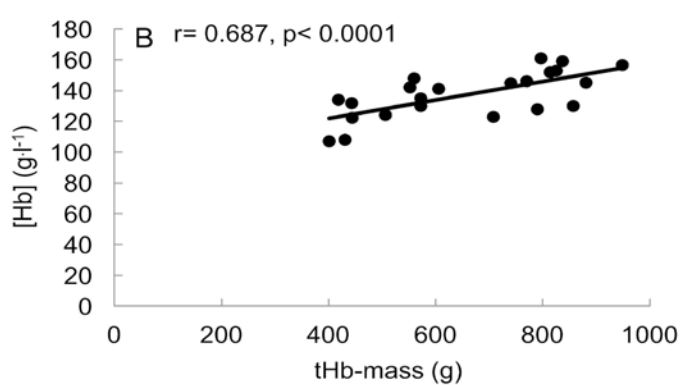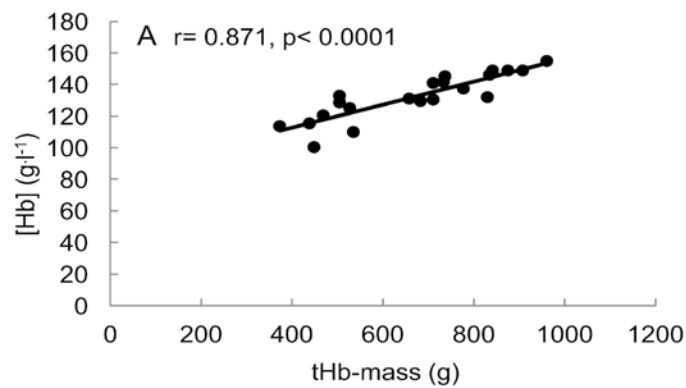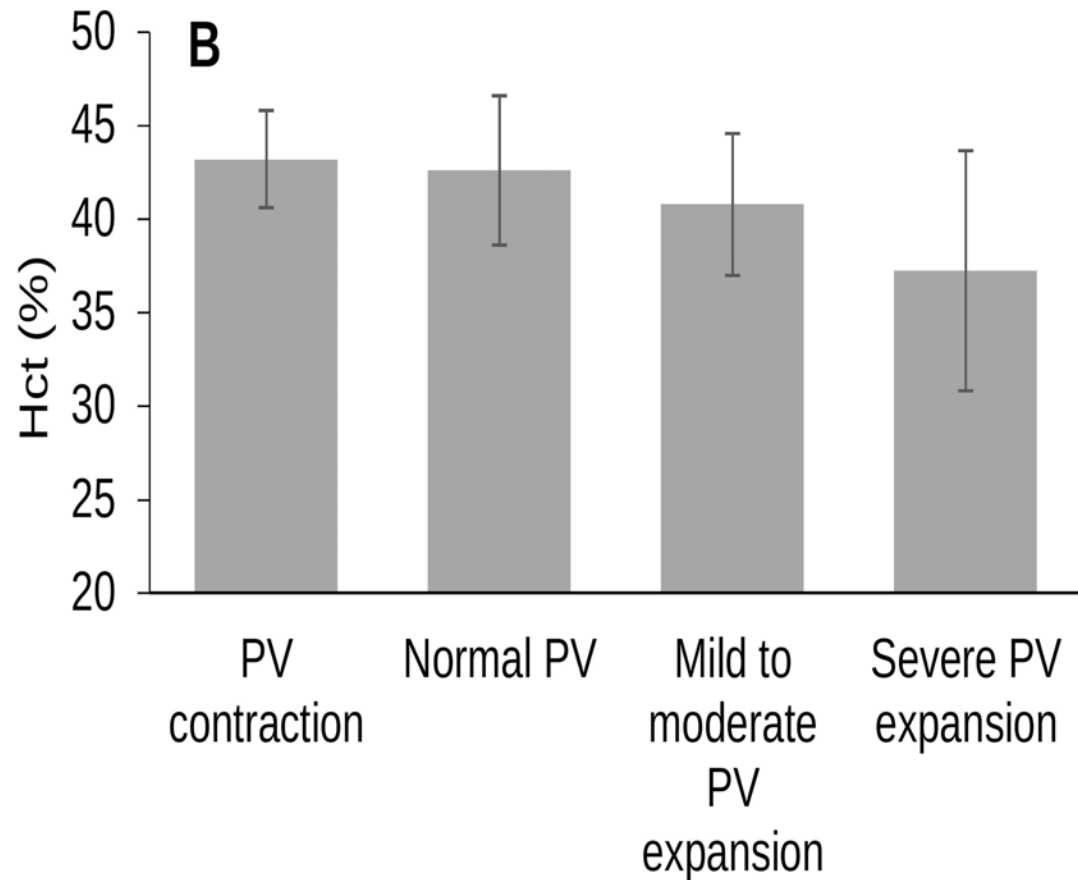