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Exaggerated systemic oxidative-nitrosative-inflammatory stress in chronic mountain sickness is associated with cognitive decline and depression.

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Running title: Redox-regulation of cerebrovascular function in highlanders

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Key points

- Chronic mountain sickness (CMS) is a maladaptation syndrome encountered at high-altitude (HA) characterised by severe hypoxaemia that carries a higher risk of stroke and migraine and is associated with increased morbidity and mortality.
- We examined if exaggerated oxidative-nitrosative-inflammatory stress (OXINOS) and corresponding decrease in vascular nitric oxide bioavailability in patients with CMS (CMS+) is associated with impaired cerebrovascular function and adverse neurological outcome.
- Systemic OXINOS was markedly elevated in CMS+ compared to healthy HA (CMS-) and low-altitude controls.
- OXINOS was associated with blunted cerebral perfusion and vasoreactivity to hypercapnia, impaired cognition and in CMS+, symptoms of depression.
- These findings are the first to suggest that a physiological continuum exists for hypoxaemia-induced systemic OXINOS in HA dwellers that when excessive is associated with accelerated cognitive decline and depression, helping identify those in need of more specialist neurological assessment and targeted support.

ABSTRACT

Chronic mountain sickness (CMS) is a maladaptation syndrome encountered at high-altitude (HA) characterised by severe hypoxaemia that carries a higher risk of stroke and migraine and is associated with increased morbidity and mortality. The present cross-sectional study examined to what extent exaggerated systemic oxidative-nitrosative-inflammatory stress (OXINOS), defined by an increase in free radical formation and corresponding decrease in vascular nitric oxide (NO) bioavailability, is associated with impaired cerebrovascular function, accelerated cognitive decline and depression in CMS. Venous blood was obtained from healthy male lowlanders (80 m, n = 17), and age and gender-matched HA dwellers born and bred in La Paz, Bolivia (3,600 m) with (CMS+, n = 23) and without (CMS-, n = 14) CMS. We sampled blood for oxidative (electron paramagnetic resonance spectroscopy, HPLC), nitrosative (ozone-based chemiluminescence), and inflammatory (fluorescence) biomarkers. We employed transcranial Doppler ultrasound to measure cerebral blood flow (CBF) and reactivity. We utilised psychometric tests and validated questionnaires to assess cognition and depression. Highlanders exhibited elevated systemic OXINOS ($P < 0.05$ *vs*. lowlanders) that was especially exaggerated in the more hypoxaemic CMS+ patients ($P < 0.05$ *vs*. CMS-). OXINOS was associated with blunted cerebral perfusion and vasoreactivity to hypercapnia, impaired cognition and in CMS+, symptoms of depression. Collectively, these findings are the first to suggest that a physiological continuum exists for hypoxaemia-induced OXINOS in HA dwellers that when excessive, is associated with accelerated cognitive decline and depression, helping identify those in need of specialist neurological assessment and support.

INTRODUCTION

The human brain has evolved a much higher rate of obligatory oxygen (O_2) consumption since, unlike most other organs, its evolutionary "drive for size" means that the brain is committed to a continually active state, demanding a disproportionate 20% of the body's basal O_2 budget in the resting state, more than 10 times that expected from its mass alone (Bailey *et al.*, 2017b). The requirement to process large amounts of O_2 over a relatively small tissue mass supports the high rate of ATP formation to fuel the maintenance of ionic equilibria and uptake of neurotransmitters for synaptic transmission (Alle *et al.*, 2009). That cognitive function is impaired by acute hypoxia is thus not surprising and an extensive literature has since documented proportional deficits in executive function, attention, mental speed, language and memory (Virues-Ortega *et al.*, 2004; Rimoldi *et al.*, 2016; McMorris *et al.*, 2017) including lasting damage to white and grey matter motor architecture (Di Paola *et al.*, 2008).

However, few studies have considered how lifelong exposure to hypoxia affects cognitive function and whether compensatory adaptations to sustain adequate tissue O_2 delivery prevent acute impairments from potentially progressing to irreversible dementia. The lack of information is surprising given an estimated 140 million high-altitude (HA) dwellers permanently living above 2,500 meters (m) through economic and social necessity (Bailey *et al.*, 2018b) and an evolving body of literature indicating that hypoxaemia is responsible for the higher prevalence of cognitive impairment and dementia observed in patients living at sea-level with cardiopulmonary disease (Peers *et al.*, 2009; Bagge *et al.*, 2018).

In the few studies published to date, cognitive function has been shown to be slightly impaired in well-adapted elderly Andean HA dwellers relative to sea-level based lowlander controls (Yan *et al.*, 2011; Hill *et al.*, 2014; Davis *et al.*, 2015b). In contrast albeit in the only study conducted to date, the authors observed an inverse relationship between altitude of residence and (age-adjusted) dementia mortality rate. However, this study examined patients living at considerably lower altitudes (up to 1,800 m) in California with no control of potential confounders such as comorbidities and air pollution, highlighting the need for additional studies.

Furthermore, the mechanism underpinning altitude-induced cognitive impairment is unclear and to what extent further impairments occur in highlanders suffering from chronic mountain sickness (CMS+), a maladaptation syndrome characterised by exaggerated erythrocytosis and more pronounced hypoxaemia (Villafuerte & Corante, 2016) has not been examined. We have

previously identified that compared to lowlander (normoxic) controls, systemic oxidative-nitrosative-stress (OXNOS), defined by an increase in free radical formation and corresponding decrease in vascular nitric oxide (NO) bioavailability, was permanently elevated in healthy well-adapted Andeans living at 3,600 m without CMS (CMS-). The observation that systemic vascular endothelial function remained intact suggested that physiological concentrations of ONS may prove hormetically beneficial for lifelong adaptation to the hypoxia of HA. In contrast, highlanders with CMS (CMS+) exhibited more exaggerated increases in ONS and impaired systemic vascular endothelial function, thus implying a potential metabolic basis to HA maladaptation (Bailey *et al.*, 2013c).

In light of these findings, we sought to determine the potential relationships between metabolic (oxidative-inflammatory-nitrosative-stress herein referred to as OXINOS), haemodynamic (cerebrovascular function) and clinical (cognition and depression) correlates in CMS+ and CMS- in order to provide more integrated mechanistic insight into the potential pathophysiology and consequences of neurological maladaptation to HA. We hypothesised that compared to lowlander controls, systemic OXINOS would be moderately elevated in CMS- in the absence of any impairments in cerebrovascular function, cognition or symptoms of depression. We further hypothesised that the systemic OXINOS response would be further exaggerated in CMS+ subsequent to more pronounced arterial hypoxaemia and associated with impairments in cerebrovascular function, cognition and symptoms of depression. Figure 1 provides a schematic summary of the proposed mechanisms.

MATERIALS AND METHODS

Ethical approval

The experimental protocol was approved by the Institutional Review Boards for Human Investigation at the University of San Andres, La Paz, Bolivia (CNB #52/04), University of Lausanne, Lasusanne, Switzerland (#89/06, #94/10), and University of Glamorgan, Pontypridd, UK (#4/07), and subsequently registered (clinicaltrials.gov; Identifier: NCT01182792). All participants were informed of the purpose/risks of the experiment and signed an informed consent form, with all procedures adhering to guidelines set forth in the Declaration of Helsinki.

Experimental design

The study was a cross-sectional population-based observational study in accordance with the STROBE statement (von Elm *et al.*, 2014). Figure 2 provides a schematic of the experimental design. The Neurovascular Research Laboratory in the UK (~80 m) was the site of investigation for the lowlanders and the Instituto Boliviano de Biologia de Altura in La Paz, Bolivia (~3,600 m) for the highlanders.

Participants

For all participants, inclusion criteria specified that they were born and had lived permanently at their resident altitude and sedentary defined as no formal recreational activity outside of everyday living (Bailey *et al.*, 2013b). Exclusion criteria included those with significant developmental delay or learning difficulties, diagnosis of any central neurological disease such as aneurysm, stroke, transient ischemic attack, epilepsy, multiple sclerosis and psychiatric disorders including any history of traumatic brain injury and hypertension. None of the participants were taking nutritional supplements including over-the-counter antioxidant or anti-inflammatory medications. We specifically chose to exclude females given our inability to control for differences in circulating estrogen known to affect cerebral blood flow (CBF) and cognition (Yao *et al.*, 2009). Prior to inclusion into the study, all participants were subject to an extensive clinical examination that consisted of a thorough medical history, chest auscultation and 12-lead ECG. Participants were subsequently familiarised with the equipment and procedures.

Highlanders

We recruited 23 male patients with primary CMS (CMS+) and 14 healthy age, gender and education (consecutive years in secondary and University education)-matched controls without CMS (CMS-) native to La Paz, Bolivia (Table 1). We scored symptoms of CMS and confirmed clinical diagnosis by an excessive erythrocytosis [haemoglobin (Hb) > 20 g/dL] in the presence of normal pulmonary function and no history of working in the mining industry (Leon-Velarde *et al.*, 2005). All participants identified themselves as Aymaras and were from similar socio-economic backgrounds having been born and bred in La Paz with Spanish spoken as their first language.

Lowlanders

We also recruited 17 age and education-matched healthy Caucasian males born and bred close to sea-level (~80 m) in the UK (Table 1) as a sea-level (normoxic) comparator.

Educational status

Data were collected on level of full time education attained.

Metabolic assessments

Participants were asked to refrain from physical activity, caffeine and alcohol and to follow a low nitrate/nitrite (NO_3^-/NO_2^-) diet 24 h prior to formal experimentation (Woodside *et al.*, 2014) and were 12 h overnight fasted when they attended the laboratory at 8 am. We obtained blood samples without stasis following 20 min of seated rest to control for plasma volume shifts.

Chemicals

All chemicals were of the highest available purity from Sigma-Aldrich® (UK).

Blood sampling

We collected blood from an indwelling cannula located in a forearm antecubital vein into Vacutainers® (Becton, Dickinson and Company, Oxford, UK) before centrifugation at 600*g* (4°C) for 10 minutes. We decanted plasma and serum samples into cryogenic vials (Nalgene®

Labware, Thermo Fisher Scientific Inc, Waltham, MA, USA) and immediately snap-frozen in liquid nitrogen (N_2) and shipped/stored under N_2 gas (Cryopak, Taylor-Wharton, Theodore, AL, USA) prior to analysis in the UK. We left samples to defrost at 37°C in the dark for 5 min before batch analysis.

Oxidative stress

Antioxidants: We assayed plasma concentrations of reduced and oxidised glutathione (GSH/GSSG) according to the methods established by N'Guessan *et al*. (N'Guessan *et al.*, 2011) with modifications (Stocker *et al.*, 2017). The intra- and inter-assay coefficients of variation (CVs) were both <5%.

