

## Global REACH 2018: Andean highlanders, chronic mountain sickness and the integrative regulation of resting blood pressure

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## RESEARCH PAPER

# Global REACH 2018: Andean highlanders, chronic mountain sickness and the integrative regulation of resting blood pressure

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## Abstract

The high-altitude maladaptation syndrome chronic mountain sickness (CMS) is characterized by excessive erythrocytosis and frequently accompanied by accentuated arterial hypoxaemia. Whether altered autonomic cardiovascular regulation is apparent in CMS is unclear. Therefore, during the 2018 Global REACH expedition to Cerro de Pasco, Peru (4383 m), we assessed integrative control of blood pressure (BP) and determined basal sympathetic vasomotor outflow and arterial baroreflex function in eight Andean natives with CMS ([Hb]  $22.6 \pm 0.9$  g·dL<sup>-1</sup>) and seven healthy highlanders ([Hb]  $19.3 \pm 0.8$  g·dL<sup>-1</sup>). R-R interval (RRI, electrocardiogram), beat-by-beat BP (photoplethysmography) and muscle sympathetic nerve activity (MSNA; micro-neurography) were recorded at rest and during pharmacologically induced changes in BP (modified Oxford test). Although [Hb] and blood viscosity ( $7.8 \pm 0.7$  vs.  $6.6 \pm 0.7$  cP;  $d = 1.7$ ,  $P = 0.01$ ) were elevated in CMS compared to healthy highlanders, cardiac output, total peripheral resistance and mean BP were similar between groups. The vascular sympathetic baroreflex MSNA set-point (i.e. MSNA burst incidence) and reflex gain (i.e. responsiveness) were also similar between groups (MSNA set-point,  $d = 0.75$ ,  $P = 0.16$ ; gain,  $d = 0.2$ ,  $P = 0.69$ ). In contrast, in CMS the cardiovagal baroreflex operated around a longer RRI ( $960 \pm 159$  vs.  $817 \pm 50$  ms;  $d = 1.4$ ,  $P = 0.04$ ) with a greater reflex gain ( $17.2 \pm 6.8$  vs.  $8.8 \pm 2.6$  ms·mmHg<sup>-1</sup>;  $d = 1.8$ ,  $P = 0.01$ ) versus healthy highlanders. Basal sympathetic vasomotor activity was also lower compared to healthy highlanders ( $33 \pm 11$  vs.  $45 \pm 13$  bursts·min<sup>-1</sup>;  $d = 1.0$ ,  $P = 0.08$ ). In conclusion, our findings indicate adaptive differences in basal sympathetic vasomotor activity and heart rate

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compensate for the haemodynamic consequences of excessive erythrocyte volume and contribute to integrative blood pressure regulation in Andean highlanders with mild CMS.

#### KEYWORDS

arterial baroreflex, blood pressure control, chronic mountain sickness, excessive erythrocytosis, muscle sympathetic nerve activity

## 1 | INTRODUCTION

Globally, between 5 and 10% of the ~140 million people living at high altitude (>2500 m) lack the ability to cope with chronic hypoxia and develop a progressively incapacitating maladaptation syndrome termed chronic mountain sickness (CMS) (León-Velarde et al., 2005). CMS, which is most prevalent in natives of the Andean plateau, is characterized by excessive erythrocytosis (EE; haemoglobin concentration [Hb]  $\geq 21$  g dl<sup>-1</sup> for men,  $\geq 19$  g dl<sup>-1</sup> for women) and is frequently accompanied by accentuated arterial hypoxaemia for the resident altitude, and, in more severe stages of the disease, pulmonary hypertension (León-Velarde et al., 2005). In addition, CMS individuals may present with a number of clinical symptoms including headache, breathlessness, sleep disturbances and cognitive impairment (León-Velarde et al., 2005; Villafuerte & Corante, 2016). Importantly, CMS is also associated with an increased cardiovascular disease risk (Corante et al., 2018), which increases with disease severity. Specifically, an increased prevalence of thrombotic events, stroke, coronary heart disease and systemic and pulmonary hypertension, which can give rise to cardiac hypertrophy and congestive heart failure, have all been reported in CMS (Monge, 1942; Peñaloza et al., 1971; Leon-Velarde & Arregui, 1994; Leon-Velarde, Rivera-Ch, Huicho, & Villafuerte, 2014). Excessive erythrocyte volume and the resulting elevations in haemoglobin and haematocrit are known to contribute to this increased risk (Corante et al., 2018; Tremblay et al., 2019). However, several other clinical conditions characterized by sustained hypoxaemia (i.e. chronic obstructive pulmonary disease) are often accompanied by arterial baroreflex dysfunction and elevated sympathetic vasomotor outflow (Andreas, Haarmann, Klarner, Hasenfuß, & Raupach, 2013; van Gestel & Steier, 2010). Such changes, which can facilitate increased blood pressure variability, elevated blood pressure, increased arterial stiffness and vascular dysfunction (Hijmering et al., 2002; Smit et al., 2002; Swierblewska et al., 2010), can all contribute to the development of cardiovascular disease. Whether arterial baroreflex dysfunction and elevated sympathetic vasomotor outflow are also apparent in CMS is unclear.

The arterial baroreflex plays a fundamental role in the control of blood pressure through its regulation of cardiac pacemaker activity and sympathetic vasomotor outflow. Previous research has found impaired baroreflex control of R-R interval (RRI) in Andean highlanders with CMS compared to healthy highlanders (Keyl et al., 2003); however, this is not a consistent finding (Gulli et al., 2007). Baroreflex control of arterial pressure also occurs via alterations in sympathetic vasomotor outflow. Previously, no difference in maximum gain (i.e. responsiveness) of carotid baroreflex control of forearm

vascular resistance (index of sympathetic vasomotor activity) was reported for CMS compared to healthy Andean highlanders (Moore et al., 2006). Nevertheless, to the best of our knowledge, no direct measurement of sympathetic vasomotor activity exists for CMS individuals. Whilst plasma catecholamine concentrations may not accurately represent sympathetic nervous system activity (Esler et al., 1988), they are reported to be either elevated (Gamboa et al., 2006) or unchanged (Antezana, Richalet, Noriega, Galarza, & Antezana, 1995) in CMS, indicating either an increased or comparable global sympathetic activation compared to their healthy Andean counterparts. On one hand, elevated sympathetic activity might be predicted in CMS if exaggerated arterial hypoxaemia is present, thus augmenting tonic peripheral chemoreflex activation. On the other hand, a larger blood volume in CMS (Claydon et al., 2004) might have a sympathoinhibitory effect on basal MSNA, as shown in healthy individuals at sea-level (Best et al., 2014; Charkoudian et al., 2004).

In light of the equivocal findings, and absence of microneurographic data for CMS, it is unclear what effect CMS has on sympathetic neural control and arterial baroreflex regulation of blood pressure in Andean highlanders. The present study, therefore, aimed to comprehensively assess integrative regulation of resting blood pressure in Andean highlanders with CMS, and to compare this with healthy highlanders. To achieve this we assessed blood volume, basal sympathetic vasomotor outflow, and arterial baroreflex control of the heart and sympathetic vasomotor outflow. Based upon limited previous reports, we hypothesized that (1) the vascular sympathetic baroreflex would operate around a higher MSNA burst incidence for CMS, with no difference in reflex gain (i.e. responsiveness), compared to healthy highlanders; (2) the cardiovagal baroreflex would operate around a shorter RRI (higher HR) in CMS with a concurrent reduction in reflex gain; and therefore (3) basal sympathetic vasomotor outflow and arterial pressure would be elevated for CMS. A secondary aim was to determine the contribution of the peripheral chemoreflex to basal MSNA and arterial baroreflex function in CMS.

