


RESEARCH PAPER

Modification of the CO-rebreathing method to determine haemoglobin mass and blood volume in patients suffering from chronic mountain sickness

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Abstract

Patients suffering from chronic mountain sickness (CMS) exhibit extremely high haemoglobin concentrations. Their haemoglobin mass (Hbmass), however, has rarely been investigated. The CO-rebreathing protocol for Hbmass determination in those patients might need to be modified because of restricted peripheral perfusion. The aim of this study was to evaluate the CO uptake and carboxyhaemoglobin-mixing time in the blood of CMS patients and to adapt the CO-rebreathing method for this group. Twenty-five male CMS patients living at elevations between 3600 and 4100 m above sea level were compared with ethnically matched healthy control subjects from identical elevations ($n = 11$) and near sea level ($n = 9$) and with a Caucasian group from sea level ($n = 6$). CO rebreathing was performed for 2 min, and blood samples were taken for the subsequent 30 min. After the method was modified, its reliability was evaluated in test–retest experiments ($n = 28$), and validity was investigated by measuring the Hbmass before and after the phlebotomy of 500 ml ($n = 4$). CO uptake was not affected by CMS. The carboxyhaemoglobin mixing was completed after 8 min in the Caucasian group but after 14 min in the groups living at altitude. When blood was sampled 14–20 min after inhalation, the typical error of the method was 1.6% (confidence limits 1.2–2.5%). After phlebotomy, Hbmass decreased from 1779 ± 123 to 1650 ± 129 g, and no difference was found between the measured and calculated Hbmass (1666 ± 122 g). When the time of blood sampling was adapted to accommodate a prolonged carboxyhaemoglobin-mixing time, the CO-rebreathing method became a reliable and valid tool to determine Hbmass in CMS patients.

KEYWORDS

high altitude, mixing time, Monge's disease, polycythaemia

1 | INTRODUCTION

In clinical practice and in the field of sports medicine, the optimized CO-rebreathing method (oCOR method; Gore et al., 2006; Prommer & Schmidt, 2007; Schmidt & Prommer, 2005) is used to determine the total haemoglobin mass (Hbmass). In comparison to previously performed dilution techniques using radioactive markers (e.g. ^{32}P , $^{99\text{m}}\text{Tc}$, ^{59}Fe , ^{51}Cr) or dye markers, such as Evans Blue (Gibson & Evans, 1937), hydroxyethyl starch (Tschaikowsky, Meisner, Durst, & Rügheimer, 1997) or Indocyanine Green (He, Tanigami, Ueyama, Mashimo, & Yoshiya, 1998), the oCOR method is less invasive and

easier to apply (Turner et al., 2014). The main differences compared with previous CO-rebreathing methods are a reduced rebreathing time of 2 min in a custom-made spirometer, fewer acquired blood samples and the possibility of using capillary blood (Schmidt & Prommer, 2005). The oCOR method is well tolerated by young trained athletes and by older subjects and patients (Ahlgrim, Schumacher, Wrobel, Waller, & Pottgiesser, 2014; Otto et al., 2017; Wachsmuth et al., 2013).

Prerequisites of the method are unrestricted diffusion of CO from the lung to the blood, the complete mixing of CO in the blood, and knowledge of the volumes of CO that have diffused out of the blood

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and bound to myoglobin or been exhaled via respiration (Garvican et al., 2010; Schmidt & Prommer, 2005). When applying the oCOR method to healthy subjects, these prerequisites are all fulfilled. In some groups of patients, however, these preconditions are not guaranteed, e.g. in patients with chronic obstructive pulmonary disease, in patients with disturbances in peripheral arterial perfusion and in polycythaemia vera (Ahlgren et al., 2014). Before applying the CO-rebreathing method to these patients, the above factors have to be verified, and the method might need to be modified.

Patients with chronic mountain sickness (CMS) or Monge's disease (Monge, 1942; Monge & Whittombury, 1976) are characterized by severe polycythaemia [concentration of haemoglobin ([Hb]) >19 g dl⁻¹ for women and >21 g dl⁻¹ for men; León-Velarde et al., 2005; Vásquez & Villena, 2001; Villafuerte & Corante, 2016]. The disease occurs at elevations >2500 m above sea level (a.s.l.; León-Velarde et al., 2005; Villafuerte & Corante, 2016), and it is supposed that peripheral chemoreceptors are desensitized and that the impaired breathing stimulus results in hypoventilation and reduced arterial partial pressure of O₂ and oxygen saturation of haemoglobin (S_{O₂}; León-Velarde & Richalet, 2006; Severinghaus, Bainton, & Carcelen, 1966). This outcome results in excessive erythropoiesis, leading to augmented red cell mass and blood viscosity (Kwaan & Wang, 2003). Significant symptoms are vascular dysfunction (Rimoldi et al., 2012), reduced cerebral blood flow velocity (Claydon et al., 2005), increased pulmonary blood pressure (Maignan et al., 2009) and cardiac failure (León-Velarde et al., 2005; Villafuerte & Corante, 2016). Therefore, it seems possible that when the CO-rebreathing method is applied to these patients, the mixing time of the CO marker in the blood might be prolonged.