Free radicals: The ascorbate free radical ($A^{\bullet-}$) was employed as a direct measure of systemic free radical formation (Buettner & Jurkiewicz, 1993). We injected 1 mL of plasma into a high-sensitivity multiple-bore sample cell (AquaX, Bruker Daltonics Inc., Billerica, MA, USA) housed within a TM_{110} cavity of an EPR spectrometer operating at X-band (9.87 GHz). We recorded samples by cumulative signal averaging of 10 scans using the following instrument parameters: resolution, 1024 points: microwave power, 20 mW; modulation amplitude, 0.65 G; receiver gain, 2×10^5; time constant, 40.96 ms; sweep rate, 0.14 G/s; sweep width, 6 G; centre field, 3486 G. We filtered spectra identically (moving average, 15 conversion points) using WINEPR software (Version 2.11, Bruker, Karlsruhe, Germany) and determined the double integral of each doublet using specialist software (OriginLab Corps, MA, USA). The intra- and inter-assay CVs were both <5 %.

Inflammatory stress

Myeloperoxidase (MPO) activity: We employed a high-throughput, sensitive and homogeneous fluorescence-based method for detection of MPO chlorination activity using 7-hydroxy-2-oxo-2Hchromene-8-carbaldehyde oxime as a selective probe for hypochlorous acid as recently described (Stocker *et al.*, 2017). The intra- and inter-assay CVs were both <5%.

Nitrosative stress

We measured plasma NO metabolites using ozone-based chemiluminescence outlined below (Bailey *et al.*, 2017a).

S-Nitrosothiols (RSNO): Plasma (400 μL) was mixed with 5% acidified sulphanilamide and left to incubate in the dark at 21º C for 15 min to remove nitrite (NO_2^-) prior to injection into tri-iodide reagent for direct measurement of RSNO.

Nitrite (NO_2^-): A separate sample (200 μL) was also injected into tri-iodide reagent for the combined measurement of NO_2^- and RSNO with NO_2^- calculated by subtracting the concentration of RSNO. We performed all calculations using Origin/Peak Analysis software. The intra- and inter-assay CVs were 7% and 10% respectively.

Total bioactive NO: Calculated as the sum of RSNO + NO_2^-.

Haemodynamic assessments

We performed all resting measurements following 10 min of seated rest breathing room air at the prevailing barometric pressures in all groups (normoxic normocapnia for lowlanders, hypoxic hypocapnia for highlanders). Measurements were also repeated following the administration of hyperoxia (F_IO_2 = 1.0, ~10 L/min for 10 min) to the inspired air in the highlanders only (hyperoxic normocapnia).

Cardiopulmonary function

We employed finger photoplethysmography (Finometer® PRO, Finapres Medical Systems, Amsterdam, The Netherlands) to monitor beat-to-beat mean arterial pressure (MAP) using the Model Flow method that incorporates participant sex, age, stature and mass (BeatScope 1.0 software; TNO; TPD Biomedical Instruments) to calculate stroke volume (SV) and cardiac output (*Q*). We corrected for vertical displacement of the finger cuff relative to heart level using a reference probe placed on the chest at the fourth intercostal space in the mid-clavicular line. We measured heart rate (HR) using a lead II electrocardiogram (Dual BioAmp; ADInstruments). We sampled end-tidal partial pressures of oxygen and carbon dioxide ($PET_{O2/CO2}$) from a leak-free mask and analysed via capnography (ML 206, ADInstruments Ltd, Oxford, UK). Pulse oximetry (Nonin 9550 Onyx II, Nonin Medical, Inc., Plymouth, MI, USA) monitored arterial oxyhaemoglobin saturation (SaO_2) on the third digit of the right hand.

Cerebrovascular function

Cerebral blood flow (CBF): We insonated the M1 segment of the right middle cerebral artery (MCA) at a depth ranging between 40-60 mm using a 2 MHz pulsed trans-cranial Doppler (TCD) ultrasound system (Multi-Dop X4, DWL Elektroniche Systeme GmbH, Sipplingen, Germany) to yield MCA velocity (MCAv). A headband device (Spencer Technologies, Nicolet Instruments, Madison, WI, USA) secured the Doppler probe over the trans-temporal window to achieve optimal insonation position and maintained in this position for the duration of the study to avoid movement artefact. We calculated cerebrovascular and total peripheral resistance (CVR and TPR) as MAP/MCAv or *Q* and cerebrovascular conductance index (CVCi) as MCAv/MAP. We calculated pulsatility index (PI) as systolic MCAv–diastolic MCAv/MCAv and further normalised the PI relative to the prevailing MAP. We calculated cerebral O_2 delivery (CDO_2) as the product of (arterial) O_2 content [$c(a)O_2$] (1.39 × Hb × SaO_2/100) and MCAv. In order to normalise for the cerebral vasoconstrictor effects of polycythaemia and hypocapnia in the highlanders, we adjusted absolute CBF values for differences in (elevated) Hct and (lower) PET_{CO2} using the following equations:

Polycythaemia: $CBF_{/Hct} = 90 \times CBF\ (measured)/(135 - Hct)$ (Severinghaus, 2001)

Hypocapnia: $CBF_{/PETCO2} = CBF\ (measured)/[1 + (PET_{CO2HYPOXIA} - PET_{CO2NORMOXIA*}) \times 0.03]$ (Bailey *et al.*, 2009b) where *refers to fixed PET_{CO2} of 40 mmHg.

Data sampling: We sampled beat-by-beat data continuously at 1 kHz using an analog-to-digital converter (Powerlab/16SP ML795; ADInstruments, Colorado Springs, CO, USA) stored on a personal computer for off-line analysis (Chart version 7.2.2, ADInstruments, Colorado Springs, CO, USA). We gave chart files a coded number (not named) by an investigator blinded to the study. We "time-aligned" the MAP and TCD channels given the time-delay (1.07 s) associated with MAP signal processing when using the Finometer® PRO.

Cerebrovascular reactivity to CO_2 (CVR_{CO2}): Following 10 min breathing room air, the inspirate was rapidly changed to 5% CO_2 with 21% O_2 and balanced nitrogen for 5 min at the prevailing barometric pressure. Following a 5 min recovery breathing room air, participants hyperventilated at 15 breaths/min for 5 min. From this, we calculated CVR_{CO2} as the % increase/decrease in MCAv from baseline per 1 mmHg increase/decrease in PET_{CO2} recorded during the final 30 s (average taken) of the hypercapneic/hypocapneic challenge having achieved steady-state:

$$CVR_{CO2}(\%) = 100 \; x \; \frac{MCA_v(final) - MCA_v(baseline)}{MCA_v(baseline)} / [PET_{CO2}(final) - PET_{CO2}(baseline)]$$

(Bailey *et al.*, 2013a)

From these data, we derived the CVR_{CO2} range as a useful indication of the cerebral circulation's combined ability to respond to differential changes in CO_2. We calculated the CVR_{CO2} range as the sum of the fractional vasodilation and vasoconstriction incurred during the respective hypercapnea and hypocapnia challenges as described:

CVR_{CO2} range (%) = $CVR_{CO2\ [Hypercapnia\ (\%)]}$ + $CVR_{CO2\ [Hypocapnia\ (\%)\ ]}$ (Bailey *et al.*, 2013a)

Dynamic cerebral autoregulatory capacity (dCA): We employed a combination of spontaneous (seated) and driven (repeated squat-stands) oscillations in blood pressure (BP) and MCAv in order to assess dCA via transfer function analysis (TFA). Following 10 min of rest in the seated position, we obtained a 5-min segment of BP and MCAv data for spectral analysis of spontaneous oscillations. In order to increase BP variability and improve the reliability and interpretation of the TFA metrics (Katsogridakis *et al.*, 2013), participants then performed 5-min periods of repeated squat-stand maneuvers at randomly assigned frequencies of 0.05 Hz [10-sec squat, 10-sec standing) and 0.10 Hz (5-sec squat, 5-sec standing) with 5 min of standing rest between frequencies (Smirl *et al.*, 2015). During these maneuvers, we instructed participants to maintain normal breathing and to avoid Valsalva. Beat-to-beat MAP and MCAv signals were calculated across each cardiac cycle, linearly interpolated, and resampled at 2 Hz for TFA (Zhang *et al.*, 1998) in accordance with the recommendations of the Cerebral Autoregulation Research Network (Claassen *et al.*, 2016). Spontaneous MAP and MCAv power spectrum

density and the mean value of TFA coherence, gain, and phase of the spontaneous oscillations were band averaged across the very low frequency (VLF: 0.02–0.07 Hz, 50 to 14.3-second cycles) and low frequency (LF: 0.07–0.2 Hz, 14.3 to 5-second cycles) ranges where CA is most operant (Zhang *et al.*, 1998). The TFA coherence, gain, and phase of the driven MAP oscillations were sampled at the driven frequencies (0.05 or 0.10 Hz). To ensure we entered robust phase and gain estimates for analysis, we averaged only those gain and phase (positive to eliminate wrap-around) values where the corresponding coherence was ≥ 0.5. Accordingly, we interpreted an increase in gain and decrease in phase to reflect impaired dCA indicative of a more pressure-passive relationship between MAP and MCAv.

Cognitive function

Psychometric tests

We conducted a battery of psychometric tests with 3 consecutive testing periods to ensure habituation and to avoid the confounding influence of learning effects as previously outlined (Marley *et al.*, 2017):

a]. *Learning & Memory*

Rey Auditory Verbal Learning consists of 2 lists containing 15 unrelated words. We read List 1 aloud at a rate of 1 word per second. The participant recalled as many of the 15 words in any order. We repeated this 4 times (total of 5 recalls, A1-A5). We read List 2 aloud and the participant recalled as many of the 15 words as possible, again in any order (B1). Finally, we asked the participant to recall as many words as possible from List 1 (delayed recall, A6) (Rey, 1958).

b]. *Working Memory*

Repetition of Digits Backwards (RDB) is based on the Repetition of Digits test (Wechsler, 1955) which comprises digits forwards (RDF) and RDB. Both tests consist of 7 pairs of random number sequences that the researcher reads aloud at the rate of one per second. RDF required the participant to recall the numbers in the correct sequence until they got the sequence wrong. The sequence began with 3 numbers and increased by 1 number every other pair. RDB required the participant to recall the numbers in the reverse order from the sequences they were presented,

until they got the sequence wrong. The sequence began with 2 numbers and increased by 1 every other pair. We scored the tests by the total number of sequences re-called. Trail Making Test B (TMT-B) is based on the Army Individual Test Battery (1944) that consists of two parts, A (TMT-A) and TMT-B constructed with 25 circles distributed over a sheet of A4 paper. In part A, the circles are numbered 1–25; the participant connected the numbers in ascending order. In part B, the circles include both numbers (1–13) and letters (A–L); again participants connected the circles in the ascending pattern, but alternating between number and letter (i.e. 1-A-2-B-3-C, etc.). We iasked the participant to complete the task as quickly as possible without removing the pen from the paper until completion. We scored the tests as the time taken in seconds to complete each trail.

c]. *Attention & Information Processing*

We assessed RDF and TMT-A as outlined above. Digit Symbol Substitution Test (DSST) consists of matching 9 digits with their corresponding symbols. We gave the participant a piece of A4 paper with 9 digits and their corresponding symbol and a grid of 93 digits, under each digit the participant drew as many of the corresponding symbols as possible within 90 seconds. We scored the test by how many they drew correctly within the alloted time (Wechsler, 1955). Stroop test (ST) consists of two parts A & B, with 24 words written in different colours on a 4 × 6 grid. Test A required the participants to read aloud the word written as quickly as possible. Test B required the participant to read aloud the colour of the word written and not what the word said as quickly as possible (Stroop, 1935). This test was assessed in the highlander subgroups only and not the lowlanders due to logistical constraints.

d]. *Visuo-Motor Coordination*

The grooved pegboard dexterity test (GPDT) consists of two parts and required the participant to place 25 pegs in a pegboard containing equally size holes with randomly positioned slots. Participants placed identical pegs with a key on one side that had to be rotated to match the hole before they could be inserted, into the 25 holes on the pegboard working from left to right, front to back. The test consisted of two parts, the first test completed using the dominant hand (GPDT-D) and repeated using the non-dominant hand (GPDT-ND). The test was scored by the time taken to complete in seconds (Klove, 1963).

