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***SENP1*, but not fetal hemoglobin, differentiates Andean highlanders with chronic mountain sickness from healthy individuals among Andean highlanders**

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Abstract

Chronic mountain sickness (CMS) results from chronic hypoxia. It is unclear why certain highlanders develop CMS. We hypothesized that modest increases in fetal hemoglobin (HbF) are associated with lower CMS severity. In this cross-sectional study, we found that HbF levels were normal (median = 0.4%) in all 153 adult Andean natives in Cerro de Pasco, Peru. Compared with healthy adults, the borderline elevated hemoglobin group frequently had symptoms (headaches, tinnitus, cyanosis, dilatation of veins) of CMS. Although the mean hemoglobin level differed between the healthy (17.1 g/dL) and CMS (22.3 g/dL) groups, mean plasma erythropoietin (EPO) levels were similar (healthy, 17.7 mIU/mL; CMS, 12.02 mIU/mL). Sanger sequencing determined that single-nucleotide polymorphisms in endothelial PAS domain 1 (*EPAS1*) and egl nine homolog 1 (*EGLN1*), associated with lower hemoglobin in Tibetans, were not identified in Andeans. Sanger sequencing of sentrin-specific protease 1 (*SENP1*) and acidic nuclear phosphoprotein 32 family, member D (*ANP32D*), in healthy and CMS individuals revealed that non-G/G genotypes were associated with higher CMS scores. No *JAK2* V617F mutation was detected in CMS individuals. Thus, HbF and other classic erythropoietic parameters did not differ between healthy and CMS

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MMH, DC, JRC, FLV, FCV, and JFT designed and obtained approval for the study. DC, JRC, and JQO collected samples and clinical measurements from study participants. XW, IM, and ATR performed the genetic and laboratory studies. DC, JRC, and NU performed statistical analyses. MMH, DC, JRC, FCV, and JFT wrote the article. All authors read and approved the final article.

Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

individuals. However, the non-G/G genotypes of *SENPI* appeared to differentiate individuals with CMS from healthy Andean highlanders.

Chronic mountain sickness (CMS) was first described by Carlos Monge in 1925 in Cerro de Pasco, Peru at 4,338 m, and is a manifestation of chronic hypoxia characterized by excessive erythrocytosis (EE). Symptoms of EE include headache, dizziness, dyspnea, sleep disturbance, tinnitus, fatigue, alterations of memory, loss of appetite, and bone and muscle pain. Additionally, individuals with EE may have physical signs of cyanosis, venous dilatation of extremities, and clubbing of the fingers and toes. The severity of CMS is assessed using the Qinghai CMS score, which is based on the grading of symptom severity [1].

The prevalence of CMS increases with age and varies among the mountainous regions of the world. CMS is an adult disease, and its prevalence is the lowest in Ethiopians (<1%) and Tibetans (~1%), higher in South Asian Indians (6.2%) and Han Chinese relocated to high altitudes (6%), and the highest among South American Andeans (15%) [2–8]. Although a familial component has been suggested for elevated hemoglobin concentration values in high-altitude populations, no differences in classic erythropoiesis-related genes, such as *EPO* and erythropoietin receptor (*EPOR*), have been reported between healthy individuals and those with CMS at the same altitude and within the same population [9].

Single-nucleotide polymorphisms (SNPs) in *EPAS1* (also known as *HIF-2α*) and *EGLN1* (prolyl hydroxylase domain 2, *PHD2*) have been reported to be associated with lower hemoglobin levels in Tibetans [10–14]. These SNPs were first discovered through whole-genome scans and confirmed by targeted or whole-exome sequencing [10–13]. Among Andean high-altitude dwellers, detailed studies of these two genes have also been reported. Studies at high altitude have indicated that both Andeans and Tibetans exhibited positive selection in *EGLN1*, although with different signature patterns, but only the Tibetans have the protective *EPAS1* variant [11,15]. However, we do not yet know whether Andeans with CMS have different SNPs than Andeans without CMS or whether healthy highlanders exhibit a protective variant(s).