## 2 | METHODS

### 2.1 | Ethical approval

This study was part of the Global REACH high-altitude research expedition to the Universidad Peruana Cayetano Heredia's Instituto de Investigaciones de Altura (4380 m; Cerro de Pasco, Peru) in July 2018. All experimental procedures had Institutional Review Board approval from Universidad Peruana Cayetano Heredia (no. 101686; date of

approval, 20 February 2018) and conformed to the latest revision of the *Declaration of Helsinki*, except for registration in a database. Prior to participation, all experimental procedures were explained to subjects in writing, and verbally, in their native language, and written informed consent was provided. Participants took part in a number of other studies; however, care was taken to ensure adequate recovery between protocols to prevent any potential for confounding results. Furthermore, the present study addressed a distinct *a priori* research question.

## 2.2 | Participants

Twenty Andean men born at an altitude above 3250 m, permanently residing in the Cerro de Pasco area and who had at least two previous known generations of high-altitude Andean ancestry were recruited for the study. None of the subjects had travelled to an altitude lower than 3000 m in the previous 6 months and they did not have a history of working in the mining industry. None of the participants were taking prescribed medication and they had no prior history of cardiovascular, pulmonary, metabolic, neurological or renal disease. Participants attended the laboratory on two occasions, with a minimum of 24 h between visits: (1) a preliminary screening visit and (2) an experimental visit.

### 2.2.1 | Preliminary screening visit

On arrival to the laboratory, participants provided a detailed clinical history and history of high-altitude residence and ancestral background. A venous blood sample was drawn from the antecubital vein to measure [Hb], haematocrit and blood viscosity. An arterial blood sample was drawn from the radial artery (by Chris Gasho), following local anaesthesia (2% lidocaine), to determine arterial blood gases ( $P_{aO_2}$  and  $P_{aCO_2}$ ) and arterial oxygen saturation ( $S_{aO_2}$ ). Total blood volume (packed cell volume and plasma volume) was determined via the modified carbon monoxide rebreathing method as previously described in detail (Schmidt & Prommer 2005) and used previously by our group in lowland and highland natives at high altitude (Stembridge et al., 2018, 2019). Participants also performed an incremental exercise test ( $20 \text{ W min}^{-1}$ ) to exhaustion, in the semi-recumbent position, on an electronically braked cycle ergometer (Lode Angio; Lode, Groningen, The Netherlands). Breath-by-breath respiratory data were collected throughout (Oxycon Mobile; Carefusion, Hoechberg, Germany) to determine peak oxygen consumption ( $\dot{V}_{O_{2peak}}$ ).

Chronic mountain sickness scores were calculated using the Qinghai CMS questionnaire based on the presence and severity of eight signs and symptoms of CMS, as agreed by international consensus (León-Velarde et al., 2005): EE, heart palpitations, difficulty sleeping, cyanosis, parathesia, headache, tinnitus and dilated veins. A value of zero was assigned to negative answers. Positive answers were categorized as light, moderate, or severe and assigned values of 1, 2 and 3, respectively. The sum of assigned values constituted the CMS score. Subjects were diagnosed with CMS by a score  $\geq 5$  in the presence of EE ( $[\text{Hb}] \geq 21 \text{ g dl}^{-1}$ ) and individuals not meeting this criterion were categorized as healthy highlanders. The sum of the score

### New Findings

- **What is the central question of this study?**  
Does chronic mountain sickness (CMS) alter sympathetic neural control and arterial baroreflex regulation of blood pressure in Andean (Quechua) highlanders?
- **What is the main finding and its importance?**  
Compared to healthy Andean highlanders, basal sympathetic vasomotor outflow is lower, baroreflex control of muscle sympathetic nerve activity is similar, supine heart rate is lower and cardiovagal baroreflex gain is greater in mild CMS. Taken together, these findings reflect flexibility in integrative regulation of blood pressure that may be important when blood viscosity and blood volume are elevated in CMS.

defines CMS severity as absent (0–5), mild (6–10), moderate (11–14) or severe ( $\geq 15$ ). Two highlanders (both CMS) were current smokers, but refrained from smoking on the day of testing.

## 3 | EXPERIMENTAL VISIT

All participants were asked to abstain from caffeine, alcohol and vigorous exercise for at least 24 h before the experimental session and arrived at the laboratory a minimum of 4 h after a light meal. Following arrival at the laboratory, subjects rested in the supine position and an antecubital venous cannula was inserted for subsequent drug administration. Following instrumentation, acquisition of an acceptable MSNA signal and a period of stabilization, 10 min of baseline data were recorded to determine resting cardiovascular and pulmonary haemodynamics and sympathetic vasomotor activity. A modified Oxford test was then performed to assess vascular sympathetic and cardiovagal baroreflex function. Following baseline measurements, participants were then transferred to breathing 100%  $O_2$ , in an attempt to eliminate peripheral chemoreceptor drive, as used previously (Simpson et al., 2019). Subjects breathed hyperoxia, via a mouthpiece, for a period of 5 min. Following 5 min of hyperoxia, a second modified Oxford test was performed, whilst subjects continued to breathe hyperoxia, in order to determine the influence of the peripheral chemoreflex on arterial baroreflex function. Due to the unknown time course of recovery from hyperoxia, the order of conditions was not randomized. A minimum of 20 min separated each modified Oxford test.

## 4 | EXPERIMENTAL MEASUREMENTS

### 4.1 | Haematological analysis

Venous blood samples were collected into lithium heparin-coated vacutainers (Becton, Dickinson and Company, Mississauga, Canada)

and tested within 15 min of acquisition. Arterial blood samples were collected into pre-heparinized syringes (safePICO syringes, Radiometer, Copenhagen, Denmark) for immediate analyses. Whole blood viscosity was measured in duplicate at a shear rate  $225 \text{ s}^{-1}$  at  $37^\circ\text{C}$  using a cone and plate viscometer (DV2T Viscometer, Brookfield Amtek, Massachusetts, USA) and a circulating water heating bath (TC-150, Brookfield Amtek). [Hb] and haematocrit, arterial blood gases and  $\text{S}_{\text{aO}_2}$  were determined with a Radiometer ABL90 analyser (Radiometer, Ontario, Canada).

#### 4.1.1 | Cardiovascular haemodynamics

Heart rate and blood pressure were continuously recorded using a Lead II electrocardiogram and finger photoplethysmography (Finometer Pro; Finapres Medical Systems BV, Amsterdam, The Netherlands). Systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP) were calculated on a beat-by-beat basis from the finger arterial pressure waveform. Finometer values were calibrated against the average of three brachial artery blood pressure measurements taken during baseline. Stroke volume (SV) and cardiac output ( $\dot{Q}_\text{C}$ ) were estimated using the Model Flow algorithm (Wesseling, Jansen, Settels, & Schreuder, 1993) and used to estimate total peripheral resistance ( $\text{TPR} = \text{MAP}/\dot{Q}_\text{C}$ ).