Higher scores in the RAVLT-A/B, RDB/RDF tests and lower scores in the TMT-A/B, GPDT-D/GPDT-ND and Stroop A/B tests indicated superior performance.

Questionnaires

The original English questionnaires outlined below were used for the lowlanders whereas the equivalent validated Spanish versions were used for the highlanders.

Montreal Cognitive Assessment (MoCA): The MoCA measures visuospatial/executive, naming, memory, attention, language, abstraction, delayed recall and orientation with each section having a designated point value with a maximum score of 30 points (Nasreddine *et al.*, 2005). We scored the test as the number of questions answered correctly and participants with 12 years or less of education had a point added to their total score. We graded MoCA on 3 levels; normal: 25.2-29.6 points, mild cognitive impairment: 19-25.2 points and Alzheimer's disease: 11.4-21 points according to established guidelines (Nasreddine *et al.*, 2005). Since MoCA assesses executive function, it is particularly useful for patients with vascular impairment, including vascular dementia (Sheehan, 2012).

Depression: We assessed depressive symptoms using the Becks Depression Inventory (BDI) shown to be a reliable and valid measure of self-reported depression in both normal and psychiatric populations (Beck *et al.*, 1961). The BDI is a self-report inventory with 21 items assessing the behavioural and cognitive symptoms of depression. Each item consists of four statements numbered from 0-3 with higher numbers indicating more severe depressive symptoms. We scored the questionnaire by the sum of all the responses with a maximum score of 63 points. A score of 0-9 points indicated minimal depression whereas scores of 10-18 points, 19-29 points and 30-63 points indicated mild, moderate and severe depression respectively.

Statistical analysis

Power calculation: We analysed these data using G* Power 3.1 software. Assuming comparable differences and corresponding effect sizes previously observed in plasma $A^{\bullet-}$ ($\eta^2 = 0.54$) and NO_2^- ($\eta^2 = 0.67$) (Bailey *et al.*, 2013c) our primary end-outcome variables for OXNOX stress, the present study required a (minimum) sample size ranging between 24-36 participants (8-12 per group) in order to achieve a power of 0.80 at $P < 0.05$. We chose to further inflate this during recruitment given the potential for incomplete data collection.

Inferential statistics: We analysed these data using using the Statistics Package for Social Scientists (IBM SPSS Statistics Version 24.0). Shapiro-Wilk *W* tests ($P > 0.05$) confirmed that all data sets were normally distributed. We analysed demographic (Table 1), metabolic (Table 2), haemodynamic (Table 3), cognition and depression (Table 5), CVR_{CO2} (Figure 2) and TFA (Figure 3) datasets using one-way analyses of variance (ANOVAs) and *post-hoc* Bonferonni-adjusted independent samples *t*-tests if we observed a main effect. We analysed education data (Table 1) using Pearson Chi-square tests. We analysed responses to hyperoxia in the highlanders (Table 4) using two-way (subgroup × inspirate) ANOVAs and *post-hoc* Bonferonni-adjusted paired samples (within subgroups) or independent (between subgroups) samples *t*-tests if we observed an interaction effect. We determined relationships between metabolic, haemodynamic and clinical variables (Figure 4) using Pearson Product Moment Correlations. We established significance at $P < 0.05$ for all two-tailed tests (with individual *P* values for all comparisons shown) and data are expressed as mean ± standard deviation (SD).

RESULTS

Anthropometric data

All groups were of a comparable age and body mass whereas the highlanders were generally shorter with a corresponding elevation in BMI in CMS+ (Table 1).

Metabolic data

Oxygenation and erythrocytosis

As anticipated, highlanders presented with arterial hypoxaemia with the most pronounced erythrocytosis observed in CMS+ (Table 1).

Oxidative stress

We observed lower GSH:GSSG in CMS+ due to the combination of lower GSH and higher GSSG (Table 2). We observed a reciprocal elevation in $A^{\bullet-}$ that was generally more pronounced in highlanders and further exaggerated in CMS+ (Table 2). Figure 3, A-C provides typical examples of EPR doublets observed exhibiting hydrogen hyperfine coupling constants (a^H) of ~1.8 G (g = 2.0052).

Inflammatory stress

MPO activity was higher in CMS+ (Table 2).

Nitrosative stress

Plasma NO_2^- and RSNO were consistently lower in highlanders (Table 2) but not different between CMS+ and CMS-. Figure 3, D-F provides typical examples of OBC traces observed.

Haemodynamic data

Cardiopulmonary

As anticipated, highlanders were more hypocapneic whereas we observed no between group/subgroup differences in MAP, SBP, DBP, *Q*, HR, SV or TPR (Table 3). Hyperoxia and the corresponding normalisation of $Sa O_2$ decreased *Q* in the highlanders due to the combined decrease in both HR and SV and as a consequence was associated with elevated TPR (Table 4).

Cerebrovascular

Cerebral perfusion was generally lower in highlanders and was associated with a corresponding elevation in CVR and PI and reciprocal decrease in CVCi and CDO_2 that was most marked in CMS+ (Table 3). While hyperoxia failed to normalise perfusion, it tended to restore CDO_2 subsequent to an elevation in caO_2 (Table 4). The lower cerebral perfusion still persisted in CMS+ when CBF was (retrospectively) adjusted for the independent vasoconstrictor effect of hypocapnea but not polycythaemia (Table 4). Highlanders exhibited a lower CVR_{CO2} range due primarily to a blunted response to hypercapnea (Figure 4) whereas we observed no differences between CMS+ and CMS-. Figure 5 illustrates typical waveform data for a representative patient with CMS+ and corresponding spectral analysis for both spontaneous (resting) and driven (squat-stand maneuvers) oscillations in MAP and MCAv. Spontaneous and driven TFA metrics are illustrated in Figure 6 (A-F). During spontaneous TFA with moderate coherence values (Figure 6A), LF phase was higher in CMS+ (Figure 6B) with no (between group) differences observed in gain at either frequency (Figure 6C). The driven protocols while not altering PET_{CO2} ($P < 0.05$ *vs*. spontaneous, data now shown) resulted in markedly amplified oscillations culminating in an augmented signal-to-noise ratio as confirmed by the 106-238 fold (0.05 Hz) and 665-1,320 fold (0.10 Hz) increase in BP spectral power and 133-374 fold (0.05 Hz) and 424-1,039 fold (0.10 Hz) increase in MCAv spectral power (compared to spontaneous VLF/LF measures at the respective frequency of interest) resulting in TFA coherence values exceeding 0.95 AU in all cases (Figure 6D). Despite no differences in phase (Figure 6E), we observed consistently lower (35-40 %) point-estimates of VLF and LF gain in the highlanders with no differences observed between CMS+ and CMS- (Figure 6F).

Clinical data

Cognitive function

We observed consistently lower performances on RAVLT, RDB, RDF, TMT-B and DSST in the highlanders with the lowest performance recorded for RAVLT-A6 and Stroop Task-A in CMS+ (Table 5). We observed comparable MoCA scores between CMS- and lowlanders and consistently lower scores in CMS+ with 87 % of the group (20/23 participants) scoring ≤ 25 points (Table 5).

Depression

Likewise, we observed comparable BDI scores between CMS- and lowlanders and consistently lower scores in CMS+ (Table 5).

Correlations

We observed inverse relationships between $A^{\bullet-}$ and the following variables: $Sa{O_2}$, total bioactive NO, MCAv and $CVR_{CO2Hyper}$ (Figure 7 A-D). We observed positive relationships between total bioactive NO and the following variables: CDO_2 and MoCA (Figure 7 E-F) and some aspects of cognitive function, namely performance on the RAVLT-A1-A5 (r = 0.438, P = 0.001), RAVLT-B1 (r = 0.358, P = 0.008), RAVLT-A6 (r = 0.270, P = 0.048), RDF (r = 0.536, P = <0.001), RDB (r = 0.565, P = <0.001) and DSST (r = 0.266, P = 0.052).

DISCUSSION

More than 150 million HA dwellers are permanently exposed to hypoxaemia, a problem predisposing lowlanders suffering from cardiopulmonary disease to cognitive dysfunction and dementia. Here, we show for the first time that systemic OXINOS was permanently elevated in healthy well-adapted (CMS-) highlanders and accompanied by a proportional decrease in cerebral perfusion and blunted reactivity to hypercapnia. These alterations were associated with a mild decrease in cognitive performance with learning/memory and attention/information processing the domains most affected, but without any clinical evidence for depression. Second and in stark contrast, the sustained elevation in systemic OXINOS was further exaggerated in maladapted (CMS+) highlanders and accompanied by more pronounced impairments in cerebrovascular function, cognition and clinical symptoms of depression. Collectively, these findings are the first to suggest that a physiological continuum may exist for hypoxaemia-induced systemic OXINOS that when excessive, is associated with accelerated cognitive decline and depression, helping identify those in need of more specialist neurological follow-up and targeted support.

Metabolic function

Since the concentration of ascorbate in human plasma is orders of magnitude greater than any oxidising free radical combined with the low one-electron reduction potential for the $A^{\bullet-}$ /ascorbate monanion (AH^-) couple ($E^{\circ\prime}$ = 282 mV) (Williams & Yandell, 1982), any oxidising species ($R^{\bullet}$) generated within the systemic circulation will result in the one-electron oxidation of ascorbate to form the distinctive EPR-detectable $A^{\bullet-}$ doublet ($AH^- + R^{\bullet} \rightarrow A^{\bullet-} + R\text{-}H$, Supplemental Figure 2 A-C) (Buettner, 1993). Thus, the elevations observed in plasma $A^{\bullet-}$ provide direct evidence that systemic free radical formation was permanently elevated in the highlanders, approximately double that observed in the lowlanders, with the highest concentrations recorded in the comparatively more hypoxaemic CMS+ patients.

These observations combined with the inverse relationships consistently observed between $A^{\bullet-}$ and SaO_2 suggest that hypoxaemia may have been the upstream stimulus for oxidative catalysis thus confirming our original hypothesis proposed in Supplemental Figure 1. Our findings agree with an evolving body of literature indicating that hypoxaemia catalyses not only systemic but also local cerebral free radical formation (Bailey *et al.*, 2009c; Bailey *et al.*, 2018a) classically

attributed to mitochondrial superoxide ($O_2^{\bullet-}$) release by complex III of the electron transport chain notwithstanding additional contributions from other (i.e. extra-mitochondrial) sources (Waypa *et al.*, 2010). Systemic free radical formation coincided with the selective oxidation of GSH, the most abundant intracellular thiol found in the brain that likely reflects an attempt to constrain OXINOS through the targeted scavenging of reactive oxygen, nitrogen and carbon-centered species (Rae & Williams, 2017).