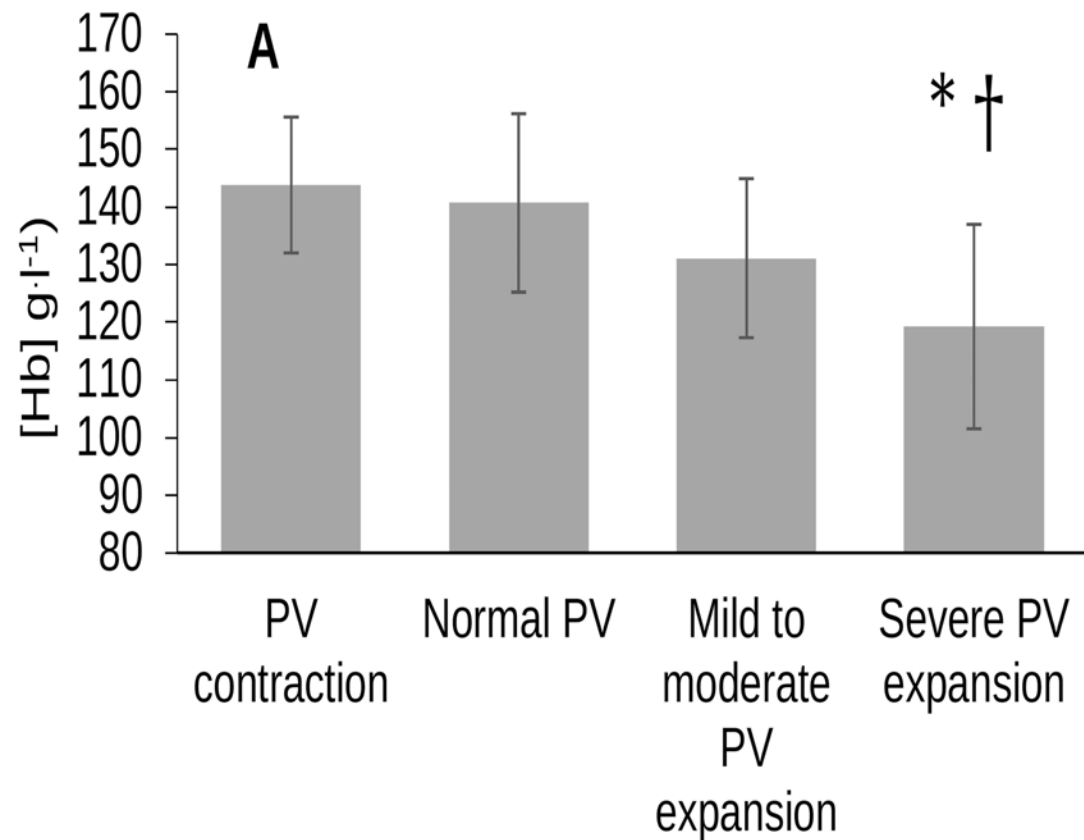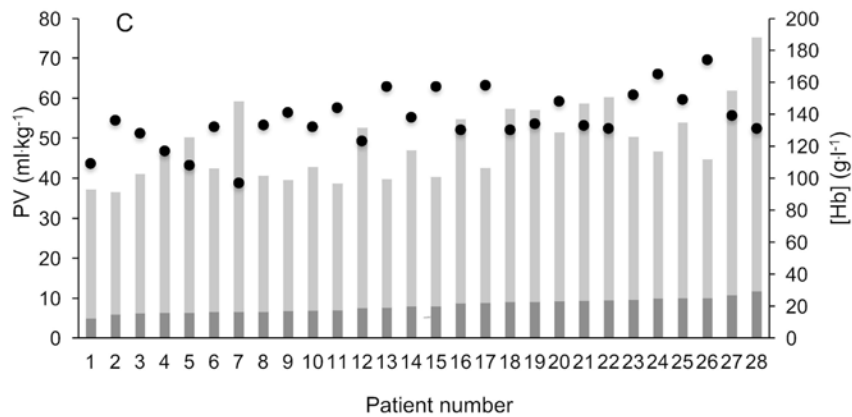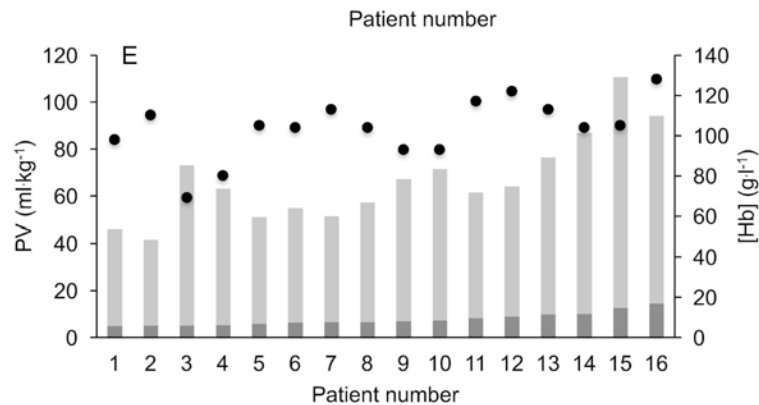