#### 4.1.2 | Pulmonary haemodynamics

Echocardiography was used to assess pulmonary artery systolic pressure (PASP). Images were obtained using a commercially available system (Vivid Q, GE, Fairfield, CT, USA) and stored for subsequent off-line analysis. PASP was quantified as the maximum systolic pressure gradient across the tricuspid valve added to right atrial pressure estimated from the collapsibility of the inferior vena cava, in line with the guidelines of the American Society of Echocardiography (Rudski et al., 2010). To derive pressure, the modified Bernoulli equation ( $4V^2$ ) was applied to the peak systolic regurgitation jet velocity measured via continuous wave Doppler (Rudski et al., 2010).

#### 4.1.3 | Muscle sympathetic nerve activity

Multi-unit MSNA was recorded from the peroneal nerve via micro-neurography as previously described (Hagbarth & Vallbo, 1968; Sundolf & Wallin, 1978). The MSNA signal was confirmed by pulse-synchronous activity that responded to end-expiratory apnoea but not to startle stimuli or skin stroking. Nerve signals were acquired (Neuro-amp EX headstage, ADInstruments, Sydney, Australia), amplified ( $\times 100,000$ ), filtered (band pass 700–2000 Hz), rectified and integrated (decay constant 0.1 s) (LabChart Pro v8.3.1, ADInstruments). No adverse events or complications occurred during or following the microneurography procedure in any subject.

#### 4.1.4 | Assessment of sympathetic and cardiac baroreflex function

Baroreflex function was assessed from the MSNA and RRI (and HR) responses during arterial blood pressure perturbations induced by the modified Oxford test (Rudas et al., 1999). Briefly, this involved bolus injection of sodium nitroprusside (SNP), followed 90 s later

by phenylephrine (PE). Prior to experimental testing, bolus doses of SNP and PE that evoked  $\sim 15$  mmHg perturbations above and below resting arterial blood pressure were determined for each individual. Briefly, individualized doses of vasoactive drugs were calculated based on total blood volume ( $20 \mu\text{g}\cdot\text{l}^{-1}$  SNP;  $30 \mu\text{g}\cdot\text{l}^{-1}$  PE), which were adjusted if insufficient BP perturbations were achieved. Identical doses were administered during all trials in the same individual. Doses of vasoactive drugs injected were similar in CMS (SNP,  $1.66 \pm 0.35 \mu\text{g}\cdot\text{kg}^{-1}$ ; PE,  $2.48 \pm 0.25 \mu\text{g}\cdot\text{kg}^{-1}$ ) and healthy highlanders (SNP,  $1.48 \pm 0.30 \mu\text{g}\cdot\text{kg}^{-1}$ ,  $P = 0.30$ ,  $d = 0.5$ ; PE,  $2.34 \pm 0.42 \mu\text{g}\cdot\text{kg}^{-1}$ ,  $P = 0.49$ ,  $d = 0.42$ ) and induced similar total blood pressure perturbations in both groups (CMS,  $26 \pm 5$  mmHg; healthy highlanders,  $25 \pm 9$  mmHg;  $P = 0.72$ ,  $d = 0.11$ ).

## 4.2 | Data analyses

All haemodynamic data were sampled at 1 kHz using commercial data acquisition software (LabChart Pro v8.3.1, ADInstruments) and stored on a laboratory computer for offline analysis. The raw MSNA signal were sampled at 10 kHz. Multi-unit bursts of MSNA were identified using an automated detection algorithm (Chart Pro 8.3.1) and confirmed by a trained observer (S.A.B./L.L.S.), using established criteria (White, Shoemaker, & Raven, 2015). To account for sympathetic baroreflex latency, MSNA data were shifted backwards (average shift: CMS  $-1.23 \pm 0.05$  s, healthy highlanders  $-1.17 \pm 0.05$  s) so that the peak of each sympathetic burst coincided with the diastolic period which initiated it (Simpson et al., 2019). To account for differences in microelectrode positioning, burst amplitude data were normalized by assigning a value of 100 to the largest burst observed during baseline. All other bursts were calibrated against this value. Resting sympathetic vasomotor activity was quantified as MSNA burst frequency ( $\text{burst}\cdot\text{min}^{-1}$ ) and total activity (mean burst amplitude  $\times$  burst frequency [ $\text{a.u}\cdot\text{min}^{-1}$ ]) as it reflects the amount of neurotransmitter release and thus vasoconstrictor drive to the vasculature over a given time period (Charkoudian & Wallin, 2014).

Baroreflex control of MSNA was assessed from the relationship between DBP and MSNA burst probability. DBP was used because MSNA correlates more closely with DBP than with SBP (Sundlof & Wallin, 1978). All DBP values during the modified Oxford test were assigned to a 3 mmHg bin to reduce the statistical impact of respiratory related oscillations (Eckberg & Eckberg, 1982). The percentage of cardiac cycles associated with a burst of MSNA (ranging from 0 to 100%) was calculated for each DBP bin to give values of burst probability. Non-linear saturation and threshold regions, if present, were excluded through visual inspection of data points by agreement of two observations. The slope of the linear relationship was determined by weighted linear regression analysis, and this value provided an index of vascular sympathetic vascular baroreflex gain. Only slopes with (1) at least five data points and (2)  $R \geq 0.5$  were included in the group mean data (Hart et al., 2011). Vascular sympathetic baroreflex gain for rising and falling pressures were not determined independently. The operating point of the vascular sympathetic baroreflex was taken as the average value for MSNA burst

incidence (bursts per 100 heart beats (HB)) and DBP during the baseline period immediately before the modified Oxford test. In contrast to burst frequency, burst incidence, which is temporally independent, is an index of reflex control and baroreflex 'gating' of sympathetic bursts. Baroreflex control of the heart was assessed from the relationship between SBP and RRI/HR during the modified Oxford test. SBP was used as it correlates more closely with RRI and HR than does DBP (Sundlof & Wallin, 1978). Values were averaged over 3 mmHg SBP bins. Baroreflex delays were accounted for by associating SBP values with either the concurrent heartbeat (resting RRI > 800 ms, HR < 75 bpm) or subsequent heartbeat (resting RRI < 800 ms, HR > 75 bpm) (Eckberg & Eckberg, 1982). Saturation and threshold regions were excluded by visual inspection; slopes were determined by weighted linear regression analysis and only slopes with at least five data points and  $R \geq 0.8$  were included in the group mean data (Taylor et al., 2015). To minimize the potential effects of hysteresis, we restricted data analysis to the rising arm of SBP and used values from the nadir to the peak SBP response (Hunt & Farquhar, 2005). The operating point was taken as the average values for RRI/HR and SBP during the resting period prior to the modified Oxford test.