One such protective trait could be fetal hemoglobin (HbF). A relationship between HbF levels and hypoxia has long been recognized, with increased HbF levels noted in a variety of clinical settings, including intrauterine hypoxia [16], maternal smoking [17], anemia of prematurity [18], birth at high altitude [19], and postnatal hypoxemia from congenital heart disease [20]. Most notably, adult alpacas living at high altitude in Peru were found to have HbF levels of 55% [21], and young baboons had increases in HbF after hypobaric hypoxia [22]. Although the magnitude of the HbF response appears to be genetically determined—for example, baboons have a more robust HbF response than rhesus macaques [23] and some patients with sickle cell disease (SCD) respond better to hydroxyurea, HbF levels could be maintained long-term by continued erythropoietic stress such as repeated phlebotomy-induced anemia [24].

It has been proposed that hypoxia and HbF are linked through the hypoxia-inducible factor (HIF) system, where HIF- α is an essential component of the oxygen-sensing mechanism.

Under normoxic conditions, HIF- α isoforms are hydroxylated by prolyl hydroxylases (PHDs) and subsequently degraded by the ubiquitin and proteasomes. Under hypoxic conditions, HIF- α isoforms are not hydroxylated; they heterodimerize with HIF, bind to hypoxic response element motifs, and induce transcription of hypoxia-responsive genes to ameliorate the effects of hypoxia [25]. HIF can also be pharmacologically stabilized using competitive PHD inhibitors. We previously tested a potent oral PHD inhibitor (FG-2216) in rhesus macaques to stabilize HIF at sea level. FG-2216 induced EPO production and significant erythrocytosis and prevented anemia induced by weekly phlebotomy. Modest increases in red cells and reticulocytes containing HbF were observed by flow cytometry [26]. These data suggest that hypoxia, or its simulation pharmacologically, induces HbF, and lack of such a response could explain CMS. Increased HbF levels in high-altitude dwellers would validate this mechanism as a potential target of exploration for the treatment of hemoglobinopathies.

We then reasoned that modest increases in the percentage of red cells containing HbF might be sufficient to prevent some highlanders from developing CMS. As the primary aim of this study, we proposed to test whether there are modest increases in HbF levels in healthy highlanders, thus distinguishing them from highlanders with CMS. The secondary aims were to determine levels of plasma factors relevant in high-altitude natives, such as EPO and pro-BNP, and perform genotyping by Sanger sequencing of genes in the HIF and related pathways and the presence of *JAK2* V617F mutation, in healthy highlanders and those with CMS to investigate other causes of erythrocytosis.

Methods

Participants

One hundred fifty-three native adult high-altitude dwellers residing near the study area in Cerro de Pasco, Peru (4,338 m) were evaluated. Exclusion criteria included red blood cell (RBC) transfusion, erythropoietin or similar medications in the last 6 months, evident malnutrition, smoking (>5 cigarettes per day for >1 month), history of chronic diseases (asthma, chronic obstructive pulmonary disease, cardiovascular, and/or renal diseases), history of phlebotomy within the last year (>500 mL every 8 weeks), ongoing pregnancy, and visits to lower altitudes (<3,000 m) with total absences longer than 1 year or within the last 6 months. The protocol and consent were reviewed and approved by the institutional ethics/review boards of UPCH in Lima, Peru, and the National Institute of Diabetes, Digestive, and Kidney Diseases in Bethesda, Maryland (clinicaltrials.gov, NCT00695123). All individuals signed informed consents before enrollment.

Clinical evaluation CMS assessment and definition of subgroups

Blood pressure (BP) was measured using a conventional sphygmomanometer after a 15-min rest in the supine position. Arterial oxygen saturation (SaO₂) was measured by digital pulse oximetry (Nellcor OxiMax N-560).

The presence and severity of CMS were assessed using the Qinghai CMS score (Supplementary Table E1, online only, available at www.exphem.org). The severity of each

symptom was assessed according to the clinical criteria (Supplementary Table E2, online only, available at www.exphem.org). Symptoms were graded on a scale from 0 (absence) to 3 (severe). A hemoglobin value ≥ 21 g/dL (hematocrit $\geq 63\%$) in men and ≥ 19 g/dL (hematocrit $\geq 57\%$) in women was assigned 3 points, and lower hemoglobin values were assigned 0 points. CMS was calculated as the sum of the points given for symptoms and hemoglobin: 0–5 = no CMS; 6–10 = mild CMS; 11–14 = moderate CMS; ≥ 15 = severe CMS. Also, healthy adults were further divided into two groups to detect differences in clinical features and a possible progression to CMS: healthy adults (HAs) had hemoglobin levels ≥ 18 g/dL (men) and ≥ 16 g/dL (women); healthy borderline adults (BDLs) had hemoglobin levels of 18.1–20.9 g/dL (men) and 16.1–18.9 g/dL (women).