In general, the [Hb] and haematocrit (Hct) depend on the Hbmass or red cell volume and the magnitude of plasma volume. In patients suffering from CMS, the size of the blood compartments is rarely investigated. There exists only one study of 11 CMS patients from 4350 m a.s.l. using Evans Blue for the determination of plasma volume (Claydon et al., 2004). Those authors described an almost normal plasma volume and calculated a massively increased red cell volume compared with healthy high-altitude residents. Although their study provided valuable information, there were some limitations influencing the validity of the data. The results were obtained from a small group of patients, and the red cell volume was determined indirectly, by measuring the plasma volume and calculating the red cell volume. Given that the cell factor describing the different distribution of cell mass and plasma in peripheral and central blood may change at altitude (Sánchez, Merino, & Figallo, 1970), direct measurements of Hbmass and/or red cell volume should be used. Exact Hbmass data would be valuable for quantifying the amount of haemoglobin and distinguishing between haemoconcentration attributable to reduced plasma volume and haemoconcentration attributable solely to an enhanced erythrocyte volume.

When applying the oCOR method for healthy subjects to CMS patients, the altered haemodynamics will probably affect the mixing time for the administered CO, leading to inaccurate or even false results. It is therefore mandatory to determine the exact mixing time to adjust the time points for blood sampling (Ahlgren et al., 2014) and

New Findings

- **What is the central question of this study?**

Is it necessary to modify the CO-rebreathing method to acquire reliable measurements of haemoglobin mass in patients with chronic mountain sickness?

- **What is the main finding and its importance?**

The CO-rebreathing method must be modified because of the prolonged CO-mixing time in patients with chronic mountain sickness. After adaptation of the blood sampling method, reliable and valid results were attained. With this modification, it is possible to quantify the extent of polycythaemia and to distinguish between a haemoconcentration and an exclusive enhancement of erythrocyte volume.

to examine the amount of CO taken up by the blood and the amount of CO that diffuses from blood to myoglobin.

Additionally, the CO uptake during the rebreathing period might be altered, as can be assumed from the delayed CO uptake (Herbert et al., 1965), microembolisms in pulmonary arteries (Arias-Stella, 1971; Burgess & Bishop, 1963) and elevated blood volume in the pulmonary vascular bed (Greening, Patel, Goolden, Munro, & Hughes, 1982) observed in patients suffering from polycythaemia vera. In addition, a third source of error might be the loss of CO owing to diffusion from the vascular bed to myoglobin, which must be quantified, especially in CMS patients, for whom no related data are available.

The aim of the study was therefore to evaluate and, if necessary, to adapt the currently used CO-rebreathing method to be used in CMS patients. We analysed the optimal time points for blood sampling, we checked the extraction of CO from the lung and determined the loss of CO from blood to myoglobin. With the information obtained, we modified the CO-rebreathing method and demonstrated its reliability and validity for CMS patients.

2 | METHODS

2.1 | Ethical approval

The subjects were informed about the requirements and possible risks of the study before giving written consent to participate. The subjects volunteered to participate in the study and were free to withdraw at any time without needing to provide a reason. The study conformed to the standards set by the *Declaration of Helsinki*, except for registration in a database. The study was approved by the Ethics Committee of the Universidad Mayor San Andres in La Paz, Bolivia.

2.2 | Subjects

In total, 51 male subjects participated in the study. Twenty-one of the subjects lived at high altitude (between 3600 and 4100 m a.s.l.) and were classified as CMS patients. Another four subjects living at

TABLE 1 Subject characteristics

Subject group	Altitude of residence (m a.s.l.)	n	Age (years)	Height (cm)	Weight (kg)	BMI (kg m ⁻²)	Hct (%)	[Hb] (g dl ⁻¹)
CMS patients	3600–4100	21	56.9 ± 8.8	166.1 ± 6.2	84.6 ± 10.5	30.7 ± 3.5	67.8 ± 3.5	21.8 ± 1.1
Intermediates	3600–4100	4	42.0 ± 17.8	166.6 ± 3.5	77.2 ± 17.0	27.9 ± 6.6	60.8 ± 3.0	19.3 ± 0.3
Controls-LP	3600–4100	11	43.2 ± 15.6	163.6 ± 6.8	76.4 ± 11.1	28.6 ± 4.4	51.7 ± 3.2	17.0 ± 0.9
Controls-SZ	300–500	9	47.8 ± 16.2	166.2 ± 8.8	73.6 ± 13.4	26.8 ± 5.6	46.1 ± 2.9	14.6 ± 0.8
Controls-BT	300–500	6	24.5 ± 4.2	180.7 ± 6.5	75.1 ± 6.1	23.0 ± 1.3	–	15.3 ± 1.4

Values are presented as means ± SD. Abbreviations: a.s.l., above sea level; BMI, body mass index; BT, Bayreuth; Hct, haematocrit; LP, La Paz; and SZ, Santa Cruz.

the same altitude who did not completely fulfil the criteria for CMS were classified as 'intermediates'. Twenty-six subjects without CMS symptoms were recruited as control subjects; 11 of them lived at the same altitude as the CMS patients (Controls-LP). Another 15 subjects lived near sea level; nine had moved from altitude >2 years ago (Controls-SZ), and six were native sea-level dwellers (Controls-BT). Some of the subjects participated in various parts of this study. The characteristics of the participants are presented in Table 1.

2.3 | Study design

The project was divided into two substudies. The first part of the project dealt with the determination of the optimal blood sampling time points, which might differ from the known oCOR method owing to the possibly delayed CO mixing in CMS patients. The prerequisite for this calculation is knowledge about the amount of CO that diffuses from the blood to myoglobin and the amount of CO that is exhaled after disconnection of the subject from the spirometer. Taking these factors into account, we calculated the Hbmass every 2 min until 20 min and then every 5 min until 30 min after inhalation of the CO bolus. When the calculated Hbmass values reached a plateau, the CO mixing in blood was assumed to be complete. Based on these data, the time points for blood sampling were modified.