### 4.3 | Statistical analyses

Differences between groups (CMS vs. healthy highlanders) and between conditions (baseline vs. hyperoxia) were assessed using pre-planned contrasts. To address hypotheses 1, 2 and 3, differences in arterial baroreflex function, basal sympathetic vasomotor activity and arterial pressure, between CMS and healthy highlanders, were assessed using an independent Student's *t* test. To address our secondary aim and examine the contribution of the peripheral chemoreflex mechanism, differences in arterial baroreflex function, sympathetic vasomotor activity and arterial pressure in CMS and healthy highlanders between baseline and hyperoxia were assessed using a dependent *t* test. Significant cardiovagal baroreflex slopes ( $R \leq 0.8$ ) were not obtained in one CMS participant and one healthy highlander; therefore cardiovagal baroreflex gain analyses at baseline were based on seven CMS participants and six healthy highlanders. As a result of MSNA signal losses, repeated measures comparisons for cardiovascular haemodynamics and sympathetic neural activity during hyperoxia were performed on six CMS participants and six healthy highlanders. Furthermore, during hyperoxia, cardiovagal baroreflex slopes did not meet the inclusion criteria ( $R \leq 0.8$ ) in one out of six healthy highlanders; therefore, repeated measures comparisons for cardiovagal baroreflex gain are limited to five healthy highlanders and six CMS individuals. Multiple *t* tests were chosen to maximize the number of subjects included in statistical analyses. To correct for multiple comparisons, *a priori*  $\alpha$  was adjusted, using the experiment-wise error rate (Hinkle, Wiersma, & Jurs, 2003) as used previously (Busch et al., 2018; Simpson et al., 2019). Statistical significance was set at  $P < 0.05$ . Furthermore, due to a small sample size, Cohen's *d* effect sizes are also reported with  $d \geq 0.8$  indicative of large effects (Cohen, 1988). Normality was assessed using the Shapiro–Wilk test, and data that were not normally distributed underwent  $\log_{10}$  transformation

prior to analysis. All statistical analyses were performed using Prism 7.03 (GraphPad Software Inc., La Jolla, CA, USA). Data are presented as means  $\pm$  SD. Differences between groups and conditions are also reported as mean difference and 95% confidence interval.

## 5 | RESULTS

### 5.1 | Participant characteristics

Although 20 participants were recruited for the study, an MSNA signal could not be obtained in five of them; therefore data are presented for 15 participants. We tested eight CMS individuals with a mean  $\pm$  SD CMS score of  $8 \pm 2$  (range 5–11) and seven healthy highlanders with a CMS score of  $1 \pm 1$  (range 0–3). Seven CMS participants were classified as having mild CMS and one was classified as having moderate CMS. CMS participants were similar in age ( $40 \pm 12$  years), weight ( $69 \pm 12$  kg), height ( $1.61 \pm 0.06$  m) and body mass index (BMI;  $26.4 \pm 4.9$  kg·m<sup>-2</sup>) to healthy highlanders ( $45 \pm 12$  years;  $d = 0.42$ ,  $P = 0.39$ ;  $71 \pm 11$  kg;  $d = 0.18$ ,  $P = 0.79$ ;  $1.61 \pm 0.03$  m;  $d = 0$ ,  $P = 0.97$ ;  $26.5 \pm 3.8$  kg·m<sup>-2</sup>;  $d = 0.21$ ,  $P = 0.74$ ).  $\dot{V}_{O_{2peak}}$  values were also similar in CMS and healthy highlanders ( $32.9 \pm 10.5$  vs.  $28.7 \pm 8.8$  ml kg<sup>-1</sup> min<sup>-1</sup>;  $d = 0.43$ ,  $P = 0.49$ ).

### 5.2 | Resting cardiovascular haemodynamics, basal sympathetic neural activity

$S_{aO_2}$  and  $P_{aO_2}$  were lower and  $P_{aCO_2}$  was higher in CMS compared to healthy highlanders (Table 1). As expected, haemoglobin concentration, haematocrit and blood viscosity ( $7.8 \pm 0.7$  vs.  $6.6 \pm 0.7$  cP;  $d = 1.7$ ,  $P = 0.01$ ) were all higher in CMS (Table 1). Although not statistically significant, total blood volume tended to be larger in CMS compared to healthy highlanders ( $101 \pm 25$  vs.  $85 \pm 16$  ml·kg<sup>-1</sup>;  $d = 0.8$ ,  $P = 0.2$ ), which was due to a larger total red blood cell volume, with a similar plasma volume between groups (Table 1, Figure 1). CMS also tended to exhibit a greater SV ( $76 \pm 13$  vs.  $64 \pm 19$  ml;  $d = 0.8$ ,  $P = 0.32$ ), and had a lower HR ( $64 \pm 10$  vs.  $74 \pm 4$  bpm;  $d = 1.4$ ,  $P = 0.03$ ) compared to healthy highlanders, with a similar  $\dot{Q}_C$  in both groups ( $4.8 \pm 0.7$  vs.  $4.7 \pm 1.3$  l·min<sup>-1</sup>;  $d = 0.1$ ,  $P = 0.83$ ). TPR was also similar between CMS and healthy highlanders ( $19.2 \pm 6.2$  vs.  $19.9 \pm 5.5$  mmHg·L<sup>-1</sup>·min<sup>-1</sup>); however, CMS exhibited a lower MSNA burst frequency ( $33 \pm 11$  vs.  $45 \pm 13$  burst·min<sup>-1</sup>;  $d = 1.0$ ,  $P = 0.08$ ) compared to healthy highlanders (Figure 1). Because  $\dot{Q}_C$  and TPR were comparable, MAP was also similar in CMS compared to healthy highlanders (Figure 1). Unfortunately, due to the difficulty in identifying the tricuspid valve regurgitant jet in four participants we could only obtain PASP measurements in seven CMS and four healthy highlanders. PASP values were not significantly different between groups (Table 1).

### 5.3 | Arterial baroreflex function

Vascular sympathetic baroreflex gain (i.e. slope of the DBP–MSNA burst probability relationship) was comparable in CMS and healthy

**TABLE 1** Haematological variables and resting pulmonary haemodynamics in CMS ( $n = 8$ ) and healthy highlanders ( $n = 7$ )

	CMS	Healthy highlanders	<i>P</i>	Cohen's <i>d</i>	Mean difference (95% CI)
<i>Haematological variable</i>					
Haemoglobin (g·dl <sup>-1</sup> )	22.6 ± 0.9	19.3 ± 0.8	<b>&lt;0.01</b>	3.9	3.3 (2.2 to 4.2)
Haematocrit (%)	65 ± 5	57 ± 3	<b>&lt;0.01</b>	2.0	8 (3 to 13)
S <sub>aO<sub>2</sub></sub> (%)†	82 ± 2	87 ± 3	<b>0.01</b>	2.0	-5 (-9 to -2)
P <sub>aO<sub>2</sub></sub> (mmHg)†	47 ± 2	51 ± 4	<b>0.05</b>	1.4	-4 (-8 to 0)
P <sub>aCO<sub>2</sub></sub> (mmHg)†	34 ± 1	29 ± 4	<b>0.02</b>	1.9	5 (1 to 9)
RBC volume (ml·kg <sup>-1</sup> )	57 ± 14	48 ± 8	0.19	0.8	9 (-5 to 21)
Plasma volume (ml·kg <sup>-1</sup> )	41 ± 9	42 ± 9	0.87	0.1	-1 (-11 to 9)
<i>Pulmonary haemodynamics</i>					
PASP (mmHg)‡	29 ± 7	33 ± 7	0.4	0.6	-4 (-14 to 6)

Data presented as means ± SD. †Values based on six CMS and five healthy highlanders; ‡values based on seven CMS and four healthy highlanders. Statistical comparisons performed using independent *t*-tests. *P*-values shown in bold are considered statistically significant. RBC, red blood cell.

highlanders ( $-2.5 \pm 0.9$  vs.  $-2.7 \pm 1.1\%$  mmHg<sup>-1</sup>;  $d = 0.2$ ,  $P = 0.69$ , mean diff. 0.2 (-0.9 to 1.3)). The operating DBP was also similar in both groups ( $71 \pm 4$  vs.  $74 \pm 9$  mmHg;  $d = 0.5$ ,  $P = 0.41$ , mean diff. -3 (-11 to 4)). The MSNA set-point appeared lower in CMS compared to healthy highlanders ( $51 \pm 12$  vs.  $62 \pm 17$  bursts·100 HB<sup>-1</sup>;  $d = 0.75$ ,  $P = 0.16$ , mean diff. -11 (-28 to 8)), although this was not statistically significant (Figure 2a).