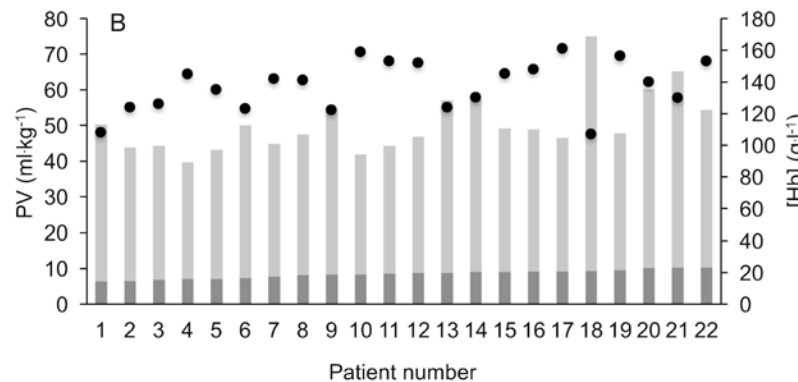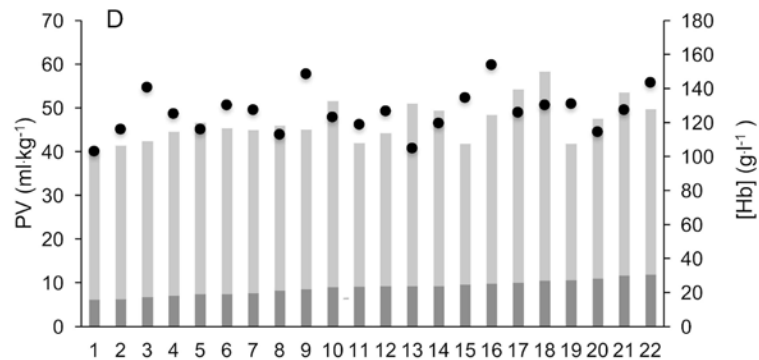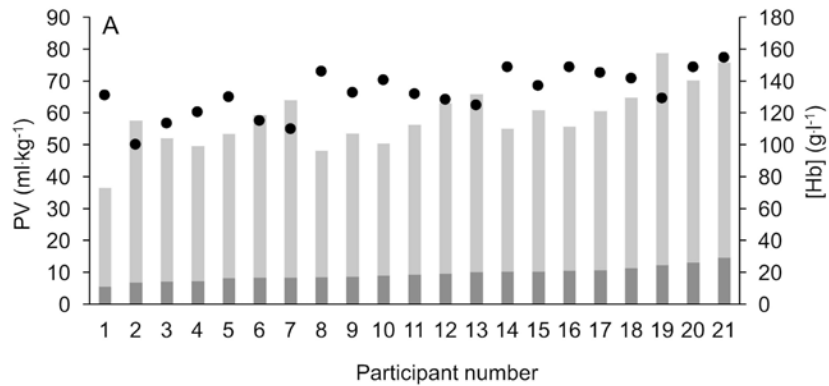
Supplementary Information