Oxidative stress coincided with both inflammatory and nitrosative stress confirmed by the combined, sustained elevation in MPO and lower NO_2^- and RSNO respectively, culminating in OXINOS that was most pronounced in CMS+. These findings extend our previous observations of a permanent and graded elevation in systemic OXNOS stress ranging from moderate in CMS- to severe in CMS+ (Bailey *et al.*, 2013c). Since plasma NO_2^- is a source of NO with conversion catalyzed by deoxyhaemoglobin-mediated reduction and acidic disproportionation (Gladwin *et al.*, 2000; Cosby *et al.*, 2003) we interpreted the more exaggerated decrease in CMS+ to be one of the contributory factors responsible for the observed impairment in systemic vascular function in the form of blunted flow-mediated dilation and increased arterial stiffness (Bailey *et al.*, 2013c). To further extend these observations, we sought to determine if the same concept applied more locally to the hypoxic cerebrovasculature, including to what extent exaggerated OXINOS associates with the neurological deficits underpinning CMS.

Haemodynamic function

Consistent with previous studies (Jansen & Basnyat, 2011), highlanders, in particular CMS+, exhibited ~30 % lower cerebral perfusion notwithstanding the interpretive constraints associated with TCD ultrasound (Liu *et al.*, 2017) and corresponding decrease in CDO_2 that persisted even following correction for the independent vasoconstrictor effects of polycythaemia-induced alteration in blood rheology and hypocapnia. The decrease in cerebral perfusion coincided with an impaired ability of the cerebrovasculature to respond to vasodilator stimuli, notably hyperoxia and hypercapnia, the latter consistently more pronounced in CMS+. The consistent relationships observed between total bioactive NO, MCAv and $CVR_{CO2Hyper,}$ though not disassociating cause from effect, support a potential contributory role for OXINOS and corresponding decrease in vascular NO availability, consistent with previous studies that have highlighted endothelial-derived NO as an important, albeit not exclusive, mediator of cerebral (hyper)perfusion (Lavi *et*

al., 2003). Interestingly, OXINOS appeared to exert more of an effect on the cerebral, than systemic circulation given the lack of difference in MAP and TPR in CMS+.

Furthermore, we have previously identified that NO plays an important role in the maintenance of cerebral vasomotor tone and blood-brain barrier integrity by dynamically buffering changes in CBF in response to spontaneous changes in MAP (Bailey *et al.*, 2009a; Bailey *et al.*, 2009c; Bailey *et al.*, 2011) justifying a complementary examination of the pressure-flow coupling dynamic. Contrary to our original expectations based on the published literature (Jansen *et al.*, 2000), the elevated LF phase observed in CMS+ during spontaneous oscillations was indicative of improved pressure-flow coupling potentially related to the elevated CVR or hypocapnia as observed subsequent to changes in the respiratory chemoreflex (Ogoh *et al.*, 2010) or equally, to increased sympathetic tone (Lundby *et al.*, 2018). The phase and gain findings associated with the elevated coherence during the driven oscillations revealed a similar mechanism, namely, gain was found to be lower in the highlanders with the lowest values observed in CMS+. Lower gain is consistent with findings in Himalayan Sherpa (Smirl *et al.*, 2014) and may reflect an improved ability to buffer perfusion during rapid alterations in BP to protect against vasogenic oedema in the face of exaggerated OXINOS. However, it would seem unlikely that this constitutes a functionally neuroprotective adaptation given that our participants, in particular CMS+, exhibited clinical signs of neurodegeneration (see below).

Clinical function

Researchers have suggested that the Andean model of HA living, defined by cerebral hypoperfusion and polycythaemia, is phenotypically maladaptive given that it carries a higher risk of stroke and migraine and is associated with increased morbidity and earlier mortality (Virues-Ortega *et al.*, 2009; Jansen & Basnyat, 2011). In support, Bolivians born and bred at the same altitude and location to that employed in the present study exhibited slower psychomotor speed in attention and digit symbol coding tasks that persisted across the lifespan reflecting a "speed-accuracy" trade-off such that slower may be surer (Hill *et al.*, 2014). The present findings further extend the albeit limited literature identifying impairments in learning/memory and attention/information processing that were especially pronounced in CMS+.

Thus, it would appear that the highlander's brain, especially with CMS+, is unable to compensate for its hypoperfusion-induced CDO_2 constraint. Any global or local cerebral O_2

deficit has the potential to impact cognition potentially due to impaired neurotransmitter release that animal studies suggest is related to depressed monoamine synthesis (Freeman & Gibson, 1988), elevated glutamate excitotoxicity (Hota *et al.*, 2008) and perturbations in choline acetyltranserase/acetyl cholinesterase expression (Guerra-Narbona *et al.*, 2013).

The observation that clinical symptoms of depression were absent in CMS- and only apparent in CMS+, in whom cognitive/haemodynamic impairments were most pronounced, suggests that a physiological continuum for hypoxaemia-induced systemic OXINOS may potentially exist that when surpassed may prove maladaptive and contribute to the neurological complications associated with CMS. This hypothesis is not unreasonable given that hypoxia and OXINOS contribute to the pathophysiology of dementia (Sun *et al.*, 2006; Wojsiat *et al.*, 2018) and depression (Salim, 2014) in lowlander patient groups and extends our previous observations albeit confined to the systemic vascular circulation (Bailey *et al.*, 2013c). Alternatively, if systemic OXINOS/hypoxaemia are less pronounced it may simply take more time to develop clinical symptoms. Furthermore, our measurements obtained at rest may have underestimated the true magnitude of hypoxic stress encountered given that hypoxaemia in CMS+ is further compounded by physical activity (Stuber *et al.*, 2010; Pratali *et al.*, 2012) and sleep-disordered breathing (Rexhaj *et al.*, 2016).

We observed a similar profile for performance on MoCA with consistently lower scores recorded in CMS+. Although originally developed as a measure of global cognitive function (Nasreddine *et al.*, 2005), MoCA is frequently used as a clinical screening tool for the dementias (Ballard *et al.*, 2013) with a cut-off score of 25 points or lower widely used as the threshold for detecting mild cognitive impairment and possible dementia (Davis *et al.*, 2015a). Thus, with 87 % of the CMS+ group fulfilling these clinical criteria, our findings may help identify those HA dwellers most “at risk” and in need of more specialist neurological assessment to diagnose an emerging dementia syndrome. Clinical diagnosis is inherently complex depending on the triad of cognitive function, patient report and informant history, notwithstanding complementary assessments of cerebrospinal fluid, blood-borne and structural biomarkers to exclude inflammatory, infective and malignancy related causes of dementia (Burns & Iliffe, 2009; Robinson *et al.*, 2015).

Experimental limitations

We need to consider several limitations when interpreting the present findings. First, the

OXINOX assays employed, despite taking advantage of the most direct analytical techniques currently available, ultimately rely on *ex-vivo* detection of relatively stable reactants confined to circulating plasma/RBC formed downstream of the primary source/reaction pathway that we assume reflects dynamic events *in-vivo* (Bailey *et al.*, 2009a). Given that these metabolites, especially GSH:GSSG, partition heterogenously across different tissues, our conclusions only apply to what we observed in circulating blood. In addition, there remains the inevitable translational challenge when attempting to determine the clinical outcomes associated with elevated OXINOS given our current inability to differentiate between physiologically adaptive and pathologically maladaptive concentration thresholds (Bailey *et al.*, 2013c). Furthermore, we encourage interventional studies incorporating targeted antioxidant prophylaxis to disassociate cause from effect and confirm the mechanisms proposed herein. Second, we need to be cautious when interpreting the perfusion data in HA dwellers given that we relied on differences in MCAv as an indirect surrogate of global CBF that fails to take into account the antagonistic dilatory/constricting effects caused by prevailing hypoxia/hypocapnea (Wilson *et al.*, 2011). Finally, future researchers need to consider more specialist follow-up neurological assessments to complement the current approaches taken to determine the prevalence of dementia in the most vulnerable CMS+ patient subgroup.

Conclusions

Notwithstanding the experimental limitations as outlined, these findings indicate that a chronic state of disequilibrium potentially exists between free radical formation and antioxidant defence in highlanders causing systemic OXINOS to be permanently elevated and especially exaggerated in more hypoxaemic CMS+ patients. OXINOS was associated with blunted perfusion and reactivity to hypercapnia, impaired cognition and in CMS+, symptoms of depression. Collectively, these findings are the first to suggest that a physiological continuum may exist for hypoxaemia-induced systemic OXINOS that when excessive, is associated with accelerated cognitive decline and depression, helping identify those HA dwellers, especially CMS+, who may require more specialist neurological follow-up and targeted support. Future investigators need to consider the potential neuroprotective benefits of targeted antioxidant prophylaxis and cognitive training in this patient population. Finally, since arterial hypoxaemia is a hallmark feature of circulatory diseases including those that affect the brain, the current findings in

Aymaras may provide complementary insight into the pathophysiology and treatment of patients suffering from early-onset neurodegeneration at sea-level.

Competing interests

The authors declare that they have no competing interests.

Author contributions

DMB, US and CS conceived and designed the research. DMB, US and CS obtained funding. All authors contributed to data collection and analysis. All authors interpreted the results of the experiments. DMB drafted the manuscript and revisions thereof. All authors edited and revised the manuscript(s) and approved the final version submitted for publication. All authors agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part 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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LEGENDS

Table 1. Demographics

Values are mean ± SD; CMS-/CMS+, highlanders without/with chronic mountain sickness; Hb, haemoglobin; Hct, haematocrit; S*a*O_2, arterial oxyhaemoglobin saturation; *ca*O_2, arterial oxygen content; CMS, chronic mountain sickness; BMI, body mass index. *different *vs*. Lowlanders ($P < 0.05$); †different *vs*. CMS- ($P < 0.05$). Clinical and anthropometric data analysed using one-way analyses of variance and *post-hoc* Bonferonni-adjusted independent samples *t*-tests. Education data analysed using Pearson Chi-square tests.

Table 2. Metabolic data

Values are mean ± SD; CMS-/CMS+, highlanders without/with chronic mountain sickness; GSH/GSSH, reduced/oxidised glutathione; $A^{\bullet-}$, ascorbate radical; NO, nitric oxide; NO_2^-, nitrite; RSNO, *S*-Nitrosothiols; Total bioactive NO (NO_2^- + RSNO); MPO, myeloperoxidase; N/A, not assessed. *different *vs*. Lowlanders ($P < 0.05$); †different *vs*. CMS- ($P < 0.05$). Data analysed using independent samples *t*-tests (where lowlander data were unavailable) and one-way analyses of variance with *post-hoc* Bonferonni-adjusted independent samples *t*-tests.