Blood sampling and testing

Hematocrit was obtained by puncturing the side of a finger with a lancet, collecting the blood into heparinized capillary tubes, centrifuging at 11,500 rpm for 10 min, and reading using a microhematocrit reader. Peripheral blood was collected into EDTA-containing tubes. Hemoglobin electrophoresis was performed with a Bio-Rad high-performance liquid chromatograph for HbF. Plasma samples were used for EPO and pro-N-terminus BNP determinations with an IMMULITE 2000 XPI analyzer.

Sanger sequencing

DNA was extracted using the MagNA Pure automated instrument (Roche) according to the manufacturer's protocol. Samples with low DNA yield were amplified using the GenomiPhi V2 DNA Amplification Kit for whole-genome amplification. SNP identification was carried out by polymerase chain reaction (PCR) of the four candidate gene regions (*EPAS1*, *EGLN1*, *ANP32D*, and *SENPI*; PCR primer sequences are listed in Supplementary Table E3, online only, available at www.exphem.org). The sequence was aligned with program Sequencher (Gene Codes, Ann Arbor, MI), and SNP was picked. Probability values were calculated with a published Excel program [27] and an online calculator (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). JAK2 V617F mutation analysis was performed using the Ipsogen JAK2 Muta-Quant™ Kit (Qiagen, Gaithersburg, MD) for quantification of the JAK2 V617F/G1849T allele in genomic DNA. The assays were run per kit protocol. Absence of the JAK2 V617F/G1849T mutation does not exclude the presence of other JAK2 mutations. Results were expressed as JAK2 V617F % = $[\text{CN V617F}/(\text{CN V617F} + \text{CN WT})] \times 100$.

Statistical analysis

Normally distributed variables are expressed as the mean $\pm$ SD or median with range in states of non-normality as identified by the Kolmogorov–Smirnov test. Hemoglobin levels were used to define groups for analysis: healthy, borderline, and CMS (Tables 1 and 2). Nonparametric variables were expressed as the median with range, and group comparisons were performed using the Kruskal–Wallis test. Groups were compared on the basis of CMS scores using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test (Table 3). A stepwise multivariable regression model was built to identify independent predictors of low CMS score. A p value < 0.05 was considered to indicate statistical significance in all tests. Data analysis was performed using PASW Statistics (Version 17.0, IBM, Armonk, NY).

Results

Demographics

One hundred fifty-three adults, 96 men (63%) and 57 women (37%), participated in the study. The mean age for all adults was 37.6 ± 13.1 years (range: 18–86). HAs included 21 men and 26 women; BDLs, 26 men and 22 women; and the CMS group, 49 men and 9 women (4 were premenopausal and 5 postmenopausal). Ten BDLs had mild CMS scores. HAs were taller and weighed less, but these differences were not statistically significantly between groups or sexes (Table 1).

Description of individuals with CMS

Adults with CMS had higher BP (Table 1). Seventeen percent of those with CMS had systolic BPs (SBPs) >140 mm Hg versus 10% of BDLs and 0% of HAs. Nineteen percent with CMS had diastolic BPs (DBPs) >90 mm Hg compared with 8% of BDLs and 4% of HAs. These increases may have resulted from the higher hemoglobin levels. CMS participants also had lower SaO₂ and higher heart rate (HR). SaO₂ levels $<80\%$ were measured in 6 participants with CMS, 2 BDLs, and 1 HA.

The prevalence of symptoms among HAs, BDLs, and individuals with CMS are detailed in Table 2. Approximately one-half of men and two-thirds of women had no symptoms. Thirty-one adults rated their symptoms as mild (26 men, 5 women), 22 as moderate (20 men, 2 women), and 5 as severe (3 men, 2 women). The most frequent symptoms were cyanosis, dilatation of vessels, and headaches in men, and cyanosis, headaches, and tinnitus in women. Dizziness was the least common symptom. Adults with CMS had a mean score of 10. Hemoglobin levels >25 g/dL were observed in seven participants; all were in men with CMS. Ten participants had previous phlebotomies.

Fetal hemoglobin

In our first analysis, the entire group of 153 individuals had normal HbF levels: median of 0.4% (range: 0.2%–1.9%, normal level: $<1\%$). We then compared men and women as well as HAs and BDLs with individuals with CMS; there were no statistical differences in both analyses. Thus, HbF did not sufficiently differ and was not included in the subsequent statistical analyses.

