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ORIGINAL ARTICLE

High serum zinc and serum testosterone levels were associated with excessive erythrocytosis in men at high altitudes

Gustavo F. Gonzales · Vilma Tapia · Manuel Gasco · Julio Rubio · Cynthia Gonzales-Castañeda

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Abstract Chronic mountain sickness (CMS), a lack of adaptation to altitude characterized by excessive erythrocytosis (EE), is a health problem associated with life at high altitude. The erythropoietic process is regulated by both erythropoietin and testosterone. Zinc (Zn) is known to be related with testosterone and hemoglobin levels; meanwhile, nitric oxide was also associated with adaptation to high altitude. The aim of this study was to determine the relationship of hemoglobin and CMS score with serum levels of zinc, total testosterone (TT), calculated free testosterone (cFT), bioavailable testosterone (BAT), hemoglobin, and nitric oxide in men at high altitude with or without EE. Men residing in Lima (150 m) and Cerro de Pasco (4,340 m), Peru, were divided into three groups: (1) low altitude, (2) high altitude without EE (hemoglobin < 21 g/dl), and (3) high altitude with EE (hemoglobin ≥ 21 g/dl). Adjusted multivariable regression models showed that serum testosterone (total or free) and Zn levels were independently correlated with increased hemoglobin levels. Similarly, hemoglobin was positively related with signs/symptoms of CMS; however, both increased the serum Zn and the nitric oxide levels correlated with reduced risk for signs/symptoms of CMS. In conclusion, higher serum testosterone levels and Zn levels were associated with EE, and low scores of signs/symptoms of CMS were associated with higher Zn and nitric oxide levels.

Keywords Testosterone · Zinc · Nitric oxide · High altitude · Chronic mountain sickness signs/symptoms · Excessive erythrocytosis

Abbreviations

BMI	Body mass index
HA	High altitude
LA	Low altitude
Hb	Hemoglobin
EE	Excessive erythrocytosis
CMS	Chronic mountain sickness
TT	Total testosterone
cFT	Calculated free testosterone
BAT	Bioavailable testosterone
Epo	Erythropoietin
NO	Nitric oxide
Zn	Zinc
ALAD	Delta-aminolevulinic acid dehydratase
HIF	Hypoxia-inducible factor

Introduction

Chronic mountain sickness (CMS), first described in 1925 in Peru, is one of the health problems associated with life at

G. F. Gonzales · M. Gasco · J. Rubio · C. Gonzales-Castañeda
Laboratory of Endocrinology and Reproduction, Faculty of Sciences and Philosophy, Universidad Peruana Cayetano Heredia, Lima, Peru
e-mail: manuel.gasco@upch.pe

J. Rubio
e-mail: julio.rubio.m@upch.pe

C. Gonzales-Castañeda
e-mail: dah_182@hotmail.com

G. F. Gonzales · V. Tapia
Instituto de Investigaciones de la Altura, Universidad Peruana Cayetano Heredia, Lima, Peru
e-mail: tapiavilma@yahoo.es

G. F. Gonzales (✉)
Universidad Peruana Cayetano Heredia, Av. Honorio Delgado 430, Lima 31, Peru
e-mail: gustavo.gonzales@upch.pe

high altitudes (HAs) [1]. The CMS is defined as a lack of adaptation to altitude [2] and is characterized by excessive erythrocytosis (EE). In Cerro de Pasco at 4,340 m, Peru, excessive erythrocytosis is defined when a subject has a level of hemoglobin higher than 21 g/dl [3]. The physiopathology of CMS includes the following sequence: blunted respiratory response to hypoxia, hypoventilation, excessive hypoxemia, and EE [4].

The prevalence of CMS in HA dwellers ranges from 1.2% in native Tibetans to 5.6% in Chinese Han; 6–8% in male residents of La Paz, Bolivia; and 15.6% in the Andes [5–7]. Differences in rates of CMS seem to be explained by the antiquity in which populations reside at HAs [8]. Populations with more multigenerational residence at HA could be more adapted to live in this environment and show fewer rates of CMS than those with less generational residence [8]. CMS leads to cardiac failure and/or neurologic disorders and clinical signs include headache, fatigue, dizziness, sleep disturbances, dyspnea, loss of memory, and digestive complaints [9].

Erythropoiesis, i.e., red blood cell production, is a process that is hormonally regulated by two hormones: erythropoietin and testosterone [10, 11]. Both higher erythropoiesis and hypoventilation are related with testosterone actions [12, 13]. Therefore, it is suggested that testosterone may be involved in HA adaptation acting on both processes associated with CMS [14].