Table 3. Haemodynamic data

Values are mean ± SD; CMS-/CMS+, highlanders without/with chronic mountain sickness; HR, heart rate; MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; *Q*, cardiac output; SV, stroke volume; TPR, total peripheral resistance; S*a*O_2, arterial oxyhemoglobin saturation; $PET_{O2/CO2}$, end-tidal partial pressure of oxygen/carbon dioxide; MCAv, middle cerebral artery velocity; SMCAv/DMCAv, systolic/diastolic MCAv; $MCAv_{/Hct}$, MCAv (retrospectively) adjusted for differences in haematocrit (Hct) and $MCAv_{/PETCO2}$, MCAv (retrospectively) adjusted for differences in end-tidal partial pressure of carbon dioxide, see Methods; AU, arbitrary units; CVR, cerebrovascular resistance; CVCi, cerebrovascular conductance index; CDO_2, cerebral oxygen delivery. *different *vs*. Lowlanders ($P < 0.05$); **different *vs*. hypoxia for given Highlanders subgroup ($P < 0.05$); †different *vs*. CMS- for given inspirate ($P < 0.05$). Data analysed using one-way analyses of variance and *post-hoc* Bonferonni-adjusted independent samples *t*-tests.

Table 4. Responses to hyperoxia in highlanders

Values are mean ± SD; CMS-/CMS+, highlanders without/with chronic mountain sickness; HR, heart rate; MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; *Q*, cardiac output; SV, stroke volume; TPR, total peripheral resistance; SaO_2, arterial oxyhemoglobin saturation; $PET_{O2/CO2}$, end-tidal partial pressure of oxygen/carbon dioxide; MCAv, middle cerebral artery velocity; SMCAv/DMCAv, systolic/diastolic MCAv; $MCAv_{/Hct}$, MCAv (retrospectively) adjusted for differences in haematocrit (Hct) and $MCAv_{/PETCO2}$, MCAv (retrospectively) adjusted for differences in end-tidal partial pressure of carbon dioxide, see Methods; AU, arbitrary units; CVR, cerebrovascular resistance; CVCi, cerebrovascular conductance index; CDO_2, cerebral oxygen delivery. **different *vs*. hypoxia for given subgroup ($P < 0.05$); †different *vs*. CMS- for given inspirate ($P < 0.05$). Data analysed using two-way (subgroup × inspirate) analyses of variance and *post-hoc* Bonferonni-adjusted paired samples (within subgroups) or independent (between subgroups) samples *t*-tests.

Table 5. Cognition and depression

Values are mean ± SD; CMS-/CMS+, highlanders without/with chronic mountain sickness; *n*, number correct; s, seconds. *different *vs*. Lowlanders ($P < 0.05$); †different *vs*. CMS- ($P < 0.05$). Data analysed using one-way analyses of variance and *post-hoc* Bonferonni-adjusted independent samples *t*-tests with the exception of MoCA cut-point (≤ 25 points) analysed using Pearson Chi-square tests with standardised residuals.

Figure 1. Functionally-integrated translational hypothesis

CMS-/CMS+, highlanders without/with chronic mountain sickness; +++comparatively more hypoxaemic; OXINOS, oxidative-inflammatory-nitrosative stress.

Figure 2. Experimental design

CMS-/CMS+, highlanders without/with chronic mountain sickness; NO, nitric oxide; O_2, oxygen; CVR, cerebrovascular reactivity; CO_2, carbon dioxide; dCA, dynamic cerebral autoregulation.

Figure 3. Typical electron paramagnetic resonance (EPR) spectra of the plasma ascorbate radical (A-C) and ozone-based chemiluminescence-based detection of bioactive (nitrite +

***S*-nitrosothiols) nitric oxide metabolites (D-F) at rest in the systemic circulation of a lowlander and highlanders without (CMS-) and with chronic mountain sickness (CMS).**

A-C: Oxidation of the ascorbate monoanion (AH^-) by any free radical ($R^\bullet$) with a one electron reduction potential that exceeds +282 mV will yield $A^{\bullet-}$ (schematic illustrated above). The unpaired electron is delocalised over a highly conjugated tri-carbonyl π-system rendering it resonance stabilized thereby facilitating direct detection by EPR spectroscopy. At the current settings, $A^{\bullet-}$ appears as a (filtered) doublet with a hydrogen hyperfine coupling constant (a_H^{β}) of ~1.76 Gauss (see insert of A for simulated spectrum). D-F: Filtered traces of bioactive nitric oxide (NO) metabolites (nitrite + *S*-nitrosothiols) generated via ozone-based chemiluminescence involving the reaction of NO with ozone (O_3) that yields a photon (*hv*) and subsequent conversion to a potential difference. Insert top right highlights the composite signals (before and after sulphanilamide incubation) for separate measurement of nitrite (NO_2^-) and *S*-nitrosothiols (RSNO). Note general elevations in the signal intensity (AU, arbitrary units) of $A^{\bullet-}$ and reciprocal decrease in the circulating concentration of bioactive NO metabolites in the highlanders especially in the patient with CMS. Spectra chosen to best reflect the average signal intensities observed in each of the respective groups.

Figure 4. Cerebrovascular reactivity to carbon dioxide (CVR_{CO2})

Values are mean ± SD; CMS-/CMS+, highlanders without/with chronic mountain sickness; *different *vs*. Lowlanders for respective challenge ($P < 0.05$). Individual hypocapnia and hypercapnia datasets analysed using one-way analyses of variance and *post-hoc* Bonferonni-adjusted independent samples *t*-tests.

Figure 5. Typical waveforms and spectral analysis of haemodynamic responses observed spontaneously during spontaneous (A) and repeated squat-stand maneuvers (B-C) in a representative patient with chronic mountain sickness.

BP, blood pressure; MCAv, middle cerebral artery velocity; PSD, power spectral density; V/LF, very/low frequency. Note the markedly amplified and coherent oscillations in MAP and MCAv during the repeated squat-stand maneuvers compared to resting (spontaneous) measures leading to improved estimation of transfer function of dynamic cerebral autoregulation at the frequency of interest.

Figure 6. Transfer function analysis of the cerebral pressure–flow relationship during spontaneous (A-C) and driven (D-F) oscillations in blood pressure and middle cerebral artery velocity

Values are mean ± SD; CMS-/CMS+, highlanders without/with chronic mountain sickness; *different *vs*. Lowlanders for given frequency ($P < 0.05$). Individual frequency data sets analysed using using one-way analyses of variance and *post-hoc* Bonferonni-adjusted independent samples *t*-tests.

Figure 7. Relationships between metabolic, hemodynamic and clinical correlates in lowlanders and highlanders with and without chronic mountain sickness (CMS).

$A^{\bullet-}$, ascorbate radical; $Sa O_2$, arterial oxyhemoglobin saturation; NO, nitric oxide; MCAv, middle cerebral artery blood flow velocity; CDO_2, cerebral oxygen delivery; $CVR_{CO2Hyper}$, cerebrovascular reactivity to carbon dioxide in the hypercapnic range; MoCA, Montreal Cognitive Assessment. Data analysed using a Pearson Product Moment Correlation.

Table 1. Demographics

Group:	**Lowlanders**	**Highlanders**		***P* values**		
Subgroup:	**Controls (*n* = 17)**	**CMS- (*n* = 14)**	**CMS+ (*n* = 23)**	**CMS- *vs.* Controls**	**CMS+ *vs.* Controls**	**CMS+ *vs.* CMS-**
Clinical						
Age (y)	56 ± 18	52 ± 12	56 ± 11	0.614 (between groups)		
Hb (g/dL)	15.0 ± 1.3	17.2 ± 1.1*	20.9 ± 1.7*†	<0.001	<0.001	<0.001
Hct (%)	46 ± 4	52 ± 4*	63 ± 6*†	0.002	<0.001	<0.001
$Sa O_2$ (%)	97 ± 0	91 ± 5*	88 ± 4*†	<0.001	<0.001	0.044
$ca O_2$ (mg/dL)	20.2 ± 1.8	21.6 ± 1.8	25.5 ± 2.3	0.147	<0.001	<0.001
CMS score (points)	0 ± 0	2 ± 2	8 ± 5*†	0.369	<0.001	<0.001
Anthropometrics						
Body mass (kg)	82.0 ± 13.4	71.6 ± 8.9	78.2 ± 11.8	0.053 (between groups)		
Stature (m)	1.77 ± 0.06	1.63 ± 0.04	1.62 ± 0.06	<0.001	<0.001	1.000
BMI (units)	26 ± 4	27 ± 4	30 ± 4*	1.000	0.025	0.122
Waist:Hip	0.93 ± 0.08	0.95 ± 0.05	0.99 ± 0.04*	1.000	0.019	0.162
Education						
Secondary (*n*/%)	16/94	13/93	20/87	0.697 (between groups)		
University (*n*/%)	8/47	4/29	5/22	0.225 (between groups)		

Table 2. Metabolic data

Group:	**Lowlanders**	**Highlanders**		***P* values**		
Subgroup:	**Controls (*n* = 17)**	**CMS- (*n* = 14)**	**CMS+ (*n* = 23)**	**CMS- *vs.* Controls**	**CMS+ *vs.* Controls**	**CMS+ *vs.* CMS-**
Oxidative stress						
GSH (μM)	N/A	549 ± 154	412 ± 151†	N/A	N/A	0.012
GSSG (μM)	N/A	176 ± 48	197 ± 34†	N/A	N/A	0.018
GSH:GSSG (AU)	N/A	3.3 ± 1.1	2.1 ± 0.9†	N/A	N/A	0.049
$A^{\bullet-}$ (AU)	29,450 ± 6,929	54,451 ± 20,722*	59,729 ± 18,133*†	<0.001	<0.001	0.042
Inflammatory stress						
MPO (μg/L)	N/A	609 ± 12	894 ± 168†	N/A	N/A	0.016
Nitrosative stress						
NO_2^- (nM)	249.1 ± 65.1	139.9 ± 76.7*	130.7 ± 83.6*	0.001	<0.001	1.000
RSNO (nM)	5.4 ± 3.0	5.0 ± 2.7	4.7 ± 3.9*	0.850 (between groups)		
Total bioactive NO (nM)	254.5 ± 64.2	144.9 ± 78.0*	135.4 ± 83.7*	0.001	<0.001	1.000