Cardiovascular baroreflex gain (i.e. slope of the relationship between RRI and SBP) was greater in CMS compared to healthy highlanders ( $17.2 \pm 6.8$  vs.  $8.8 \pm 2.6$  ms·mmHg<sup>-1</sup>;  $d = 1.8$ ,  $P < 0.01$ , mean diff. 8.4 (2.7 to 15.0); Figure 2b). These findings were similar regardless of whether RRI or HR was used. Operating SBP was similar in both groups (CMS,  $109 \pm 8$  vs. healthy highlanders,  $113 \pm 15$  mmHg;  $d = 0.4$ ,  $P = 0.5$ , mean diff. -4 (-17 to 8)); however CMS participants operated around a longer RRI ( $960 \pm 159$  vs.  $817 \pm 50$  ms;  $d = 1.4$ ,  $P = 0.04$ , mean diff. -143 (-7 to -279); Figure 2).

### 5.4 | Arterial baroreflex-peripheral chemoreflex interactions

In CMS participants exposed to 100% O<sub>2</sub>, HR significantly decreased and  $\dot{Q}_C$  also tended to decrease, although this did not achieve significance ( $d = 0.6$ ,  $P = 0.07$ ). Oxygen administration had no effect on any other cardiovascular haemodynamic variable in CMS. HR and  $\dot{Q}_C$  both significantly decreased in healthy highlanders exposed to 100% O<sub>2</sub>. This reduction in  $\dot{Q}_C$  was accompanied by an increase in TPR, with no significant effect on BP. The reduction in HR in both groups was accompanied by a lowering of MSNA burst frequency, with no effect on burst amplitude (Table 2).

Administration of oxygen had no significant effect on vascular sympathetic baroreflex gain, operating DBP or MSNA set-point in either CMS or healthy highlanders. Administration of oxygen had no effect on cardiovagal baroreflex gain ( $18.8 \pm 9.7$  to  $20.3 \pm 7.4$  ms·mmHg<sup>-1</sup>;  $d = 0.2$ ,  $P = 0.7$ ; Figure 3) or operating SBP in CMS, but

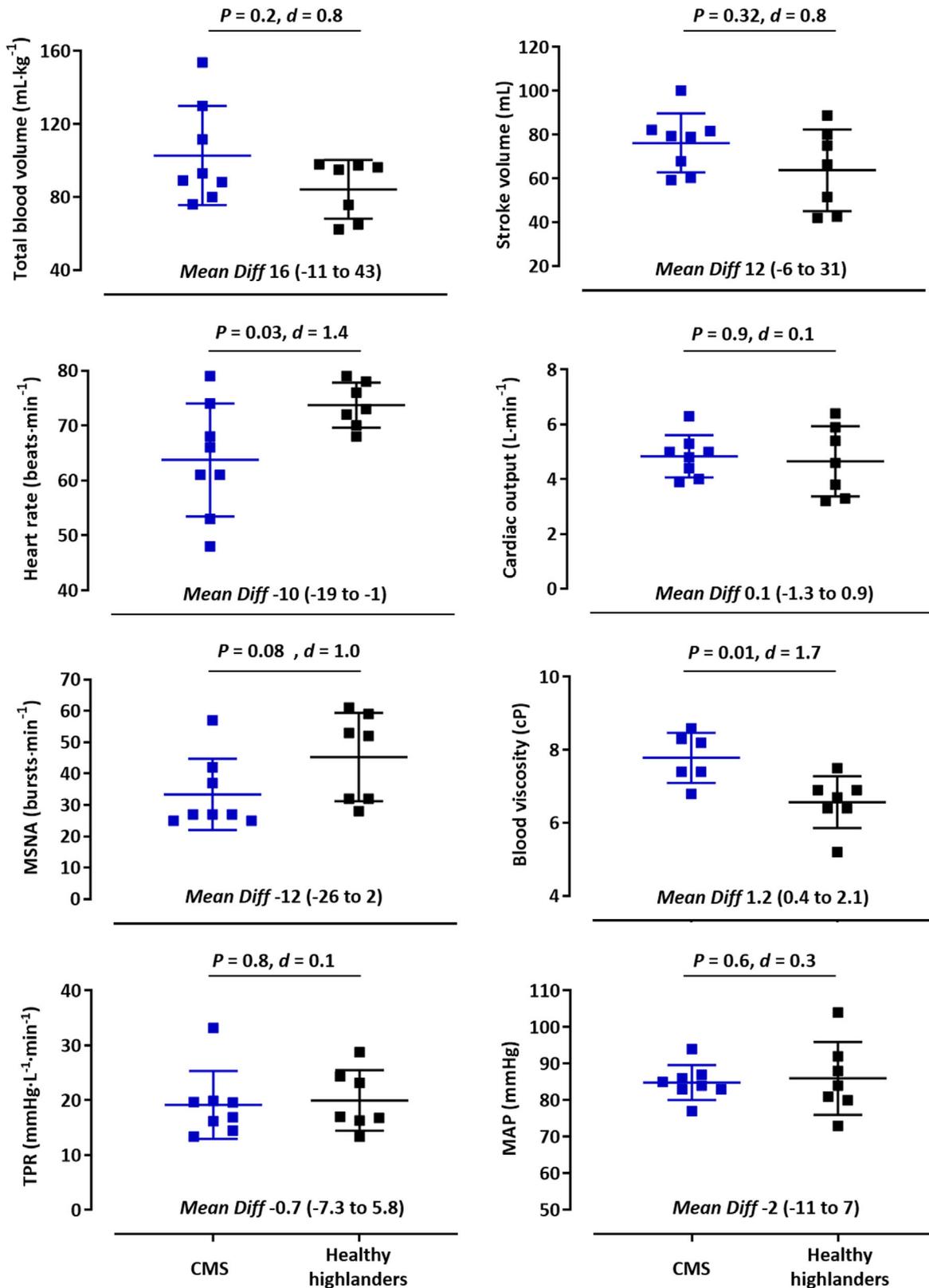
RRI was longer. In healthy highlanders administration of oxygen also increased RRI; cardiovagal baroreflex gain was greater ( $8.0 \pm 2.6$  to  $14.1 \pm 4.9$  ms·mmHg<sup>-1</sup>;  $d = 1.6$ ,  $P = 0.01$ ), with no change in operating SBP (Figure 3).