Supplemental Methods: Participant recruitment and testing sites, eligibility criteria, blood sampling, calculation of total haemoglobin mass and normal plasma volumes

Table S1: Surgical specialty for planned surgical procedure

Table S2: Characteristics and aetiology of inflammatory bowel disease patients

Table S3: Characteristics, medications and aetiology of liver disease patients.

Table S4: Characteristics, medications and aetiology of chronic heart failure patients.

Table S5: Relationship between total haemoglobin mass and haemoglobin concentration in sub-groups based on plasma volume status.

Participant recruitment and testing sites

Patients were recruited from those managed at University College Hospital (University College London Hospitals NHS Foundation Trust, UCLH), Southampton General Hospital (University Hospital Southampton NHS Foundation Trust) and the Royal Free Hospital (Royal Free London NHS Foundation Trust, RFH). Supplementary Figure 1 shows a Consolidated Standards of Reporting Trials (CONSORT)- style flow diagram indicating included and excluded patients and specific sub-groups. HV, patients with IBD and those awaiting surgery were recruited from and tested at UCLH only. Patients with CLD were recruited from and tested at all three sites, and those with CHF at UCLH and Southampton. Healthy volunteers were members of staff at UCLH or University College London (UCL) recruited by word of mouth or email advertisement. Patients were identified and recruited at outpatient clinics or when attending other routine clinical appointments.

Eligibility criteria

Inclusion criteria for all participants were, age >18 years, and ability give informed consent and to comply with basic breath-holding instructions. Healthy volunteers were additionally free from known chronic disease or use of regular medications. In CHF (clinically diagnosed and under treatment, confirmed- as clinically indicated- by the presence of a circulating concentration of B-type natriuretic peptide (BNP) >200 pg ml^{-1}), additional inclusion criteria were echocardiographic or cardiac magnetic resonance imaging (cMRI) evidence of left ventricular (LV) systolic impairment as defined by a left ventricular ejection fraction of less than 50%; New York Heart Association (NYHA) class II-IV; and not fully stabilised, i.e. still symptomatic with shortness of breath and/or peripheral oedema and/or clinical signs of bibasal crepitations or peripheral oedema.

Patients with CLD all had a diagnosis of alcoholic liver disease, primary sclerosing cholangitis, hepatitis C or cryptogenic cirrhosis. A confirmed diagnosis of IBD (either Crohn's disease or ulcerative colitis) was required for inclusion into this group. For surgical patients, inclusion was based on a patient being scheduled to undergo elective major surgery, regardless of specialty.

Excluded were prisoners, or those with a baseline %COHb greater than 5% (as can occur in smokers), presence of haemoglobinopathy.

Blood sampling

At UCLH and RFH, [Hb] was determined from a fingertip capillary blood sample (Hb 201 Microvette, Hemocue AB, Angelholm, Sweden), analysed immediately (HemoCue® Hb 201+, Hemocue AB, Angelholm, Sweden) when [Hb] could not be obtained from venous blood at the time of testing or was not available on the same day as tHb-mass measurement from hospital electronic records. At UCLH and RFH, fingertip capillary samples (200 µl) were collected before and 6- and 8-min after the start of CO rebreathing (Na-heparinized 200 µl RAPIDLyte Multicap Capillary tubes, Siemens Healthcare Diagnostics Inc, Deerfield, USA), with samples analysed within 15 minutes for percent carboxyhaemoglobin (%COHb) using a blood gas analyser (Hemoximeter; Cobas b 221 POC system, Roche Diagnostics Ltd, Switzerland). At Southampton, [Hb] was measured in venous blood. An intravenous cannula was inserted prior to tHb-mass testing at Southampton, allowing venous blood samples (200 µl) to be collected before and at 6- and 8-mins after CO rebreathing via a Na-heparinized blood gas syringe (RAPIDLyte, Siemens Healthcare Diagnostics Inc, Deerfield, USA). %COHb was determined at Southampton using the RAPIDPoint 500 Blood Gas System (Siemens Healthcare Diagnostics Inc, Deerfield, USA). All blood samples for the determination of [Hb],