In the second part of the project, the reliability of the modified CO-rebreathing method was calculated by performing test-retests and calculating the typical error of the method. Furthermore, the validity of the method was determined by measuring the Hbmass before and after withdrawal of ~500 ml of blood. The distribution of the subjects to the different substudies is illustrated in Table 2.

2.4 | Part I: Modification of the CO-rebreathing method

Haemoglobin mass was measured using the optimized CO-rebreathing method (Gore et al., 2006; Prommer & Schmidt, 2007; Schmidt & Prommer, 2005) with modifications concerning blood sampling and measurements of the end-tidal CO concentration. A CO bolus followed by 3.5 l of pure oxygen was rebreathed through a glass spirometer (SpiCo, Bayreuth, Germany) for 2 min. The volume of CO administered was adjusted to altitude according to the barometric pressure and to the expected value of Hbmass. The CO bolus for the control subjects from sea level (SZ and BT) was calculated as 1.0 ml (kg body mass)⁻¹. For all measurements at altitude (3600 m a.s.l.),

TABLE 2 Participants in the substudies

Subject group	Part I	Part II	
	Modification of the method	Reliability	Validity
CMS patients	9	19	4
Intermediates	–	4	–
Controls-LP	11	5	–
Controls-SZ	9	–	–
Controls-BT	6	–	–
Total	35	28	4

Abbreviations: BT, Bayreuth; CMS, chronic mountain sickness; LP, La Paz; and SZ, Santa Cruz.

the decrease in barometric pressure (~500 mmHg) was considered. Given that Hbmass is ~20% higher in natives to high altitude than in subjects from sea level (Heinicke et al., 2003), the CO bolus was increased to 1.5 ml (kg body mass)⁻¹ in the control subjects from altitude (LP). For CMS patients and intermediates, the CO bolus was calculated according to the measurements in a pilot study as 1.7 ml (kg body mass)⁻¹. In overweight subjects, the theoretically derived body mass corresponding to a body mass index of 25 kg m⁻² was used for the calculation. Capillary blood was sampled from an earlobe three times before and once every second minute after starting the rebreathing period until the 20 min mark and at the 25 and 30 min marks. From each blood sample, three replicates were analysed for carboxyhaemoglobin (COHb) using a CO haemoximeter (OSM3; Radiometer, Copenhagen, Denmark).

To calculate the amount of CO not taken up during the inhalation, a CO analyser (Draeger, Luebeck, Germany) was used to determine the CO remaining in the spirometer system. Additionally, the end-tidal CO concentration was measured 2, 6, 13 and 23 min after disconnecting the subject from the spirometer. Owing to the hyperventilation at altitude, the alveolar ventilation of controls-LP was adjusted by 10% (Hultgren, 1997). This adjustment was not performed for the CMS patients owing to the lack of increased hypoxic ventilation (Villafuerte & Corante, 2016). Unlike the Bolivian subjects, in controls-BT, the volume and the CO concentration of exhaled air were measured continuously after CO rebreathing up to 30 min. The Hbmass was determined using the following formula (Equation 1):

$$\text{Hbmass (in grams)} = K \times \text{MCO} \times 100(\Delta\text{COHb}\% \times 1.39)^{-1} \quad (1)$$

Where K = current barometric pressure $\times 760^{-1} \times [1(0.003661 \times$ current temperature)]; $MCO = CO_{adm} - (CO_{system+lung \text{ after disconnection}} + CO_{exhaled \text{ after disconnection}}) - M_{Hb}$; CO_{adm} = CO volume administered into the system; $CO_{system+lung \text{ after disconnection}}$ = CO concentration in the spirometer \times (spirometer volume + lung residual volume); $CO_{exhaled \text{ after disconnection}} = \Delta \text{end-tidal CO concentration} \times \text{alveolar ventilation} \times \text{time}$; M_{Hb} = CO diffusing to myoglobin; $\Delta COHb\%$ = difference between basal COHb% and COHb% in the blood samples after CO administration; and 1.39 = Hüfner's number (in millilitres of CO per gram of Hb; e.g. Gorelov, 2004). The Hbmass values were calculated every second minute until 20 min and at 25 and 30 min. To quantify the diffusion of CO from blood to myoglobin, three different factors were used. We assumed that in the case of the best-fitting factor, the most constant plateau of Hbmass was achieved. The following three formulae (Equations 2–4) for calculating CO diffusion to myoglobin were used to verify the appropriate correction factor:

$$M_{Hb}0.002 = 0.002 \times \text{time} \times \text{CO administered}$$

(Schmidt & Prommer, 2005), (2)

$$M_{Hb}0.003 = 0.003 \times \text{time} \times \text{CO administered}$$

(Prommer & Schmidt, 2007); (3)

$$M_{Hb}CHA = [(0.091 \times \text{time}) + (0.013 \times \text{CO administered})]$$

(Chada & Bruce, 2012). (4)

When using OSM3 (Radiometer, Denmark) to analyse blood samples, one must take into account that percentage carboxyhaemoglobin (COHb%) values also depend on the S_{O_2} (Hütler, Beneke, Littschwager, & Böning, 2001). The majority of the tests were performed at high altitude, where the S_{O_2} in arterialized blood is reduced. After inhaling 3.5 l of pure oxygen during the rebreathing procedure, the S_{O_2} increases, which requires an adjustment in COHb%. We used an individual correction factor for every subject according to the recommendations of Hütler et al. (2001): venous (see next paragraph) and capillary blood samples were taken simultaneously before the CO-rebreathing test and were analysed for COHb% and S_{O_2} , and a correction factor for the real COHb% in the main test was obtained (see Equation 5):

$$\text{Factor COHb} = (\text{COHb}\%_{\text{capillary}} - \text{COHb}\%_{\text{venous}}) / (S_{O_2 \text{ capillary}} - S_{O_2 \text{ venous}})$$

(5)

To determine the [Hb] and Hct, venous blood samples were drawn into an EDTA tube from a cubital vein after the subject had been sitting for ≥ 10 min and after the tourniquet was removed. The [Hb] was measured spectrophotometrically using the cyanmethaemoglobin

method, and the Hct was measured by the microhaematocrit technique at 12,000 r.p.m. for 5 min.