## 6 | DISCUSSION

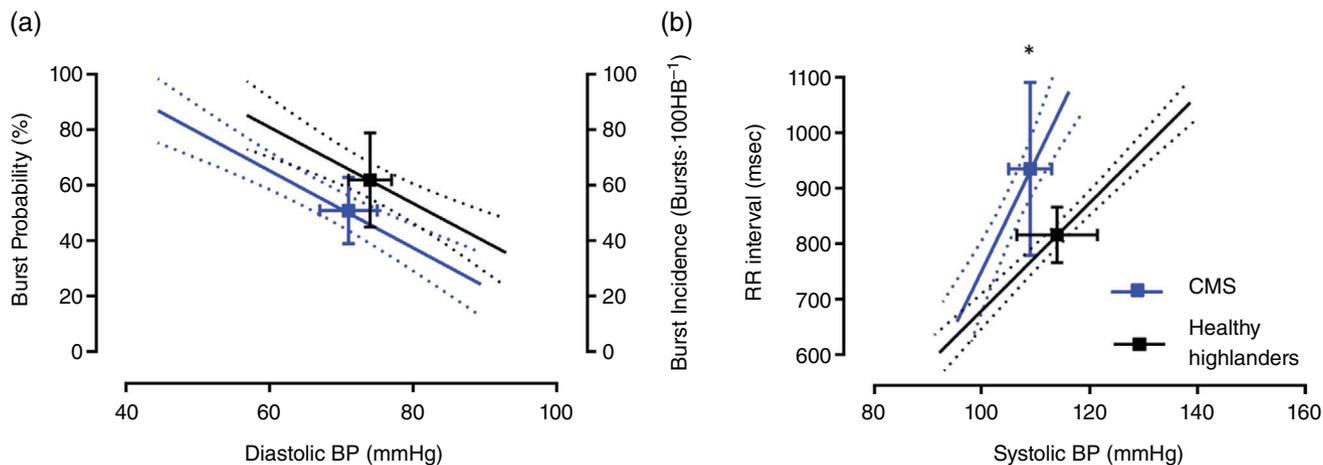
The major findings of the present study are threefold: (1) CMS individuals and healthy highlanders exhibit similar vascular sympathetic baroreflex gain (i.e. responsiveness), operating diastolic pressure and MSNA set-point (i.e. MSNA burst incidence); (2) however, in mild CMS, the cardiovagal baroreflex operates around a longer RRI (lower heart rate) with a greater reflex gain; and (3) CMS individuals have comparable cardiac output, total peripheral resistance, and thus, arterial pressure compared to healthy highlanders. However, CMS individuals exhibit a greater haemoglobin concentration, total blood volume and blood viscosity, and lower basal vasomotor sympathetic activity (i.e. MSNA burst frequency) compared to healthy highlanders. Taken together, these findings indicate adaptive changes in autonomic regulation of blood pressure homeostasis in Andean highlanders with mild CMS.

### 6.1 | Basal sympathetic vasomotor activity in Andeans

The one previous study that has assessed resting sympathetic vasomotor activity in Andean high-altitude natives found comparable basal MSNA in healthy Bolivian Andeans (Aymara) and acclimatizing lowlanders (Lundby, Calbet, van Hall, Saltin, & Sander, 2018). However, we are the first to assess basal sympathetic vasomotor activity in Peruvian (Quechua) Andeans, including individuals with CMS. We observe a 25% lower basal MSNA in CMS compared to healthy highlanders, as indicated by a reduced MSNA burst frequency



**FIGURE 1** Group mean ( $\pm$ SD) and individual data for haematological and cardiovascular haemodynamic variables in CMS ( $n = 8$ ) and healthy highlanders ( $n = 7$ ). Blood viscosity values based on six CMS and five healthy highlanders. Statistical comparisons performed using independent  $t$  tests.  $P$ -values are reported with Cohen's  $d$  effect sizes, and mean differences with (95% confidence intervals)



**FIGURE 2** Arterial baroreflex function. Group average regressions between (a) diastolic BP and MSNA burst probability in CMS ( $n = 8$ ) and healthy highlanders ( $n = 7$ ) and (b) systolic BP and RRI in CMS ( $n = 7$ ) and healthy highlanders ( $n = 6$ ). The set-points of the vascular sympathetic and cardiovagal limb of the baroreflex are indicated by the symbols and error bars (mean  $\pm$  SD). The arterial baroreflex set points and slopes were compared using independent  $t$  tests. \* $P < 0.05$  versus healthy highlanders. The vascular sympathetic baroreflex set-point was similar between CMS and healthy highlanders. The slope of the relationship between diastolic BP and MSNA burst probability was also similar between groups, indicating no differences in reflex gain. The cardiovagal baroreflex operated around a similar systolic BP, but a greater RRI set-point in CMS compared to healthy highlanders. The slope of the relationship between systolic BP and R-R interval was also greater in CMS, indicating a greater reflex gain

(Figure 1). This finding is in contrast to our hypothesis that basal sympathetic vasomotor activity would be greater in CMS, which was based upon previous studies reporting either comparable (Antezana et al., 1995) or elevated (Gamboa et al., 2006) plasma noradrenaline levels in individuals with CMS. A reduced glomerular filtration rate (Lozano, & Monge, 1965) and thus noradrenaline clearance, in CMS would, however, serve to overestimate sympathetic activation using this method, and potentially explain these contradictory findings. Despite this, it might be anticipated that sympathetic vasomotor activity would be elevated in CMS individuals due to several factors. These factors include exaggerated arterial hypoxaemia (lower  $P_{aO_2}$ ), reports of increased inflammation and oxidative stress (Bailey et al., 2013, Bailey et al., 2019), and a reduced NO bioavailability, all of which exert known sympathoexcitatory effects (Patel, Li, & Hirooka, 2001). Sympathetic vasomotor outflow, however, is the net effect of the integration of both excitatory and inhibitory inputs to the cardiovascular control centres in the brainstem. For example, elevations in blood volume exert a sympathoinhibitory influence on basal MSNA (Best et al., 2014, Charkoudian et al., 2004). Notably, CMS individuals in the present study exhibited a 20% greater blood volume compared to healthy highlanders (Figure 1). Whilst not statistically significantly different, the effect was large (Cohen's  $d = 0.8$ ) and the differences were comparable to those previously reported in this population (Claydon et al., 2004). Thus, lower basal sympathetic vasomotor activity in CMS could be mediated by an increase in circulating blood volume. Indeed, we have previously demonstrated a lower basal sympathetic activity in high-altitude native Sherpa, compared to acclimatizing Lowlanders (Simpson et al., 2019), with Sherpa also exhibiting a greater total blood volume (Stembridge et al., 2019). Despite this, however, there was no significant correlation between these factors in the present study (data not shown). It is

also important to note, however, that individuals with CMS were on average  $\sim 5$  years younger than healthy highlanders, although the reported  $\sim 3$  bursts  $\cdot$   $\text{min}^{-1}$  increase in basal MSNA per decade of life (Narkiewicz et al., 2005) would not exclusively explain the observed 12 bursts  $\cdot$   $\text{min}^{-1}$  difference in basal MSNA.