Sanger sequencing of EPAS1, EGLN1, ANP32D, SENP1, and JAK2

Previous studies had indicated that high-altitude dwellers in Tibet harbored protective SNPs in *EPAS1* (*HIF-2 α*) and *EGLN1* (*PHD2*). Thus, we selected a larger cohort, 26 men with the highest hemoglobin and 13 individuals with normal hemoglobin levels, and performed Sanger sequencing in these two genes. There were no differences in the *EPAS1* gene between those with high hemoglobin levels and those with normal levels; all had the C/C genotype. The T allele in *EGLN1* (rs480902) was in 69% versus 85% ($p = 0.14$).

Genotyping by Sanger sequencing of *ANP32D* (rs72644851) revealed that the G allele in those with high versus normal hemoglobin levels was 64% versus 96% ($p = 0.002$). The G allele in *SENP1* (rs7963934) was in 84% versus 100% ($p = 0.045$). Additionally, 20 samples

with high hemoglobin levels (range: 21–26 g/dL, and one sample with hemoglobin of 18 g/dL) were subjected to *JAK2* V167F mutation analysis to rule out another cause of erythrocytosis: 13 had a borderline number of wildtype DNA copies (between 1,092 and 7,142, because of low DNA concentrations), and 7 had a sufficient number of wild-type DNA copies (07,142). No mutation for *JAK2* V617F was detected.

Plasma levels of EPO and pro-BNP and multivariate analysis

Among the participants, 45 had mild CMS (score: 6–10), 22 had moderate CMS (score: 11–14), and 4 had severe CMS (score: 15). Approximately one-half to two-thirds of each group had a sufficient volume of plasma for EPO and pro-BNP determinations (Table 3). Because the hemoglobin level is a large portion of the CMS score, higher mean hemoglobin correlated with higher CMS score: 17.1 ± 0.2 g/dL in the healthy group and 22.5 ± 1.3 in the severe CMS group ($p < 0.001$). There were no differences in mean plasma EPO levels between groups ($p = 0.24$) or between men and women. In contrast, the healthy group had the lowest pro-BNP levels (27.5 ± 3.1 pg/mL), and the mild CMS group had the highest levels (66.5 ± 14.1 pg/mL), but these were still within the normal range (0–124 pg/mL).

Univariate analysis of *SENP1* showed the G/G genotypes correlated with lower hemoglobin levels ($p = 0.005$) and lower CMS scores ($p = 0.035$), but not with any plasma factors or SaO_2 . We then performed a logistic stepwise regression multivariable analysis of CMS scores using age, sex, EPO, pro-BNP, SaO_2 , *SENP1* genotypes, and *ANP32D* genotypes. The *SENP1* C/G or C/C SNPs were significantly associated with higher CMS score, compared with G/G ($p = 0.014$). When the hemoglobin level was included in the multivariate model, hemoglobin level was significantly associated with CMS scores ($p = 0.004$), and *SENP1* remained significant ($p = 0.024$).

Discussion

In this large cross-sectional study of high-altitude dwellers in Cerro de Pasco, Peru, our cohort had a higher prevalence of CMS (38%) than in previous reports, likely because of our efforts to recruit individuals with CMS. This rate was reflected in a larger proportion with mild and moderate disease (20.2% mild, 14.4% moderate, and 3.3% severe CMS). We first tested our primary hypothesis that healthy high-altitude Andeans have higher HbF levels. HbF testing in 153 individuals revealed normal values (median of 0.4%), which were similar to results at sea level. Therefore, mechanisms other than HbF are responsible for protection against CMS.

The HIF pathway has been studied at high altitude in healthy highlanders and individuals with CMS. Recent reports confirmed that the protective gene signatures in *EPAS1* and *EGLN1* have been identified in Tibetans only, and these alleles were acquired many years ago [11,15,28]. However, the results in these genetic analyses in Andeans have not been definitive. Appenzeller et al. reported correlations between the higher protein expression levels of HIF-1 α , CMS-related symptoms at high altitude, and CMS score [29]. However, Mejia et al. sequenced the SNPs in similar genes that regulate the stabilization and HIF-1 α degradation and found no genetic association between HIF-related genes and individuals with CMS or high-altitude controls [30]. This is not surprising because these *EPAS1*

genotype results were detected in Himalayan highlanders only and were likely transferred from the Denisovan genome [28,31].