haematocrit (Hct) and %COHb were collected under the same conditions, from the same anatomical site, and with patients in a seated position. Blood samples were analysed within 10-15 minutes of one another due to %COHb being analysed after the oCOR test using a blood gas machine (which takes 15 minutes). Blood sampling from venous, arterial and capillary blood yields an identical Δ%COHb and therefore identical tHb-mass values. [1]

Calculation of tHb-mass

tHb-mass was calculated using a specifically design excel spreadsheet (Microsoft Excel 2011 for Apple Macintosh) using the formula:

$$\text{tHb-mass (g)} = K \times \text{MCO(ml)} \times 100 \times (\Delta\%\text{COHb} \times 1.39)^{-1}$$

K = barometric pressure x 760^{-1} x [1(0.003661 x temperature)]

$\text{MCO} = \text{CO}_{adm} - (\text{CO}_{system + lung \text{ (after disconnection)}} + \text{CO}_{exhaled \text{ (after disconnection)}}$

CO_{adm} = CO volume administered into the system

$\text{CO}_{system + lung \text{ (after disconnection)}}$ = CO concentration in spirometer x (spirometer volume + remaining volume in the lung after disconnection)

$\text{CO}_{exhaled \text{ (after disconnection)}}$ = end-tidal CO concentration x alveolar ventilation x time

Δ%COHb = difference between baseline %COHb and %COHb post CO administration (average of 6- and 8-min %COHb values)

1.39 = Hüfners number (constant) (ml CO x g Hb^{-1})

where:

lung residual volume, 1500 ml in men, 1200 ml in women; alveolar ventilation, 5000 ml min^{-1}, CO concentration is in parts per million (ppm)

Blood volume (BV), plasma volume (PV) and red cell volume (RCV) were calculated

from mean corpuscular haemoglobin concentration (MCHC), [Hb] and tHb-mass, as below:

BV (ml) = tHb-mass (g)/[Hb] (g·dl^{-1}) • 100
RCV (ml) = tHb-mass (g)/MCHC • 100
PV (ml) = BV – RCV

When Hct and [Hb] values were obtained from capillary blood at UCLH and the RFH these values were corrected to venous conditions using the following formulas [2, 3]:

[Hb] (g·dl^{-1}) = [Hbcapillary] • 0.8787 + 1.24
Hct (%) = [Hctcapillary] • 0.8425 + 5.23

Estimation of normal adult values for PV were calculated using the formulas from the 1995 Expert Panel on Radionuclides of the International Council for Standardisation in Haematology. [4]