Table 3. Haemodynamic data

Group:	**Lowlanders**	**Highlanders**		***P* values**		
Subgroup:	**Controls (*n* = 17)**	**CMS- (*n* = 14)**	**CMS+ (*n* = 23)**	**CMS- *vs.* Controls**	**CMS+ *vs.* Controls**	**CMS+ *vs.* CMS-**
MAP (mmHg)	79 ± 21	80 ± 10	87 ± 14	0.238 (between groups)		
SBP (mmHg)	118 ± 30	118 ± 18	128 ± 22	0.309 (between groups)		
DBP (mmHg)	61 ± 18	61 ± 8	67 ± 11	0.228 (between groups)		
Q (L/min)	7.42 ± 1.33	6.99 ± 0.80	7.24 ± 1.38	0.627 (between groups)		
HR (b/min)	71 ± 14	71 ± 12	72 ± 14	0.990 (between groups)		
SV (mL)	105 ± 13	101 ± 22	103 ± 22	0.853 (between groups)		
TPR (mmHg/L/min)	10.97 ± 2.93	11.60 ± 1.73	12.41 ± 2.80	0.231 (between groups)		
SaO_2 (%)	97 ± 0	91 ± 5*	88 ± 4*†	<0.001	<0.001	0.044
PET_{O2} (mmHg)	107 ± 5	54 ± 3*	51 ± 5*	<0.001	<0.001	0.236
PET_{CO2} (mmHg)	39 ± 3	34 ± 5*	33 ± 4*	0.019	0.022	1.000
Cerebrovascular						
MCAv (cm/s)	49 ± 12	39 ± 12*	35 ± 12*†	0.044	0.001	0.048
$MCAv_{/Hct}$ (cm/s)	50 ± 11	42 ± 13*	43 ± 14*	0.039	0.037	1.000
$MCAv_{/PETCO2}$ (cm/s)	51 ± 11	48 ± 16	44 ± 15*	1.000	0.040	1.000
PI (AU)	1.04 ± 0.18	1.15 ± 0.41	1.45 ± 0.39*†	1.000	0.002	0.048
Normalised PI (AU)	0.014 ± 0.006	0.014 ± 0.005	0.017 ± 0.004	0.216 (between groups)		
CVR (mmHg/cm/s)	1.74 ± 0.69	2.27 ± 0.80	2.89 ± 0.57*†	0.667	0.010	0.037
CVCi (cm/s/mmHg)	0.70 ± 0.39	0.49 ± 0.16	0.42 ± 0.18*†	0.088	0.003	0.010
CDO_2 (mL/cm/s)	983 ± 209	850 ± 305*	889 ± 303*	0.046	0.047	1.000

Table 4. Responses to hyperoxia in highlanders

Subgroup:	**CMS- (*n* = 14)**		**CMS+ (*n* = 23)**		***P* values**		
Inspirate:	**Hypoxia**	**Hyperoxia**	**Hypoxia**	**Hyperoxia**	**Subgroup**	**Inspirate**	**Interaction**
Cardiopulmonary							
MAP (mmHg)	80 ± 10	83 ± 8	87 ± 14	87 ± 16	0.188	0.500	0.405
SBP (mmHg)	118 ± 18	121 ± 13	128 ± 22	125 ± 24	0.284	0.885	0.267
DBP (mmHg)	61 ± 8	64 ± 7	67 ± 11	68 ± 13	0.167	0.241	0.426
Q (L/min)	6.99 ± 0.80	6.11 ± 0.77	7.24 ± 1.38	6.17 ± 1.22	0.663	<0.001	0.540
HR (b/min)	71 ± 12	68 ± 11	72 ± 14	67 ± 14	0.993	<0.001	0.456
SV (mL)	101 ± 22	92 ± 21	103 ± 22	95 ± 28	0.746	0.001	0.863
TPR (mmHg/L/min)	11.60 ± 1.73	13.81 ± 2.00	12.41 ± 2.80	14.55 ± 3.99	0.385	<0.001	0.948
S*a*O_2 (%)	91 ± 5	97 ± 1**	88 ± 4†	97 ± 1**	0.043	<0.001	0.043
PET_{O2} (mmHg)	54 ± 3	374 ± 81	51 ± 5	380 ± 51	0.887	<0.001	0.699
PET_{CO2} (mmHg)	34 ± 5	35 ± 6	33 ± 4	39 ± 4	0.007	0.385	0.847
Cerebrovascular							
MCAv (cm/s)	39 ± 12	39 ± 9	35 ± 12†	34 ± 7†	0.052	0.756	0.029
$MCAv_{/Hct}$ (cm/s)	42 ± 13	43 ± 11	43 ± 14	42 ± 8	0.939	0.759	0.626
$MCAv_{/PETCO2}$ (cm/s)	48 ± 16	48 ± 14	44 ± 15	36 ± 11**†	0.045	0.009	0.011
PI (AU)	1.15 ± 0.41	0.99 ± 0.18	1.45 ± 0.39†	1.21 ± 0.27**	0.013	0.001	0.041
Normalised PI (AU)	0.014 ± 0.005	0.012 ± 0.003	0.017 ± 0.004	0.015 ± 0.006	0.068	0.010	0.803
CVR (mmHg/cm/s)	2.27 ± 0.80	2.23 ± 0.53	2.89 ± 0.57	2.67 ± 0.74	0.054	0.518	0.640
CVCi (cm/s/mmHg)	0.49 ± 0.16	0.47 ± 0.11	0.42 ± 0.18	0.40 ± 0.12	0.119	0.504	0.861
CDO_2 (mL/cm/s)	850 ± 305	909 ± 243	889 ± 303	951 ± 193	0.626	0.083	0.981

Table 5. Cognition and depression

Group:	**Lowlanders**	**Highlanders**		***P* values**		
Subgroup:	**Controls (*n* = 17)**	**CMS- (*n* = 14)**	**CMS+ (*n* = 23)**	**CMS- *vs*. Controls**	**CMS+ *vs*. Controls**	**CMS+ *vs*. CMS-**
Learning and Memory						
Rey Auditory Verbal Learning Test-A1-A5 (*n*)	48 ± 8	34 ± 9*	30 ± 9*	<0.001	<0.001	0.538
Rey Auditory Verbal Learning Test-B1 (*n*)	5 ± 1	4 ± 1	3 ± 1*	0.335	<0.001	0.116
Rey Auditory Verbal Learning Test-A6 (*n*)	9 ± 4	8 ± 3	5 ± 2*†	0.392	<0.001	0.044
Working Memory						
Repetition of Digits Backwards (*n*)	6 ± 2	4 ± 1*	4 ± 2*	<0.001	<0.001	1.000
Trail Making Test-B (s)	74 ± 31	106 ± 43	109 ± 51*	0.137	0.044	1.000
Attention/information processing						
Repetition of Digits Forwards (*n*)	8 ± 2	4 ± 2*	4 ± 2*	<0.001	<0.001	1.000
Trail Making Test-A (s)	37 ± 15	41 ± 18	48 ± 17	0.155 (between groups)		
Digit Symbol Substitution Test (*n*)	54 ± 12	42 ± 12*	37 ± 13*	0.034	<0.001	0.555
Stroop Task-A (s)	No data	14 ± 2	17 ± 5†	N/A	N/A	0.048
Stroop Task-B (s)	No data	38 ± 14	41 ± 15	N/A	N/A	0.555
Montreal Cognitive Assessment (points)	26 ± 3	24 ± 4	21 ± 5*†	0.676	0.001	0.044
Montreal Cognitive Assessment Score ≤ 25 points (*n/%*)	5/29	7/50	20/87*†	0.241	<0.001	0.014
Visuomotor coordination						
Grooved Pegboard Dexterity Test-Dominant (s)	73 ± 11	67 ± 8	71 ± 12	0.296 (between groups)		
Grooved Pegboard Dexterity Test-Non Dominant (s)	83 ± 24	72 ± 11	81 ± 23	0.309 (between groups)		
Depression						
Beck’s Depression Inventory (points)	6 ± 5	7 ± 10	16 ± 13*†	1.000	0.014	0.044



↑Free radicals → ↑Inflammation → ↓Nitric oxide
(OXINOS)

↓Cerebrovascular Reactivity + ↓Cerebral Perfusion

↓Cognition + ↑Depression

Metabolic

Haemodynamic

Clinical







Lowlanders (*n* = 17)
80 m

CMS- (*n* = 14)
3,600 m

CMS+ (*n* = 23)
3,600 m



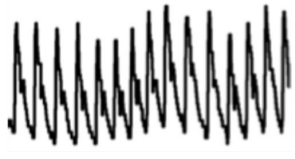
I. Demographics

- Clinical examination
- Anthropometrics
- CMS scores



II. Metabolic

- Free radicals
- Inflammation
- NO metabolites



III. Haemodynamics

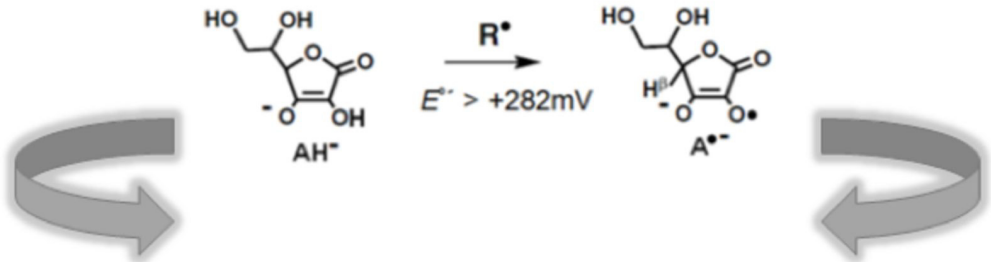
- Baseline (+/- O_2)
- CVR (+/-CO_2)
- dCA (spontaneous/driven)