2.5 | Part II: Reliability and validity of the modified method

To determine the reliability of the modified CO-rebreathing method, test-retests were performed. Based on the results of part I, the time points of blood sampling were set at 14, 16, 18 and 20 min, and the mean COHb value of this period was used for further calculations. The end-tidal CO concentration was measured before the tests and at 2, 6 and 13 min after disconnecting the subject from the spirometer. To calculate the amount of CO diffusing to myoglobin, the best-fitting factor from part I, i.e. $0.002 \text{ ml min}^{-1}$, was used.

The validity of the modified CO-rebreathing method was evaluated by measuring Hbmass immediately before and after withdrawal of ~ 500 ml blood with subsequent fluid replacement. The Hbmass was determined as described in section 2.4. The transfusion bag, including the tubes for collecting the blood, was weighed before and after the blood withdrawal, and a blood sample was taken from the transfusion bag to determine the [Hb]. The Hbmass in the bag was calculated according to the following formula (Equation 6):

$$\begin{aligned} \text{Hbmass in transfusion bag} = & (\text{weight of transfusion bag after} \\ & - \text{weight of transfusion bag before}) \\ & \times \text{specific weight for blood } (1.07 \text{ g ml}^{-1}) \times [\text{Hb}] / 100 \end{aligned} \quad (6)$$

2.6 | Statistics

Data are presented as the mean value \pm SD. Student's paired t tests were applied to compare possible differences between the time points of blood sampling and between the three calculation possibilities for Hbmass by using the different equations for CO diffusion to myoglobin (Hopkins, 2003). Furthermore, Student's paired t tests were used to compare the Hbmass values calculated for different mixing times, i.e. according to the established oCOR method (second blood sample at minute 6 + 8; Prommer & Schmidt, 2007) and the period of complete mixing obtained in this study (14–20 min). Another Student's paired t test was performed to compare the calculated and measured Hbmass lost after the blood withdrawal, which was performed to check the validity of the method. An ANOVA with repeated measurements was carried out to detect effects of group \times time interactions for the COHb kinetics after the CO bolus inhalation. When the outcomes of the ANOVA were significant ($P < 0.05$), Scheffé's tests were used to compare the individual groups with one another.

The typical error (TE) of the modified method was calculated according to Hopkins (2000). A correlation analysis was performed to check the reliability of the modified method by evaluating the relationship of Hbmass obtained in the test and retest.

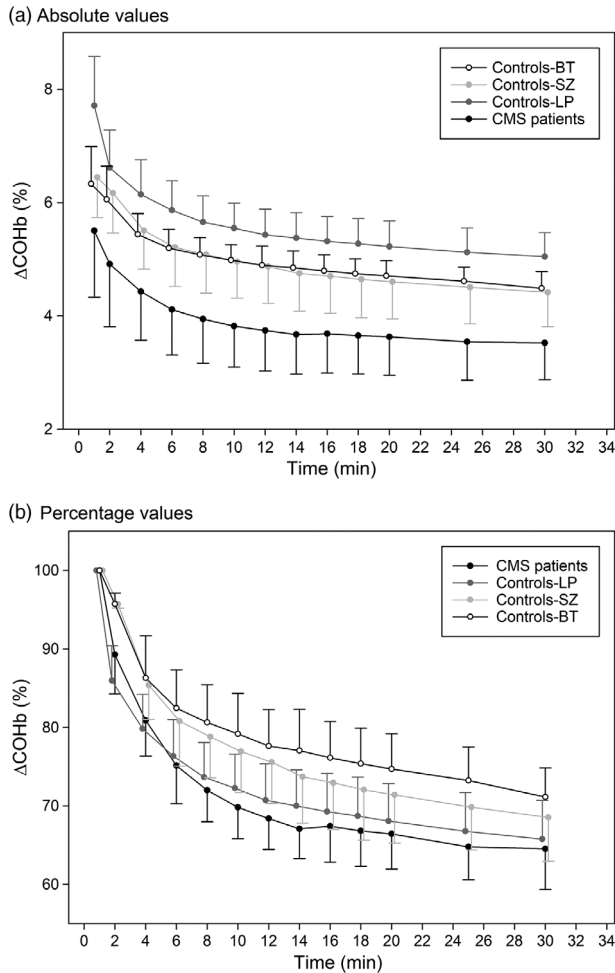


FIGURE 1 Carboxyhaemoglobin (COHb) kinetics during and up to 28 min after the CO-rebreathing protocols. The absolute differences from baseline values (a) and the percentage decrease from the peak value observed at minute 1 (b) are shown. Abbreviations: BT, Bayreuth; CMS, chronic mountain sickness; LP, La Paz; and SZ, Santa Cruz. ANOVA and *post hoc* tests (Scheffé's test) yielded significant differences in the percentage decrease between Controls-BT and CMS patients ($P < 0.01$) and Controls-LP ($P < 0.05$)