Equation 1. Estimation of normal male plasma volume

$$\text{Mean normal PV (ml)} = 1578 \times S$$

Equation 2. Estimation of normal female adult plasma volume

$$\text{Mean normal PV (ml)} = 1395 \times S$$

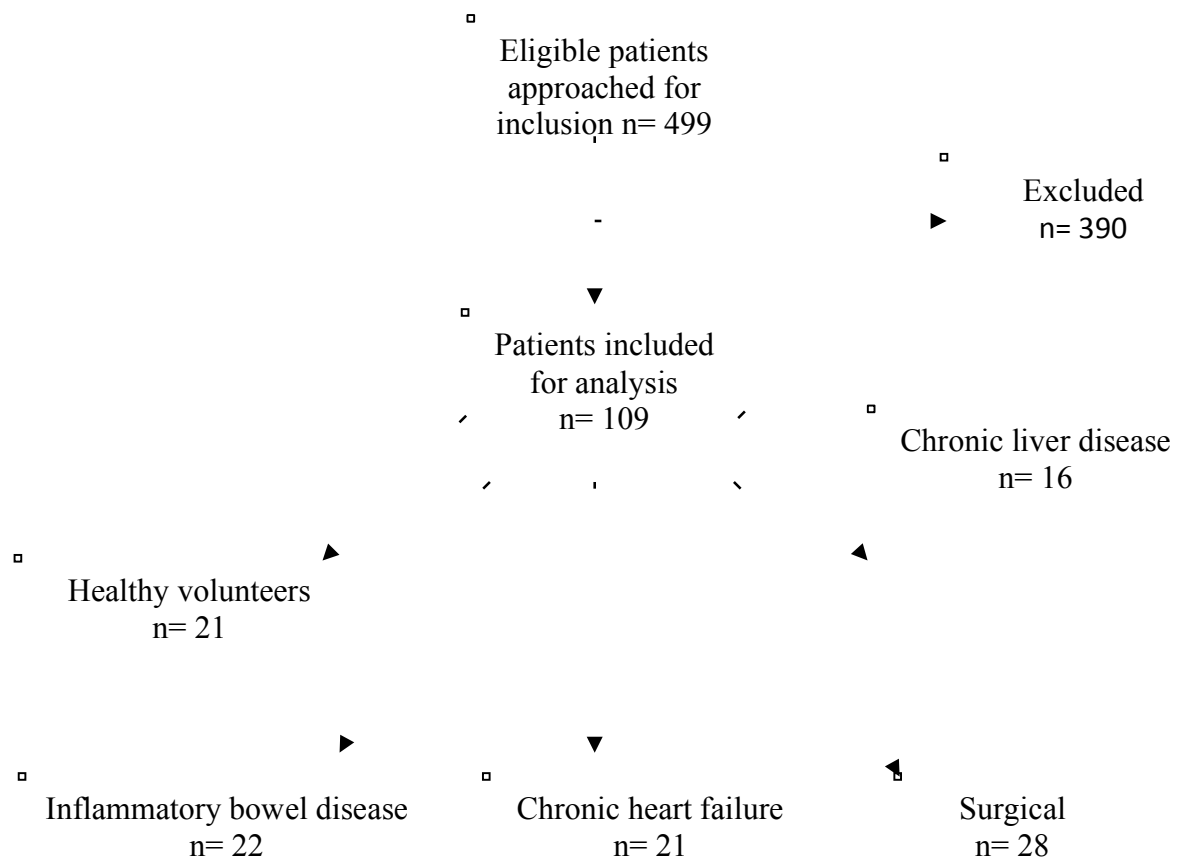Where S = body surface area (m^2) calculated using the formula of Du Bois and Du Bois. [5]

PV values were adjusted for age, sex, weight, and height using a published formula to calculate normal volumes as derived from >100,000 measurements of height and weight from Metropolitan Life tables. [6] Normal PV was classified as measured volumes within ± 8% of the expected normal volume on an individual level. Mild to moderate volume

expansion was considered >8% to ≤25% positive deviation from expected norms, and severe as >25% above the expected normal volume. [6]

Supplementary references

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Eligible patients
approached for
inclusion n= 499

Excluded
n= 390

Patients included
for analysis
n= 109

Chronic liver disease
n= 16

Healthy volunteers
n= 21

Inflammatory bowel disease
n= 22

Chronic heart failure
n= 21

Surgical
n= 28

Supplementary Figure 1. Consolidated Standards of Reporting Trials (CONSORT)- style flow diagram indicating included and excluded patients and specific sub-groups. Excluded patients are those who declined participation, failed to respond to correspondence via letter and telephone contact or could not be contacted despite multiple attempts by the research team.

Table-S1. Surgical specialty for planned surgical procedure (n= 28).

Surgical specialty	Frequency N (%)
Upper gastrointestinal	8 (28.5%)
Lower gastrointestinal	8 (28.5%)
Orthopaedic	7 (25%)
Other	3 (10.7%)
Urology	2 (7.1%)

Table-S2. Characteristics and aetiology of inflammatory bowel disease patients.