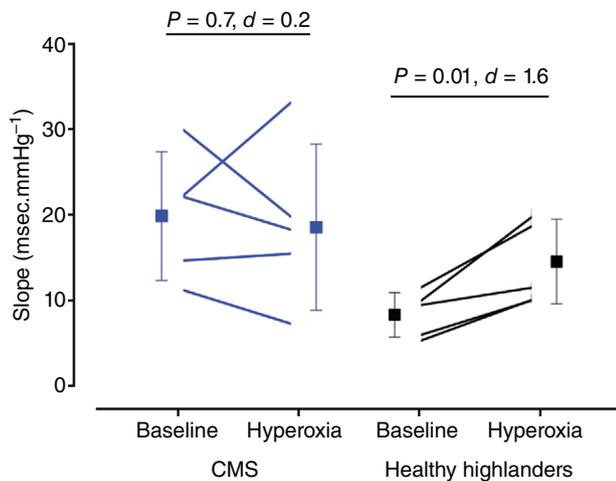
## 6.2 | Arterial baroreflex function in Andeans

This is the first study to assess baroreflex control of MSNA in Andean high-altitude natives. In addition, it is the first to simultaneously assess the vascular sympathetic and cardiovagal limbs of the arterial baroreflex in the same group. We demonstrated that both CMS and healthy highlanders exhibit a similar ability to increase and decrease MSNA in response to transient, pharmacologically induced changes in blood pressure (i.e. the vascular sympathetic baroreflex gain was unchanged). This is consistent with one previous report of a similar reflex gain for carotid baroreflex control of forearm vascular resistance (Moore et al., 2006) in both CMS and healthy highlanders. Contrary to our hypothesis, resting heart rate was lower, and the ability to alter RRI during the modified Oxford test was greater in CMS compared to healthy highlanders. The operating diastolic pressure for the vascular sympathetic baroreflex and the operating systolic pressure for the cardiovagal baroreflex were similar in CMS and healthy highlanders. Furthermore, MSNA burst incidence (i.e. vascular sympathetic baroreflex set-point) was also not significantly different between groups, meaning the probability of a burst occurring per cardiac cycle was similar between CMS and healthy highlanders. Therefore, our data indicate that CMS does not influence the arterial baroreflex control and gating of sympathetic bursts. Importantly, however, a lower resting heart rate in CMS reduces the opportunities (i.e. cardiac cycles) for a burst to occur. Thus, the interaction of a

**TABLE 2** Haemodynamics, MSNA and arterial baroreflex function at baseline and during hyperoxia in CMS ( $n = 6$ ) and healthy highlanders ( $n = 6$ )

	CMS						Healthy highlanders					
	Baseline	Hyperoxia	P	Cohen's <i>d</i>	Mean difference (95% CI)		Baseline	Hyperoxia	P	Cohen's <i>d</i>	Mean difference (95% CI)	
<b>Haemodynamics</b>												
Heart rate (bpm)	60 ± 10	54 ± 14	<0.01	0.5	-6 (-10 to -3)		69 ± 7	61 ± 6	0.03	1.2	-8 (-14 to -1)	
R-R interval (ms)	1019 ± 164	1175 ± 263	0.01	0.7	156 (52 to 262)		880 ± 89	905 ± 100	0.03	0.3	25 (17 to 207)	
Stroke volume (ml)	92 ± 6	93 ± 12	0.95	0.1	0.6 (-16 to 18)		80 ± 21	82 ± 26	0.72	0.1	1.8 (-8 to 12)	
Cardiac output (l · min <sup>-1</sup> )	5.6 ± 1.1	4.9 ± 1.2	0.07	0.6	-0.7 (-1.4 to 0.1)		5.4 ± 1.1	4.9 ± 1.4	0.05	0.4	-0.5 (-0.9 to -0.2)	
TPR (mmHg · l <sup>-1</sup> · min <sup>-1</sup> )	16.0 ± 3.2	17.9 ± 4.9	0.18	0.5	1.9 (-1.3 to 5.1)		17.6 ± 5.7	20.5 ± 7.9	0.01	0.4	2.9 (0.3 to)	
Diastolic BP (mmHg)	73 ± 12	71 ± 12	0.20	0.2	-2 (-4 to 1)		74 ± 5	77 ± 6	0.15	0.5	3 (-1 to 6)	
Systolic BP (mmHg)	118 ± 8	115 ± 10	0.13	0.3	-3 (-6 to 1)		115 ± 7	120 ± 12	0.13	0.5	5 (-2 to 13)	
MAP (mmHg)	89 ± 9	89 ± 5	0.15	0.2	-2 (-4 to 1)		87 ± 11	92 ± 7	0.11	0.5	3 (-1 to 8)	
<b>Sympathetic neural activity</b>												
Burst frequency (burst · min <sup>-1</sup> )	32 ± 13	29 ± 13	0.02	0.7	-3 (-6 to -1)		41 ± 16	33 ± 13	0.04	0.7	-8 (-16 to -0.7)	
Burst incidence (bursts · 100 HB <sup>-1</sup> )	51 ± 14	53 ± 13	0.37	0.2	2 (-2 to 5)		60 ± 20	54 ± 21	0.15	0.2	-6 (-13 to 3)	
Normalized burst amplitude (a.u.)	56 ± 8	55 ± 13	0.99	0.2	2 (-10 to 9)		57 ± 5	54 ± 13	0.78	0.2	2 (-10 to 13)	
Total activity (a.u. · min <sup>-1</sup> )	1780 ± 801	1618 ± 922	0.19	0.4	-162 (-473 to 149)		2335 ± 900	1992 ± 1026	0.08	0.4	-343 (-728 to 42)	

Data presented as means ± SD. Statistical comparisons performed using dependent *t* tests. *P*-values shown in bold are considered statistically significant.



**FIGURE 3** Cardiovascular baroreflex gain. Individual and group average slopes for the relationship between RRI and systolic blood pressure at baseline and during hyperoxia in CMS ( $n = 5$ ) and healthy highlanders ( $n = 6$ ). Administration of oxygen had no effect on cardiovascular baroreflex gain in CMS, but increased cardiovascular baroreflex gain ( $8.0 \pm 2.6$  to  $14.1 \pm 4.9$  ms $\cdot$ mmHg $^{-1}$ ;  $d = 1.6$ ,  $P = 0.01$ ) in healthy highlanders. Statistical comparisons performed using dependent  $t$  tests

similar MSNA burst incidence (i.e. vascular sympathetic baroreflex set-point) and lower resting heart in CMS reduces MSNA burst frequency (i.e. basal sympathetic vasomotor activity). It should be noted that, in contrast to our findings, several studies report higher, rather than lower, resting heart rates and/or blood pressures for CMS (Claydon et al., 2004; Keyl et al., 2003; Richalet et al., 2005; Corante et al., 2018). The reasons for these differences are unclear, although differences in posture, measurement technique, time of day for measurement, and the duration of the measurement period might all contribute. In addition, CMS severity may also be important; indeed, studies that report higher resting heart rates and blood pressures for CMS also report greater average Hct and CMS scores than those observed in the present study. Moreover, a positive correlation has been observed between [Hb] and blood pressure in the Cerro de Pasco population (Gonzales & Tapia, 2013). Importantly in the present study, cardiac output, total peripheral resistance, and thus, arterial pressure for CMS are comparable to healthy highlanders; this is despite greater blood viscosity and total blood volume, secondary to an increase in red blood cell volume, in CMS. Thus, the lower sympathetic vasomotor outflow and heart rate in CMS appear to balance the haemodynamic effects of EE, which maintains blood pressure homeostasis, at least in mild CMS studied here.