In whole-genome sequencing recently performed only in Andeans, there were no differences in *EPAS1* or *EGLN1* between 10 participants with severe CMS (score: 15) and 10 healthy high-altitude dwellers, but there was a large frequency differential in SNPs in *SENP1* and *ANP32D* and increased hypoxia-induced expression of both genes in cultured skin fibroblasts from the same individuals with CMS [32]. The higher frequency in *SENP1* SNP in individuals with CMS has recently been confirmed in a larger study [33]. *SENP1* belongs to a family of proteases that are present in the nucleus and cytoplasm, and it reverses the posttranslational modification of small ubiquitin-like modifiers (SUMOs). Higher *SENP1* expression has been hypothesized to be associated with higher androgen activity in Andean highlanders with EE [34]. Higher expression of *SENP1* has been documented to induce HIF-1 α and reduce apoptosis, thus promoting the development or progression of prostate [35] and other cancer [36,37] cells. Recently, absence of *SENP1* has been reported to produce inflammatory cytokines, leading to type 1 diabetes in a murine model [38], and reduced expression led to impaired glucosedependent insulin exocytosis in an in vitro human islet model [39]. Unfortunately, we were not able to test the protease activities of *SENP1* and *ANP32D* to correlate the genotypes and expression levels. Certainly, our understanding of *SENP1* in hypoxia, cancer development, and other disease models is growing.

Our genotyping by Sanger sequencing of *EPAS1* and *EGLN1* revealed that the SNPs associated with low hemoglobin in Tibetans were not found at high frequency among Andean highlanders. We genotyped two other genes (*SENP1* and *ANP32D*) and found that only *SENP1* was associated with CMS. This finding is similar to previous reports that defined CMS cases as individuals with a CMS score O15 [32] or O11 [33]. Our analyses differed from those reports, with the majority of our cohort having mild (score: 6–10) and moderate (score: 11–14) CMS. Therefore, we were able to further correlate the genotypes of *SENP1* and *ANP32D* with the severity of CMS. Specifically, the G/G genotype in *SENP1* was associated with a milder phenotype and could differentiate healthy Andeans from Andeans with CMS.

We then explored the possibility of blood markers that predict the severity of CMS. EPO has been reported to be present at higher levels in Andean and Oromo highlanders than in those living at sea level [40,41], but no differences have been detected between healthy high-altitude dwellers and those with EE. Other investigations have determined the diurnal patterns of EPO levels among high-altitude dwellers and found that those with EE and CMS had persistently higher EPO levels, without the usual circadian variation of peaks in the evening and low levels in the morning [42]. When we separated the CMS group into mild, moderate, and severe categories to correlate the differences in EPO levels with CMS severity, we found normal levels in all participants (Table 3). We also performed *JAK2* V617F mutation analysis and found only the normal (wild-type) allele, indicating that no other cause of EE, such as a myeloproliferative disorder, was present.

Previous studies in Han Chinese and Tibetans have reported high levels of BNP, vascular endothelial growth factor, and endothelin-1 in individuals with CMS compared with healthy

highlanders [43,44]. We measured pro-BNP levels in HAs and those with CMS, but obtained different results. The highest levels were measured in the mild CMS group, and higher CMS scores did not correlate with higher pro-BNP levels, likely because of the small number of participants with severe CMS ($N = 2$). All these levels were still within the normal range; thus, levels of pro-BNP or EPO were not able to differentiate healthy individuals from those with CMS.

Conclusions

In this cross-sectional cohort of Andean highlanders, we achieved our primary aim and concluded that modest increases in levels of HbF were not present in well-adapted individuals. The severity of CMS was associated with higher hemoglobin levels, and not with EPO or pro-BNP levels. Protective SNPs in EPAS1 and EGLN1 reported in Tibetans were not found in our cohort. *SENPI*, and not the classic HIF-regulated genes, may differentiate healthy Andean highlanders from those with CMS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Characteristics of adult Andean highlanders

Table 1.