3 | RESULTS

3.1 | Part I: Modification of the CO-rebreathing method

In all groups, peak values for COHb concentration were observed 1 min after CO bolus inhalation (Figure 1a). Thereafter, the decline in COHb showed clear effects of a treatment \times time interaction ($P < 0.01$), and *post hoc* tests (Scheffé's tests) yielded significant differences between Controls-BT and CMS ($P < 0.01$) and Controls-LP ($P < 0.05$)

TABLE 3 [CO] remaining in the spirometer after CO rebreathing

	CMS patients	Controls-LP	Controls-SZ	Controls-BT
[CO] in spirometer (p.p.m.)	187 \pm 89*†	284 \pm 90	327 \pm 123	235 \pm 124

Values are presented as means \pm SD. Abbreviations: BT, Bayreuth; LP, La Paz; and SZ, Santa Cruz. Significant differences in [CO] in the spirometer between the groups: * $P < 0.05$ compared with Controls-LP and † $P < 0.05$ compared with Controls-SZ.

(Figure 1b). Furthermore, we compared the CO content remaining in the spirometer after disconnection of the test subject to evaluate possible diffusion limitations. As demonstrated in Table 3, there were normal amounts of CO left in all groups, and in the spirometer of CMS patients the CO concentration was even lower than that of control subjects from La Paz and Santa Cruz.

3.1.1 | CO diffusion to myoglobin

Calculation of Hbmass with the three different correction factors yielded an almost identical time course of the values until 12 min after inhalation of the CO bolus. As time passed, the calculated Hbmass decreased when using the factor $M_{\text{Hb}}0.003$, indicating a non-realistically high diffusion rate, whereas the other two factors, $M_{\text{Hb}}0.002$ and $M_{\text{Hb}}\text{CHA}$, resulted in an almost constant and similar time course of Hbmass values over time. Given that there was a small and non-significant tendency of $M_{\text{Hb}}\text{CHA}$ to overestimate Hbmass in the high-altitude groups, we decided to use the factor $M_{\text{Hb}}0.002$ for all subsequent calculations of Hbmass because this factor yielded a plateau from 14 to 30 min in all four groups.

3.1.2 | CO-mixing time in blood

The calculated Hbmass values in the four groups reached their plateau at different time points after CO rebreathing. Controls-BT achieved constant Hbmass values after 6 min (Figure 2a), and Controls-SZ achieved constant Hbmass values after 8 min (Figure 2b). No significant differences from previous values were found thereafter. In the subjects from high altitude, the time to reach the plateau was delayed, and no significant difference from previous values was found from 14 to 30 min (Figure 2c,d). Given that all four groups showed a plateau between 14 and 20 min, blood samples from this period of time were used to determine the real Hbmass.

Figure 3 illustrates the difference in Hbmass between the conventional method (Prommer & Schmidt, 2007; Schmidt & Prommer, 2005) using the mean COHb values at 6 and 8 min for the calculation of Hbmass and the modified method, assuming a delayed mixing time, as mentioned above. No differences in Hbmass were found in the Controls-BT, whereas in all other groups, higher Hbmass values were obtained with the modified method (blood sampling after 14–20 min) than with the original method. The percentage differences were $4.4 \pm 2.3\%$ in Controls-SZ, $4.8 \pm 1.9\%$ in Controls-LP and $6.7 \pm 2.5\%$ in CMS patients.

3.2 | Parts II and III: Reliability and validity of the modified method

Test-retests were performed using the modified method with 28 subjects from high altitude (control subjects and CMS patients; see