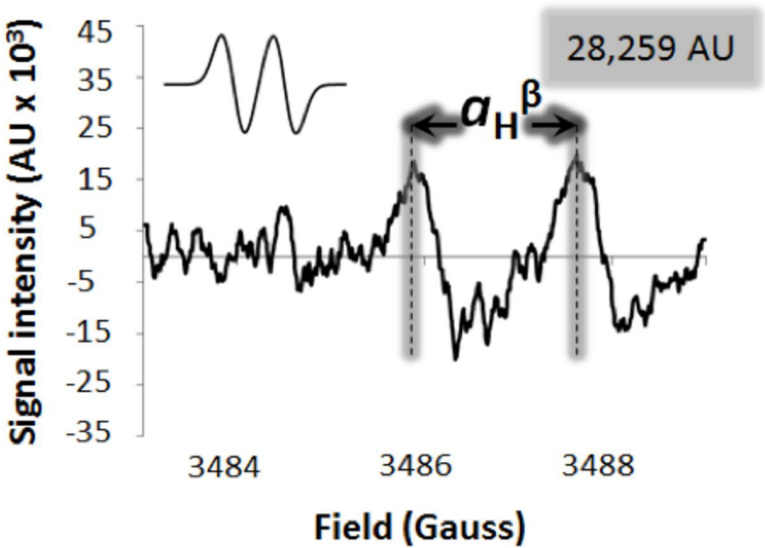
IV. Neuropsychological

- Cognitive function
- Depression

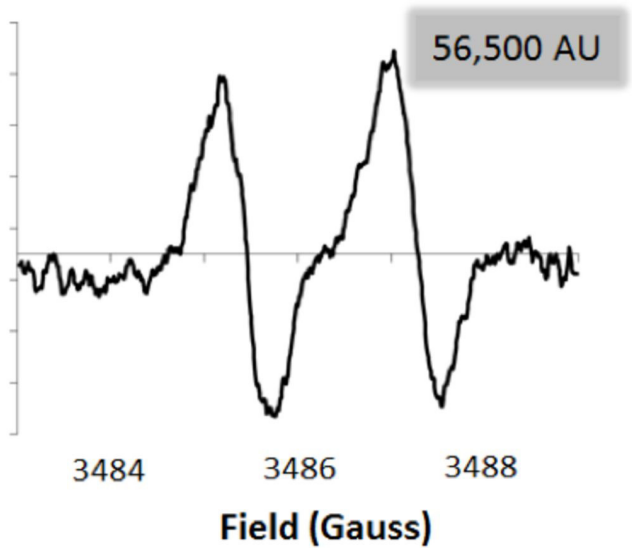
Free radicals



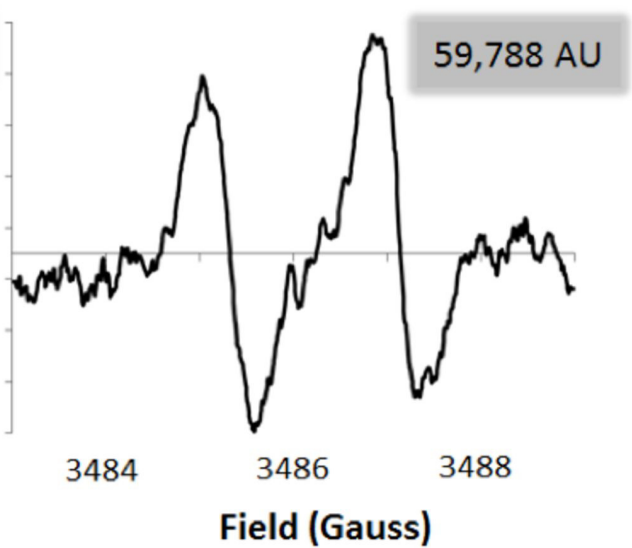
A Lowlander



B CMS-



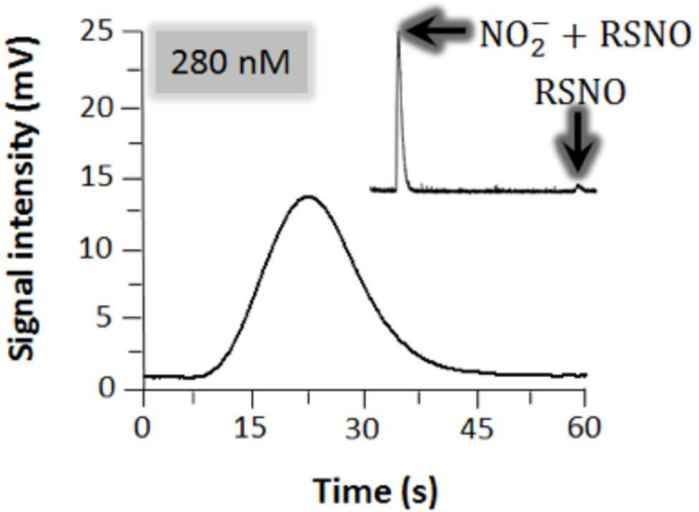
C CMS+



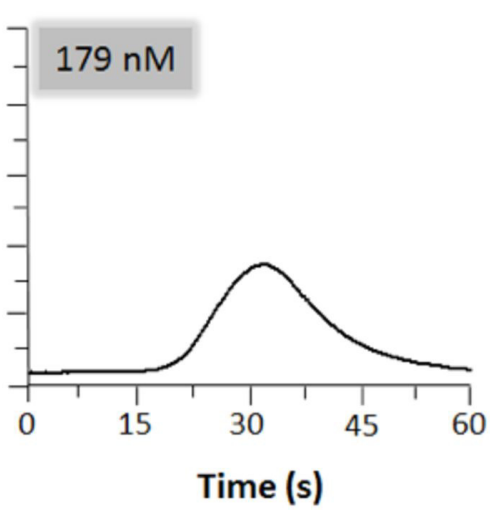
Bioactive NO

$$NO + O_3 \rightarrow NO_2^- + O_2 + hv$$

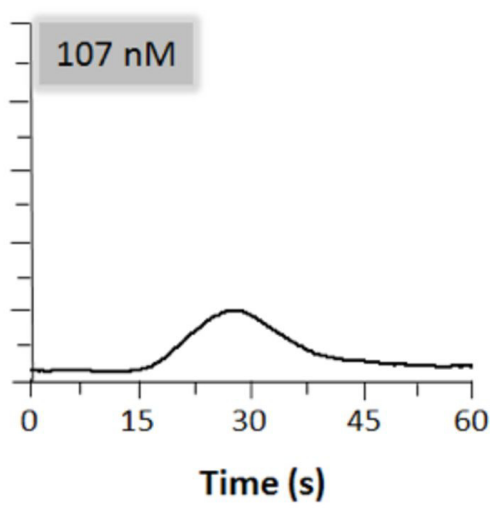
D Lowlander

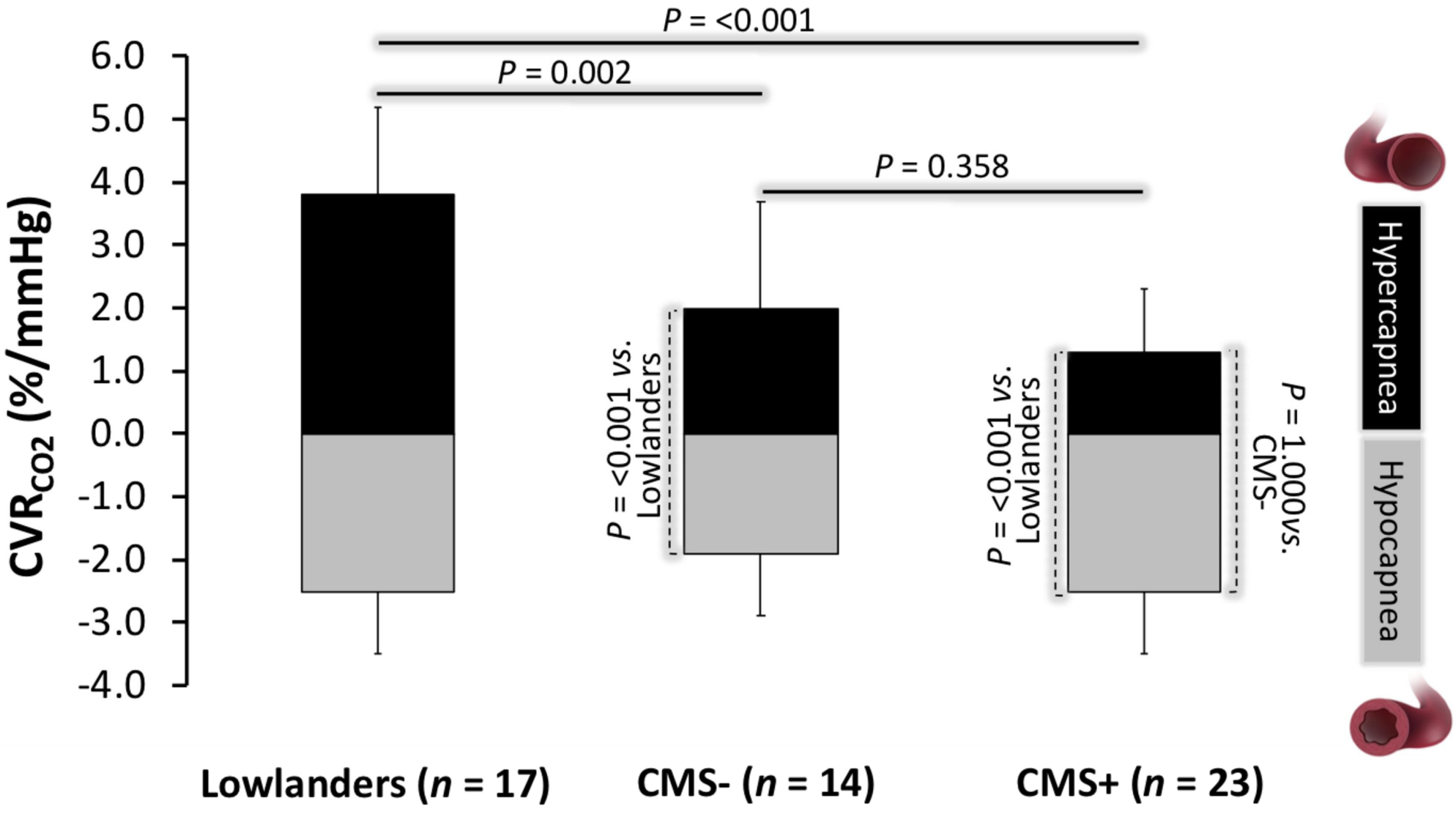


E CMS-

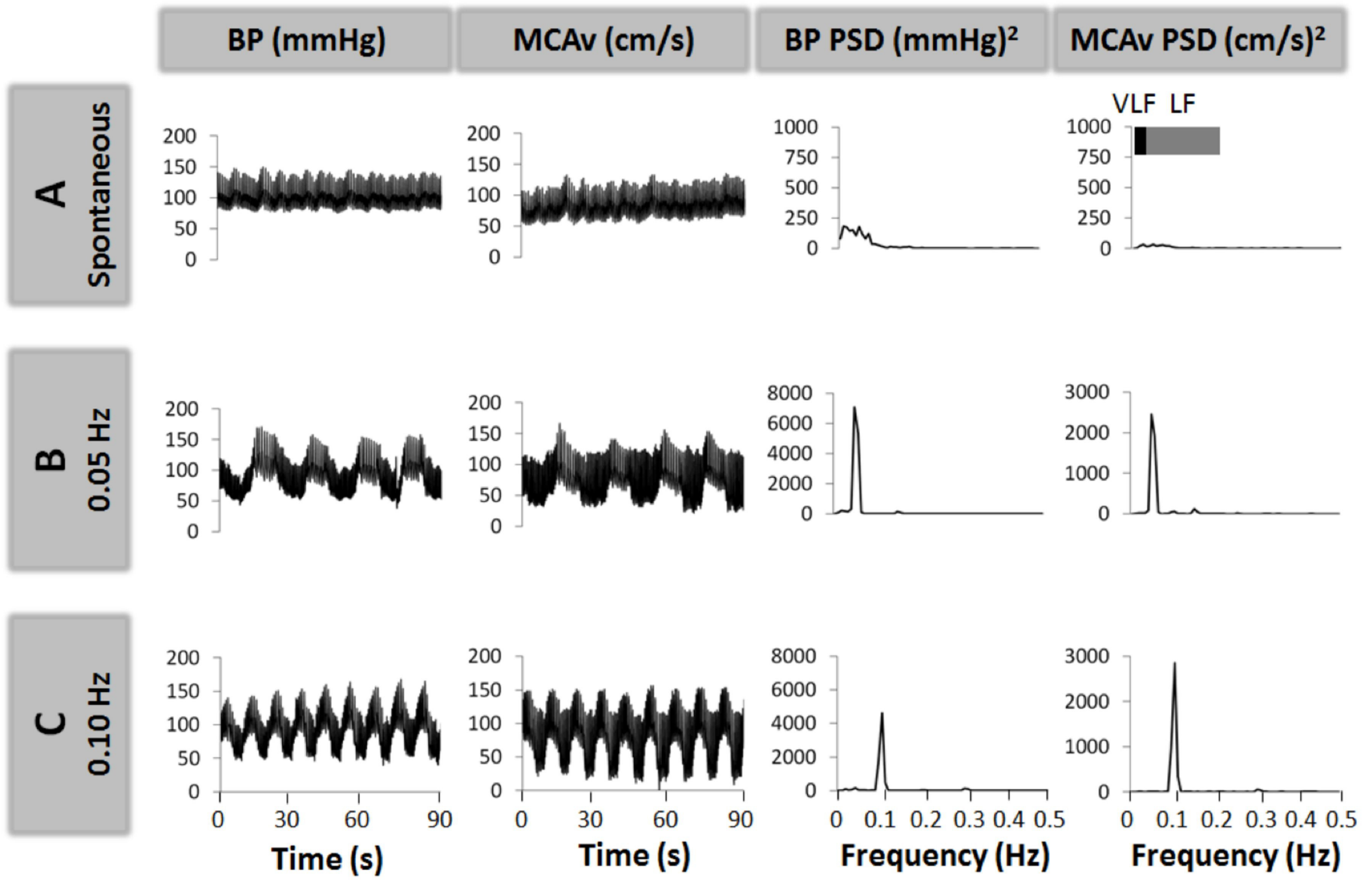


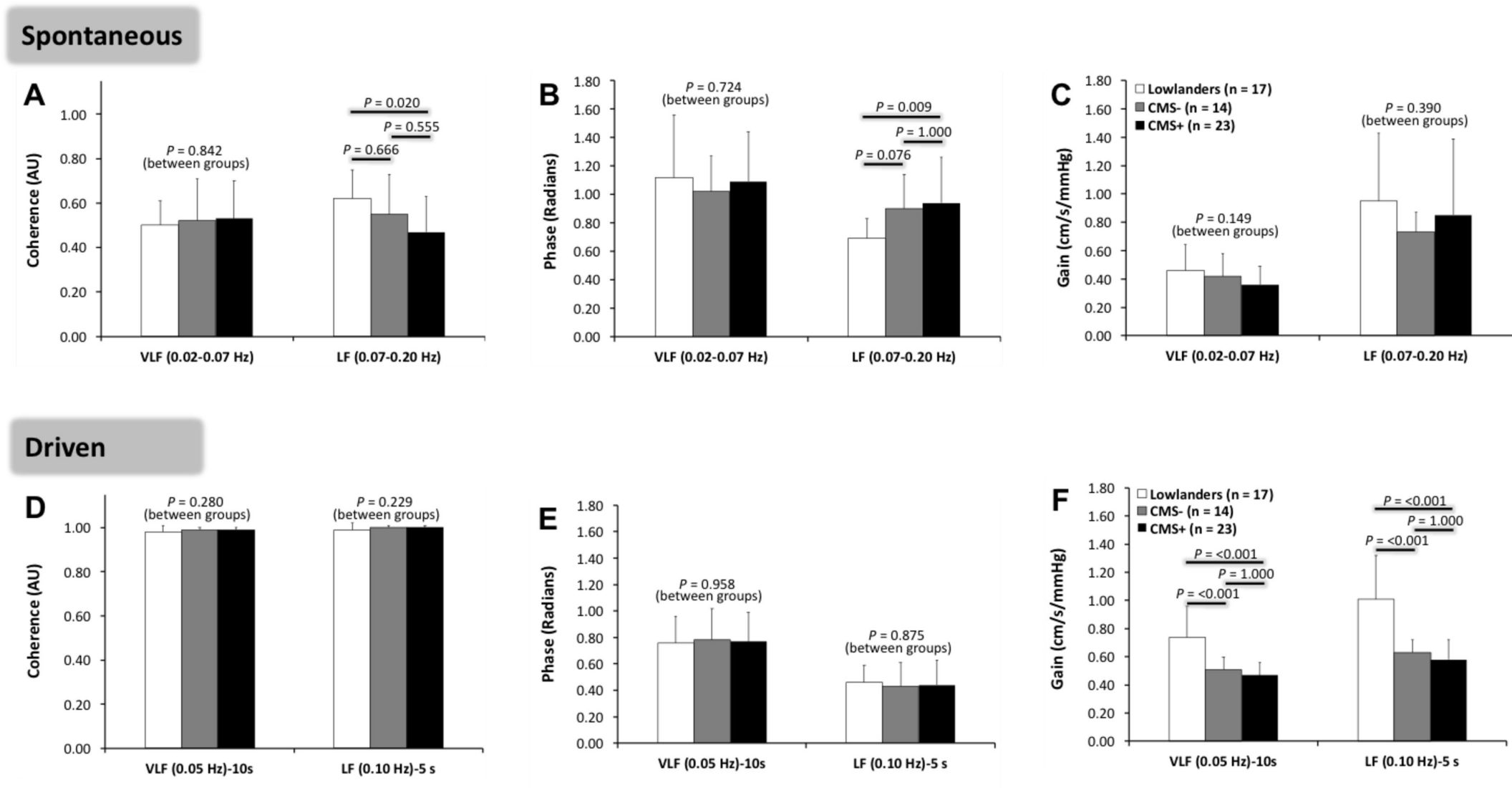
F CMS+

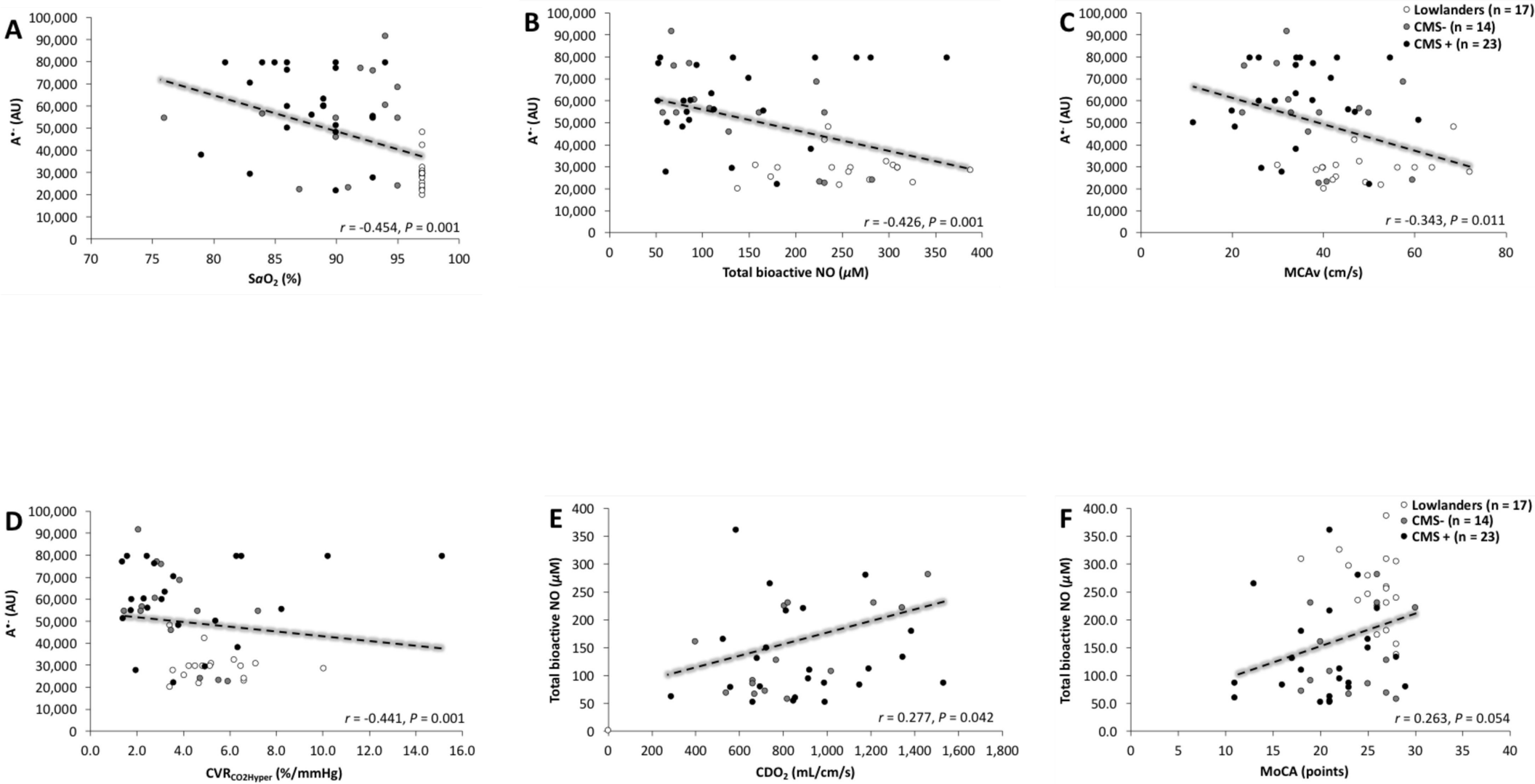




Hypocapnia: P = 0.143 (between groups)


BP (mmHg)
MCAv (cm/s)
BP PSD (mmHg)²
MCAv PSD (cm/s)²
A
Spontaneous
B
0.05 Hz
C
0.10 Hz
VLF
LF
200
150
100
50
0
1000
750
500
250
8000
6000
4000
2000
3000
0
30
60
90
0.1
0.2
0.3
0.4
0.5
Time (s)
Frequency (Hz)


Spontaneous
A
Coherence (AU)
1.00
0.80
0.60
0.40
0.20
0.00
P = 0.842
(between groups)
P = 0.020
P = 0.555
P = 0.666
VLF (0.02-0.07 Hz)
LF (0.07-0.20 Hz)
B
Phase (Radians)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
P = 0.724
(between groups)
P = 0.009
P = 1.000
P = 0.076
VLF (0.02-0.07 Hz)