	Men				Women			
	Has (Hb 18 g/dL)	BDLs (Hb 18–20.9 g/dL)	Those with CMS (Hb 21 g/dL)	Has (Hb 16 g/dL)	BDL (Hb 16–18.9 g/dL)	Those with CMS (Hb 19 g/dL)		
<i>N</i>	21	26	49	26	22	9		
Age (years)	35.1 ± 12.1	32.2 ± 0.8	43.3 ± 11.5 ^{a,e}	29 ± 9.9	38.7 ± 13.6 ^a	50.4 ± 17.7 ^d		
Height (cm)	168.6 ± 6.1	162.7 ± 3.9 ^a	163.5 ± 5.3 ^d	150.7 ± 4	149.8 ± 5.6	148.6 ± 6.9		
Weight (kg)	61.6 ± 8.9	62.7 ± 6.2	67 ± 11.6 ^a	55.2 ± 7.2	60 ± 8.9	63 ± 11.2		
Hb (g/dL)	17.4 ± 0.5 ^{e,f}	19.3 ± 0.9 ^{d,f}	22 ± 1.6 ^{d,e}	15.7 ± 0.9 ^{e,f}	17.4 ± 0.7 ^{d,f}	21.3 ± 1.6 ^{d,e}		
Median CMS score	3 ^f	4 ^f	10 ^{d,e}	2 ^{b,f}	3 ^{a,f}	10 ^{a,e}		
SBP (mm Hg)	109.3 ± 10.9	110 ± 16.3	116.6 ± 6.19	103.5 ± 11.3 ^c	109.1 ± 15.3	121.7 ± 20.9 ^a		
DBP (mm Hg)	69.1 ± 7.7 ^f	69.8 ± 13.9	76.6 ± 6.13 ^d	62.5 ± 9.6 ^c	65.9 ± 8.8	76.7 ± 16.8 ^a		
HR (beats/min)	65.3 ± 1	70.9 ± 1	70 ± 8.6	76.9 ± 1.1 ^b	70.2 ± 8 ^{a,c}	79.2 ± 9.2 ^b		
SaO ₂ (%)	88.2 ± 2.4 ^f	86 ± 4.7 ^c	83.8 ± 2.9 ^{b,d}	87.2 ± 3.1 ^f	86.4 ± 3.1 ^f	81.6 ± 3 ^{d,e}		

Values are means ± SD or medians.

^a $p < 0.05$: vs. HAs.^b $p < 0.05$: vs. BDLs.^c $p < 0.05$: vs. those with CMS, by Kruskal–Wallis test.^d $p < 0.01$: vs. HAs.^e $p < 0.01$: vs. BDLs.^f $p < 0.01$: vs. those with CMS, by Kruskal–Wallis test.

Table 2.

Distribution of symptoms and signs in high-altitude adults

Symptom or sign	Men				Women	
	HAs (N = 21)	BDLs (N = 26)	Those with CMS (N = 49)	HAs (N = 26)	BDLs (N = 22)	Those with CMS (N = 9)
Symptoms/signs used in CMS scoring						
Breathlessness and/or palpitations ^a						
Absence	11 (52)	17 (65)	18 (37)	14 (54)	15 (68)	2 (22)
Mild	7 (33)	9 (35)	23 (47)	12 (46)	5 (23)	5 (56)
Moderate	3 (14)	0 (0%)	8 (16)	0 (0)	2 (9)	2 (22)
Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Sleep disturbance						
Absence	11 (52)	17 (65)	28 (57)	21 (81)	13 (59)	4 (45)
Mild	5 (29)	6 (23)	9 (18)	4 (15)	6 (27)	3 (33)
Moderate	5 (29)	3 (12)	10 (20)	1 (4)	3 (14)	2 (22)
Severe	0 (0)	0 (0)	2 (4)	0 (0)	0 (0)	0 (0)
Cyanosis ^b						
Absence	12 (57)	7 (27)	9 (18)	19 (73)	8 (36)	1 (11)
Mild	7 (33)	12 (46)	8 (16)	6 (23)	9 (41)	1 (11)
Moderate	2 (10)	7 (27)	18 (37)	1 (4)	5 (23)	5 (56)
Severe	0 (0)	0 (0)	14 (29)	0 (0)	0 (0)	2 (22)
Dilatation of veins ^b						
Absence	14 (67)	11 (42)	10 (20)	23 (88)	14 (64)	6 (67)
Mild	6 (28)	12 (46)	21 (43)	2 (8)	8 (36)	1 (11)
Moderate	1 (5)	2 (8)	15 (31)	1 (4)	0 (0)	2 (22)
Severe	0 (0)	1 (4)	3 (6)	0 (0)	0 (0)	0 (0)
Paresthesia						
Absence	12 (57)	15 (57)	24 (49)	17 (65)	12 (54)	5 (56)
Mild	7 (33)	8 (31)	17 (35)	9 (35)	8 (36)	2 (22)
Moderate	2 (10)	3 (12)	6 (12)	0 (0)	1 (5)	2 (22)
Severe	0 (0)	0 (0)	2 (4)	0 (0)	1 (5)	0 (0)