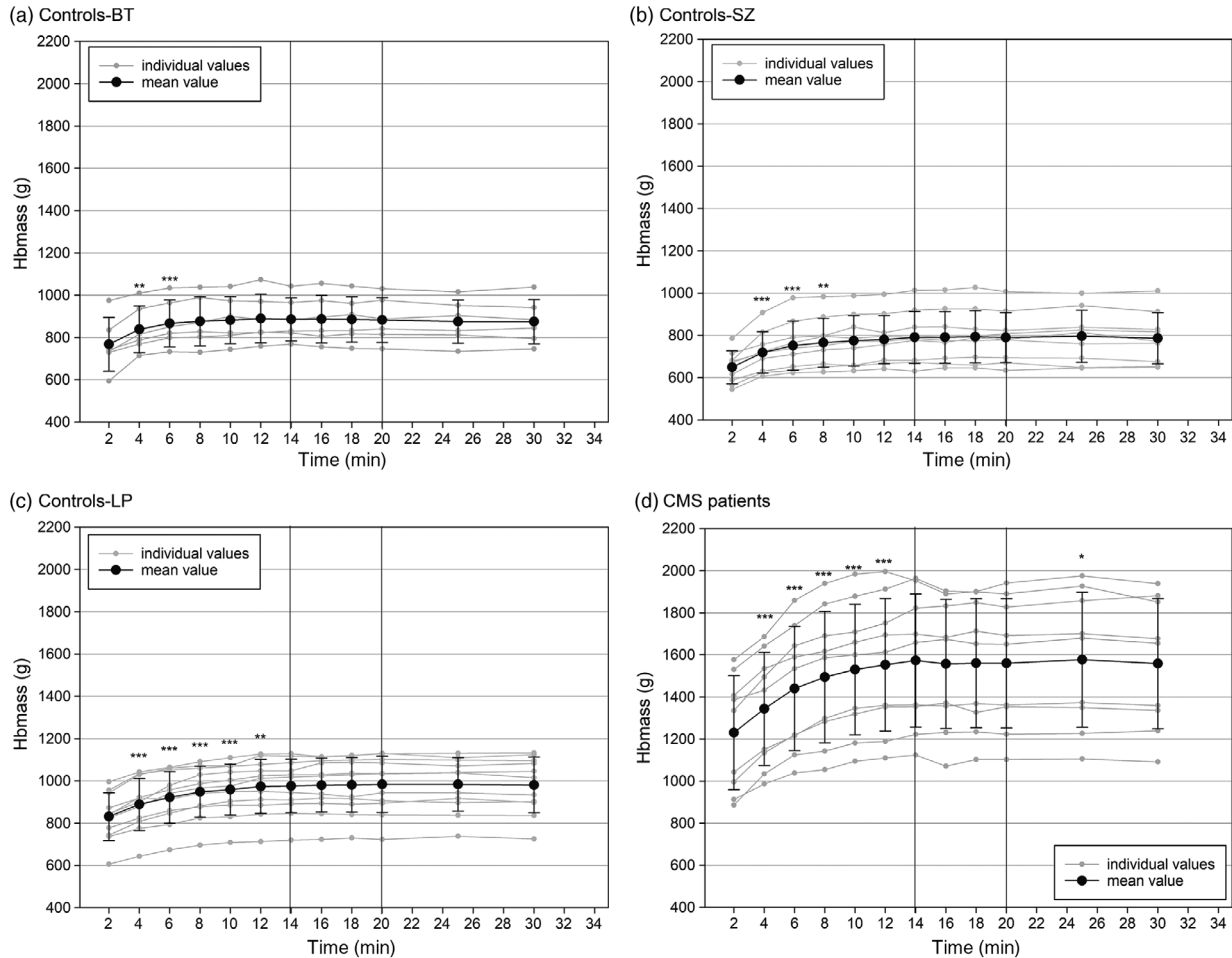


FIGURE 2 Time course of the calculated haemoglobin mass (Hbmass) after CO rebreathing in subjects from sea level (a,b) and altitude (c) and in CMS patients from La Paz (d). Presented are mean values and individual data of the Hbmass calculated for different time points of blood sampling. Abbreviations: BT, Bayreuth; CMS, chronic mountain sickness; LP, La Paz; and SZ, Santa Cruz. Significant differences from previous values: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. The time period between 14 and 20 min after CO inhalation that was used for the calculation of Hbmass is marked by vertical lines

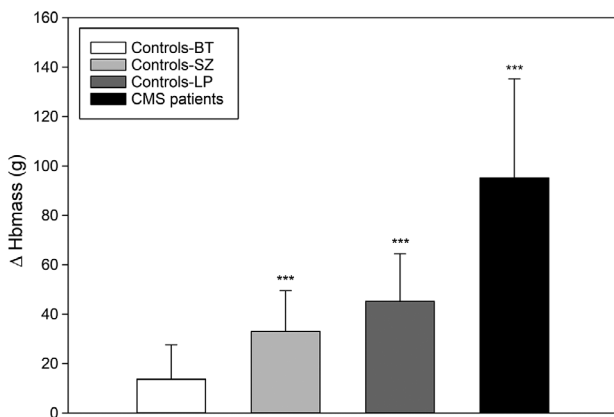


FIGURE 3 Differences in haemoglobin mass (Hbmass) using different time points for blood sampling in subjects from sea level (Controls-BT and Controls-SZ) and altitude (Controls-LP and the CMS patients). Abbreviations: BT, Bayreuth; CMS, chronic mountain sickness; LP, La Paz; and SZ, Santa Cruz. The difference between the conventional and modified methods is presented. Significant differences between the methods: *** $P \leq 0.001$

Table 2). The TE was 23.1 g (1.6%; confidence limits, 1.2–2.5%). There was an excellent correlation between the test and retest measurements of Hbmass ($r = 0.995$; $P \leq 0.001$; Figure 4).

In four CMS patients, Hbmass was measured before and after withdrawal of 500 ml of blood, and the reduction in Hbmass was compared with the reduction calculated from the [Hb] in the transfusion bag. By this measure, Hbmass was reduced from 1779 ± 123 g to 1650 ± 129 g ($7.2 \pm 1.6\%$; measured) or to 1666 ± 122 g ($6.3 \pm 0.4\%$; calculated), and no difference between the two methods of determination was found (Figure 5).

4 | DISCUSSION

4.1 | CO application and CO diffusion in the lungs

Owing to the low ambient pressure, the volume of the CO bolus inhaled by the subjects at high altitude had to be increased by 35%. A further increase of ~20% was implemented for the control subjects from

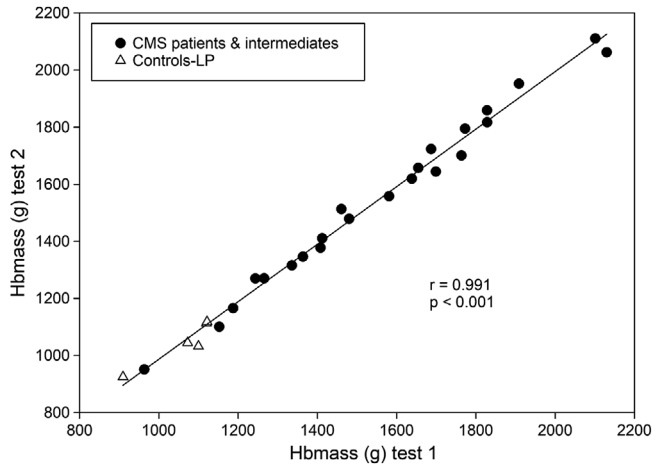


FIGURE 4 Correlation between test-retest measurements of haemoglobin mass (Hbmass; $r = 0.991$; $P \leq 0.001$) by the modified CO-rebreathing method. Abbreviations: CMS, chronic mountain sickness; and LP, La Paz