Variable	IBD (n= 22)
Gender	
Male (%)	50%
Age (yr)	50 (33-62)
Height (cm)	170.2 ± 7.3
Weight (kg)	77.7 ± 18.1
Aetiology of IBD	
Crohn's disease	4 (18%)
Small bowel CD	2 (9%)
Terminal ileal CD	1 (4.5%
Ulcerative colitis	9 (41%)
Ulcerative proctitis	3 (14%)
Pan UC	2 (9%)
Collagenous colitis	1 (4.5%)

IBD, Inflammatory bowel disease; CD, Crohn's disease; UC, Ulcerative colitis

Table-S3. Characteristics, medications and aetiology of liver disease patients.

Variable	LD (n= 16)
Gender	
Male (%)	75%
Age (yr)	51 (48-55)
Height (cm)	174.8 ± 8.9
Weight (kg)	85.3 ± 18.5
Primary aetiology of liver disease	
Hepatitis	3 (19%)
ALD	8 (50%)
Cryptogenic cirrhosis	2 (12%)
Sclerosing cholangitis	2 (12%)
Other	1 (6%)
Hypertension	1 (6%)
Medication	
Beta blocker	3 (19%)
ACE inhibitor	0 (0%)
Statin	0 (0%)
Diuretic	5 (31%)
Diuretic times per day	
None	11 (69 %)
Once	5 (31 %)
Bi daily	0

ALD, decompensated alcoholic liver disease; ACE inhibitor, angiotensin-converting-enzyme inhibitor.

Table-S4. Characteristics, medications and aetiology of chronic heart failure patients.

Variable	CHF (n= 22)
Gender	
Male (%)	82%
Age (yr)	68 (61-75)
Height (cm)	173.5 ± 8.1
Weight (kg)	80.4 ± 15.9
Aetiology of heart failure	
Ischaemic heart disease	7 (32%)
Cardiomyopathy	10 (45%)
Ischaemic	5 (22%)
Non-ischaemic	5 (22%)
Other	5 (22%)
Hypertension	7 (32%)
NHYA class	
I	2 (9%)
II	14 (64%)
III	4 (18%)
IV	2 (9%)
LVEF (%)	33.2 ± 11.4
Medication	
Beta blocker	19 (86%)
ACE inhibitor	20 (91%)
Statin	16 (73%)
Diuretic	17 (77%)
Diuretic dose (mg)	25 ± 29
Diuretic times per day	
None	5 (22%)
Once	14 (64%)
Bi daily	3 (14 %)

NYHA, New York Heart Association Class; LVEF (%), left ventricular ejection fraction; ACE inhibitor, angiotensin-converting-enzyme inhibitor

Table-S5. Relationship between total haemoglobin mass and haemoglobin concentration in sub-groups based on plasma volume status.

Sub group	Plasma volume status		
	Normal	Mild to moderate	Severe
IBD	(n= 9)	(n= 7)	(n= 5)
	r= 0.86, p= 0.003	r= 0.94, p< 0.002	r= 0.78, p= 0.118
Surgical	(n= 8)	(n= 14)	(n= 5)
	r= 0.94, p= 0.001	n= 0.78, p= 0.001	r= 0.80, p= 0.104
CHF	(n= 4)	(n= 6)	(n= 11)
	r= 0.95, p= 0.046	r= 0.86, p< 0.029	r= 0.59, p= 0.055
HV	(n=2)	(n= 8)	(n= 11)
		r= 0.91, p< 0.002	r= 0.89, p< 0.0001

r, Pearson's correlation coefficient; PV values were adjusted for age, sex, weight, and height using a published formula to calculate normal volumes as derived from >100,000 measurements of height and weight from Metropolitan Life tables. [6] Normal PV was classified as measured volumes within ± 8% of the expected normal volume on an individual level. Mild to moderate volume expansion was considered >8% to <25% deviation from expected norms, and severe as >25% of the expected normal volume. [6] Insufficient numbers to perform sub-group analysis in chronic liver disease group. IBD, inflammatory bowel disease; CHF, chronic heart failure; HV, healthy volunteers.