LF (0.07-0.20 Hz)
C
Lowlanders (n = 17)
CMS- (n = 14)
CMS+ (n = 23)
Gain (cm/s/mmHg)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
P = 0.149
(between groups)
P = 0.390
(between groups)
VLF (0.02-0.07 Hz)
LF (0.07-0.20 Hz)
Driven
D
Coherence (AU)
1.00
0.80
0.60
0.40
0.20
0.00
P = 0.280
(between groups)
P = 0.229
(between groups)
VLF (0.05 Hz)-10s
LF (0.10 Hz)-5 s
E
Phase (Radians)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
P = 0.958
(between groups)
P = 0.875
(between groups)
VLF (0.05 Hz)-10s
LF (0.10 Hz)-5 s
F
Lowlanders (n = 17)
CMS- (n = 14)
CMS+ (n = 23)
Gain (cm/s/mmHg)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
P = <0.001
P = 1.000
P = <0.001
P = <0.001
P = 1.000
P = <0.001
VLF (0.05 Hz)-10s
LF (0.10 Hz)-5 s


A
A•- (AU)
SaO2 (%)
r = -0.454, P = 0.001
B
A•- (AU)
Total bioactive NO (μM)
r = -0.426, P = 0.001
C
Lowlanders (n = 17)
CMS- (n = 14)
CMS + (n = 23)
A•- (AU)
MCAv (cm/s)
r = -0.343, P = 0.011
D
A•- (AU)
CVRCO2Hyper (%/mmHg)
r = -0.441, P = 0.001
E
Total bioactive NO (μM)
CDO2 (mL/cm/s)
r = 0.277, P = 0.042
F
Lowlanders (n = 17)
CMS- (n = 14)
CMS + (n = 23)
Total bioactive NO (μM)
MoCA (points)
r = 0.263, P = 0.054