high altitude because their Hbmass was expected to be augmented in comparison to that of subjects from sea level (Heinicke et al., 2003). Given that we did not want to expose the CMS patients to any risk, we did not increase the CO volume to the extent ($\sim 2.4 \text{ ml kg}^{-1}$) that would yield COHb concentrations matching those in the control subjects. The CMS patients, therefore, received CO at $1.7 \text{ ml (kg body mass)}^{-1}$, which resulted in a lower increase in COHb than that in the control groups (Figure 1a). To counteract a possible lower accuracy of the calculated Hbmass owing to a smaller difference in COHb after inhalation, we increased the number of replicate measures from six to nine for the baseline value and to 12 for the post-inhalation value using the four blood samples obtained between 14 and 20 min. The resulting analytical error was $\sim 1\%$ (Alexander et al., 2011).

All patients and control subjects were asked whether they felt any side-effects from the CO-rebreathing procedure; none of the subjects complained or mentioned any discomfort.

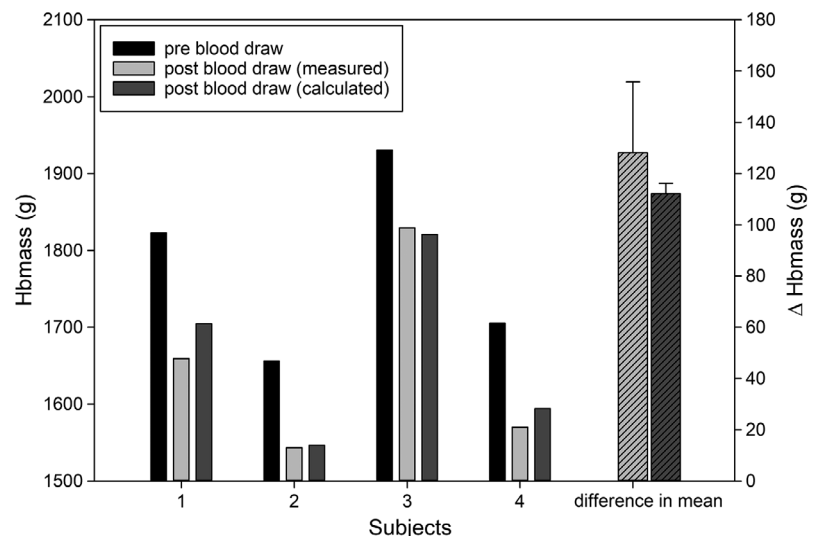
The CO concentration in the air remaining in the spirometer after cessation of the rebreathing period was significantly lower for the CMS patients than for the control groups. Given that CO is used as a routine marker for lung diffusion (Salcedo Posadas, Villa Asensi, de Mir Messa, Sardón Prado, & Larramona, 2015), this indicates the absence of diffusion barriers in the lung, as could be expected from the chronically augmented pulmonary arterial constriction (Villafuerte & Corante, 2016) or multiple pulmonary arterial thrombi occurring in patients with polycythaemia vera (Ahlgren et al., 2014; Arias-Stella, 1971). This finding, however, is in agreement with data from Groepenhoff et al. (2012), hinting at even greater CO-diffusion capacity in CMS patients than in subjects from near sea level. Therefore, we concluded that CO application to CMS patients is not limited by any problems with pulmonary diffusion.

4.2 | CO diffusion to myoglobin

When CO is inhaled and bound to haemoglobin, a small amount of CO diffuses from the blood to extravascular spaces, i.e. mostly to myoglobin (Bruce & Bruce, 2003). In the present study, we determined the Hbmass by calculating the diffusion of CO from blood to myoglobin with the fixed factors $0.2\% \text{ min}^{-1}$ (Schmidt & Prommer, 2005) and $0.3\% \text{ min}^{-1}$ (Prommer & Schmidt, 2007) for inhaled CO and by using the model from Chada and Bruce (2012). Until 12 min after starting the inhalation, the three methods showed very similar results; thereafter, in all four groups, the factor $0.3\% \text{ min}^{-1}$ slightly underestimated the Hbmass, whereas the model of Chada and Bruce (2012) slightly overestimated the Hbmass in the high-altitude subjects. Using the factor of $0.2\% \text{ min}^{-1}$ yielded an Hbmass plateau for $\geq 30 \text{ min}$ after CO inhalation, providing the best fit for all the groups investigated here.

Our calculation is, therefore, a strong approximation with low measurement error when applied to groups similar to those used in the present study, but different CO-diffusion factors might be more valid for other groups and patients.

FIGURE 5 Haemoglobin mass (Hbmass) before and after withdrawal of 500 ml of blood in four chronic mountain sickness patients. Presented are the individual values of Hbmass measured with the modified CO-rebreathing method before and after the blood withdrawal and the Hbmass measured before and calculated after the blood withdrawal. On the right side, the mean values for the measured and calculated loss of Hbmass are demonstrated



4.3 | CO-mixing time

For healthy subjects, complete mixing of an inhaled CO bolus was determined to occur within 6 min (Prommer & Schmidt, 2007; Schmidt & Prommer, 2005). Therefore, blood samples from minute 7 after CO inhalation (mean from samples drawn at minutes 6 and 8) are routinely used in the optimized CO-rebreathing method to calculate the Hbmass (Prommer & Schmidt, 2007). This concept was criticized by Keiser et al. (2013), who claimed that the mixing was not complete at this time point, thereby leading to the overestimation of Hbmass. Complete mixing of a CO bolus with high interindividual variation was found after 10 min by Garvican et al. (2010) and Gore et al. (2006), but because of only small differences compared with the findings of Prommer and Schmidt (2007), the method was not modified for healthy subjects from sea level. Both groups describing the extended period for mixing, however, recommended checking the mixing time for the investigation of special populations.