Symptom or sign ^b	Men				Women		
	HAs (N = 21)		Those with CMS (N = 49)		HAs (N = 26)		Those with CMS (N = 9)
	BDLS (N = 26)	BDLS (N = 26)	BDLS (N = 26)	BDLS (N = 26)	BDLS (N = 22)	BDLS (N = 22)	BDLS (N = 22)
Headache ^b							
Absence	12 (57)	13 (50)	8 (16)	19 (73)	11 (50)	2 (22)	2 (22)
Mild	6 (29)	10 (38)	28 (57)	6 (23)	10 (45)	2 (22)	2 (22)
Moderate	3 (14)	3 (12)	10 (20)	1 (4)	1 (5)	4 (45)	4 (45)
Severe	0 (0)	0 (0)	3 (6)	0 (0)	0 (0)	1 (11)	1 (11)
Tinnitus ^a							
Absence	12 (57)	9 (34)	20 (41)	20 (77)	17 (77)	1 (11)	1 (11)
Mild	5 (24)	15 (58)	18 (37)	6 (23)	4 (18)	4 (45)	4 (45)
Moderate	4 (19)	2 (8)	9 (18)	0 (0)	1 (5)	3 (33)	3 (33)
Severe	0 (0)	0 (0)	2 (4)	0 (0)	0 (0)	1 (11)	1 (11)
Other symptoms and signs							
Dizziness							
Absence	19 (95)	25 (97)	44 (90)	25 (96)	21 (96)	7 (78)	7 (78)
Mild	1 (5)	1 (3)	5 (10)	1 (4)	1 (4)	2 (22)	2 (22)
Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Fatigue							
Absence	11 (53)	12 (46)	18 (37)	14 (54)	12 (54)	5 (56)	5 (56)
Mild	7 (33)	11 (42)	20 (41)	8 (31)	10 (46)	2 (22)	2 (22)
Moderate	3 (14)	3 (12)	9 (18)	4 (15)	0 (0)	2 (22)	2 (22)
Severe	0 (0)	0 (0)	2 (4)	0 (0)	0 (0)	0 (0)	0 (0)
Failing memory							
Absence	8 (38)	12 (46)	19 (39)	15 (58)	11 (50)	4 (44)	4 (44)
Mild	11 (52)	10 (39)	24 (49)	9 (34)	10 (46)	4 (44)	4 (44)
Moderate	1 (5)	4 (15)	5 (10)	2 (8)	1 (4)	1 (12)	1 (12)
Severe	1 (5)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)

All values are percentages.

^a $p < 0.05$ in women.

^b $p < 0.05$ in men and women.

Table 3.

Analysis of hemoglobin, plasma EPO, and plasma pro-BNP levels

Group	CMS score	Hemoglobin (g/dL)	EPO (mIU/mL)	Pro-BNP (pg/mL)
Healthy	5	17.1 ± 0.2 (N = 101)	17.7 ± 2.4 (N = 47)	27.5 ± 3.1 (N = 47)
Mild CMS	6–10	20.6 ± 0.4 ^a (N = 45)	12.6 ± 1.6 (N = 30)	66.5 ± 14.1 ^a (N = 30)
Moderate CMS	11–14	22.3 ± 0.4 ^a (N = 22)	11.2 ± 2.3 (N = 15)	42.0 ± 9.1 (N = 15)
Severe CMS	15	22.5 ± 1.3 ^a (N = 4)	10.6 ± 2.7 (N = 2)	47.5 ± 23.5 (N = 2)

The normal range for EPO at our institution is 3.7–31.5 and 0–124 for pro-BNP. Blood samples were drawn throughout the day. Men and women were included in each group. The distribution of male and female individuals is 1:1 for healthy, 2:1 for mild CMS, 4:1 for moderate CMS, and for severe CMS, 1 man and 1 woman. There were no statistical differences between men and women.

^a $p < 0.001$ when compared with healthy group by one-way ANOVA, Tukey–Kramer method.

^b $p = 0.005$ when compared with healthy group by one-way ANOVA, Tukey–Kramer method.