### 6.3 | Influence of peripheral chemoreflex on arterial baroreflex function in Andeans

Lower  $P_{aO_2}$  in CMS individuals would be expected to increase peripheral chemoreflex activation and potentially reset the arterial baroreflex to operate at higher heart rates, arterial pressures and level of MSNA (Halliwill & Minson, 2002; Steinback, Salzer,

Medeiros, Kowalchuk, & Shoemaker, 2009). Importantly, however, peripheral chemoreceptor ventilatory responsiveness to hypoxia is reported to be blunted in CMS individuals (León-Velarde & Richalet, 2006; Severinghaus, Bainton, & Carcelen, 1966), contributing to alveolar hypoventilation (higher  $P_{aCO_2}$ ) reported in this population (León-Velarde & Richalet, 2006). Despite a blunted ventilatory responsiveness reported in CMS, the peripheral chemoreflex mechanism did not appear to contribute to the lower HR in CMS, as acutely eliminating peripheral chemoreceptor drive, via 100% oxygen administration, had comparable effects on HR in both groups. Interestingly, MSNA burst incidence remained unchanged for CMS during acute hyperoxia, whilst it was reduced ( $\sim 6$  bursts $\cdot$ 100 HB $^{-1}$ ) for healthy highlanders. However, this reduction in MSNA burst incidence occurred alongside a small increase in both arterial pressure ( $\sim 3$  mmHg) and stroke volume; therefore, such reductions were likely arterial baroreflex-mediated. In addition, whilst other haemodynamic responses to hyperoxia were comparable between groups, there was a significant reduction in TPR in healthy highlanders; this was not observed in CMS. This may indicate different intrinsic control and regulation of vascular tone; i.e. healthy highlanders possess a greater vascular responsiveness to hypoxia compared with CMS. Indeed, this could contribute, in part, to the observed difference in basal MSNA (i.e. extrinsic control) under ambient hypoxic conditions. However, any potential difference in local control cannot be determined from the data presented here.

An inhibitory relationship exists between the peripheral chemoreflex and baroreflex mechanisms (Somers, Mark, & Abboud, 1991), whereby an acute increase in peripheral chemoreflex activation is consistently shown to inhibit baroreflex control of the heart (Heistad et al., 1971; Sagawa et al., 1997; Steinback et al., 2009; Niewinski, Tubek, Banasiak, Paton, & Ponikowski, 2014; Mozer, Holbein, Joyner, Curry, & Limberg, 2016) with inconsistent effects on baroreflex control of MSNA (Halliwill & Minson, 2002; Simpson et al., 2019). In the present study, during ambient air breathing, we observed a greater cardiovascular baroreflex responsiveness for CMS compared to healthy highlanders. Furthermore, we observed no change in cardiovascular baroreflex responsiveness for CMS during acute hyperoxia, but demonstrated a 75% increase in reflex gain for healthy highlanders. These findings indicate a peripheral chemoreflex-mediated inhibition of cardiovascular baroreflex responsiveness in healthy Andeans at high altitude, which does not appear to be present in CMS. This raises an interesting possibility that whilst a blunted peripheral chemoreflex responsiveness may contribute to the exaggerated arterial hypoxaemia in CMS, it may, paradoxically, prevent the reduced cardiovascular baroreflex gain normally observed during sustained high-altitude exposure (Bourdillon et al., 2018; Simpson et al., 2019; Yazdani et al., 2016).

### 6.4 | Implications

Our findings imply that elevated sympathetic vasomotor outflow and arterial baroreflex dysfunction do not contribute to the elevated cardiovascular disease risk reported in mild CMS, since autonomic

control of blood pressure is well maintained in the group studied here. Therefore, other factors may predispose individuals with CMS to cardiovascular disease. However, we cannot exclude the possibility that elevated sympathetic vasomotor outflow and/or arterial baroreflex dysfunction may develop in more severe CMS, with elevated pulmonary arterial pressure (Simpson et al., 2020), which may contribute to the greater cardiovascular disease risk reported in moderate and severe CMS.

## 6.5 | Experimental limitations

There are several limitations in the present study that should be acknowledged. First, due to time constraints, only small opportunistic samples could be studied. Therefore, meaningful differences between groups may not have been detected due to low statistical power. Indeed, insufficient statistical power likely prevented a meaningful 20% difference in both total blood volume between groups from being detected, despite a similar magnitude of difference to previous studies (Claydon et al., 2004). Second, given the time constraints associated with expedition research, it was not possible to control for the time of day that participants were tested; therefore, diurnal variations in basal MSNA, blood pressure and cardiovagal baroreflex gain (Taylor et al., 2011) are a consideration in our interpretation. Notably, our analysis indicates that time of day was not a significant covariate. Third, two CMS individuals were light to moderate smokers. It is reported that tobacco smoking leads to increased basal MSNA and attenuates vascular sympathetic baroreflex gain (Middlekauff et al., 2014), which may have influenced our results. However, this would have potentially overestimated resting MSNA in CMS, which would not have altered the interpretation of our results (i.e. lower sympathetic vasomotor outflow in CMS). Fourth, due to a lack of CMS positive female volunteers, we only studied males, meaning that the findings cannot be generalized to females, who likely exhibit differences in blood pressure control mechanisms. Last, we did not assess vascular sympathetic baroreflex gain to rising and falling pressure independently, due to an insufficient number of data points to construct baroreflex slopes that met the criteria for inclusion. We acknowledge that this fails to take baroreflex hysteresis into account (Rudas et al., 1999).

## 7 | CONCLUSION

Contrary to our hypotheses, elevated sympathetic vasomotor outflow and arterial baroreflex dysfunction are not apparent in mild CMS. In fact, basal sympathetic vasoconstrictor drive and heart rate are lower in CMS, with enhanced cardiovagal baroreflex gain, compared to healthy highlanders. Such changes appear to be adaptive physiological responses to the elevations in red blood cell volume, which allow blood pressure homeostasis to be maintained. Furthermore, whilst a blunted peripheral chemoreflex is reported to be a possible mechanism responsible for accentuated arterial hypoxaemia in CMS, it may, paradoxically, augment cardiovagal baroreflex responsiveness compared to healthy highlanders.

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## COMPETING INTERESTS

None

## AUTHOR CONTRIBUTIONS

L.L.S., J.P.M., M.S., C.D.S., P.N.A. and F.C.V. contributed to conception and design of the work. L.L.S., J.P.M., M.S., C.D.S., J.S.L., G.M., V.L.M., C.G., A.S., S.A.B., T.G.D., G.A.V., R.J.F., C.H., M.M.T. and S.J.O. contributed to acquisition analysis, or interpretation of the data. L.L.S., J.P.M., M.S., C.D.S., F.C.V., P.N.A., J.S.L., G.M. and M.M.T. contributed to the drafting of the work or revising it critically for important intellectual content. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons included as an author qualify for authorship, and all those who qualify for authorship are listed.

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