In the present study, complete mixing was achieved after 6 min in the control subjects from near sea level (Controls-BT) but complete mixing was not achieved until 12 min in the CMS patients and control subjects from high altitude. For the CMS patients, this delay might be explained by the impaired haemodynamics owing to higher blood viscosity (Kwaan & Wang, 2003) as a consequence of haematocrit values ranging between 63 and 78%; this impairment probably results in reduced peripheral and organ perfusion, as can be assumed from the reduced blood flow velocity in CMS patients (Claydon et al., 2005).

According to the model of Bruce and Bruce (2003), CO mixing and CO distribution in the body depends on five compartments, four of which are vascular: arterial blood, mixed venous blood, muscle tissue and non-muscle tissue. The time for blood to pass through each of these compartments and therefore to become mixed depends on the volume of the compartment and the blood flow through each compartment (Bruce & Bruce, 2003; Garvican et al., 2010). Despite the higher blood volume (Claydon et al., 2004), cardiac output is decreased in CMS patients (León-Velarde, Villafuerte, & Richalet, 2010; Maignan et al., 2009); given the high viscosity of the blood and the low perfusion rate in all compartments, the mixing time is prolonged. Premature sampling of either capillary or venous blood might therefore yield spurious results that falsely inflate the COHb%. This results in an underestimation of Hbmass, as presented in Figure 2 for blood samples obtained until 12 min after bolus inhalation. A plateau for the calculated Hbmass, indicating the occurrence of complete mixing, was achieved in all patients by 14 min after CO inhalation. When comparing Hbmass values for this plateau with values derived from the established protocol, in which blood sampling occurs at 7 min, the difference in Hbmass amounts to ~100 g, which corresponds to 6.7% (Figure 3). Therefore, these data strongly suggest that the oCOR method should be modified when it is applied to patients suffering from erythrocytosis.

A delayed mixing time was also observed in control subjects from high altitude, who have haematocrit values between 46 and 58%. Although the difference in calculated Hbmass between the two

protocols was smaller (~50 g), we recommend the modified protocol for further investigation of subjects from high altitude.

A possibility for reduction of the mixing time might be to conduct the CO-rebreathing tests in the supine position, in which increased venous return from the lower extremities (e.g. Truijen, Bundgaard-Nielsen, & Van Lieshout, 2010) and more homogeneous lung perfusion could facilitate CO mixing.

4.4 | Reliability and validity

Test-retest with the modified protocol was performed on subjects from high altitude, i.e. CMS patients, patients with previous CMS diagnoses who did not fulfil the criterion $[Hb] > 21 \text{ g dl}^{-1}$ and control subjects. As shown in Figure 4, the reliability was very good in all groups, resulting in a TE of 1.6%. Similar TE values were also described for CO-rebreathing tests at sea level [TE between 1.1 (Gore et al., 2006) and 2.3% (Eastwood, Bourdon, Withers, & Gore, 2009)] and at altitude [TE = 1.4% (Wachsmuth et al., 2013)], proving the reliability of the modified method when applied to high-altitude populations, similar to the established method.

The validity of the modified method was checked by phlebotomy of a known blood volume and known Hbmass. The difference between the measured and calculated volumes (0.9%) was in the same range as that reported by Schmidt and Prommer (2005) immediately after a blood donation. Both the reliability and the validity of the modified method for CMS patients showed that the quality of data was the same as that achieved using the established CO-rebreathing method for healthy subjects and patients at sea level. Unfortunately, we did not determine validity after blood sampling at minutes 6–8, which could have confirmed the prolonged mixing time in CMS patients.

4.5 | Possible practical importance

As our preliminary data show, the mean Hbmass of CMS patients was expanded twofold, and the individual Hbmass was increased threefold, compared with healthy subjects from sea level. This increase might be accompanied by blood volume expansion, which in turn strongly influences cardiovascular function via volume overloading and hyperviscosity. Similar pathophysiological mechanisms might exist in polycythaemia vera, in which plasma volume is also frequently expanded, which masks the high red cell volume (Spivak, 2019). The modified CO-rebreathing method might therefore represent a simple tool for diagnosing the degree of erythrocytosis and the risk of possible negative consequences in CMS patients and in patients with polycythaemia of other origins, e.g. polycythaemia vera.

4.6 | Conclusions

Application of the CO-rebreathing method to CMS patients and healthy residents from high altitude (3600–4100 m a.s.l.) requires modification of the established CO-rebreathing method. Although CO uptake in the lungs and CO diffusion from blood to myoglobin are similar between CMS patients and healthy subjects from sea level, the

CO-mixing time is considerably delayed. If this delay is considered for blood sampling after CO inhalation, the reliability and validity of the modified method are as good as those of the established method; thus, the modified method can be used to investigate patients suffering from CMS or from other forms of polycythaemia.

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COMPETING INTERESTS

W.S. is a managing partner of the company 'Blood tec GmbH', but he is unaware of any direct or indirect conflict of interest with the contents of this paper. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

W.S., R.S. and N.W. were involved in the conception and design of the study, the acquisition of data, the analysis and interpretation of the data and the drafting of the manuscript. J.J. was involved in the acquisition of data and the critical revision of the manuscript. R.S. unfortunately passed away after finishing the first draft of the manuscript. W.S., N.W. and J.J. approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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