On the other hand, several studies have found that zinc (Zn), a micronutrient involved in structural and regulatory functions in mammalian cells [15], could also be associated with erythrocytes production, hemoglobin [16], and serum testosterone levels [17, 18].

Recent evidence suggests that nitric oxide was also associated with adaptation to HA [19]. It is still unknown whether the relationship between serum testosterone and hemoglobin at HA [14] is also associated with Zn and nitric oxide levels.

For these reasons, the aim of the present study was to determine the circulating levels of testosterone, calculate free testosterone (cFT), bioavailable testosterone (BAT), hemoglobin, nitric oxide, and Zn in men with or without EE living at Cerro de Pasco, 4340 m in Peru, and the associations between them. The relationships between these variables and CMS signs and symptoms were also assessed.

Subject and methods

Design

The study design was cross sectional in 92 adult men aged 30–61 years old. From these individuals, 62 men were living in Cerro de Pasco, Peru (4,340 m), and 30 men in Lima, Peru (Low altitude, 150 m). Men voluntarily accepted to participate in the study and signed an informed consent form. The study was approved by an Institutional Review Board at the Universidad Peruana Cayetano Heredia (SIDISI: 57085) in Lima, Peru.

Men at HAs were recruited into the study based on hemoglobin values, and two groups were conformed: one with hemoglobin $\geq$ 21 g/dl which included 33 men (these subjects were classified as EE, a cardinal sign of CMS), and the second with hemoglobin $<$ 21 g/dl included 29 men (these subjects were classified as men without EE).

Volunteers were recruited through advertising on radio, local health offices, and academic centers. From those men living at Cerro de Pasco, 96.6% of men without EE, and 98.4% of men with EE were born in the same place ($P > 0.05$). Men from the group in Lima were all natives at low altitude (LA) except one who was born in Cutervo, Cajamarca at 1800 m.

Men from the low-altitude group were residing in Lima for 33.9 $\pm$ 14.2 years (mean $\pm$ SD), whereas those from the HA group without EE were residing in Cerro de Pasco for 36.8 $\pm$ 8.9 years, and those with EE were residing in Cerro de Pasco for 37.8 $\pm$ 12.9 years ($P > 0.05$).

Men were not receiving any medication for at least three months before the study nor did they have phlebotomy during the year preceding the study.

Subjects were excluded from the study if they had chronic obstructive pulmonary disease, cardiovascular or renal diseases, or if they had received medication during the previous 3 months. None of the patients were affected by prostate cancer and/or were undergoing androgen deprivation therapy.

Experimental protocol

A basal venous blood sample was drawn by a trained professional from an antecubital vein of each subject between 08:00 and 11:00 h to avoid the circadian and diurnal variation of hormones. In most cases, men were in a fasting state.

Survey

The sociodemographic variables were recorded through a questionnaire that included age, alcohol, and/or tobacco consumption, and time of residence at HA.

During the study, it was noticed that the men were non-smokers or smoked sporadically (less than one cigarette packet a year). Alcohol drinking was recorded as a dichotomic variable. No differences were observed in alcohol consumption between men with and without EE at HA ($P > 0.05$).

Body mass index (BMI)

Height and weight were measured from each subject in the laboratory, in Lima for the low-altitude group, and in Cerro de Pasco for the HA group; and, BMI was calculated in each of the subjects and expressed as Kg/m^2.

Serum hormone measurements

Blood samples were centrifuged at $1{,}000 \times g$ for 10 min at room temperature, and serum from each sample was drawn and placed in a vial. Samples were immediately stored at −20°C until assayed for hormonal analysis. The serum samples were assessed no longer than 4 months after having been collected. The following hormones were determined in serum: follicle-stimulating hormone (FSH), luteinizing hormone (LH), total testosterone (TT), erythropoietin, and sex hormone-binding globulin (SHBG).

Serum total testosterone levels (nmol/l) (Siemens Medical Solutions Diagnostics, Los Angeles, USA) were measured by radioimmunoassay (RIA) using commercial kits. Serum LH (IU/l) and FSH (IU/l) levels were measured by immuno radiometric assay (IRMA) using commercial kits from Siemens Medical Solutions Diagnostics (Los Angeles, CA, USA). All the assays required ^{125}I-labeled analyte. Testosterone values in ng/dl were transformed to nmol/l multiplying by 0.0347. All the assays were performed in the same laboratory located in Lima. Minimal detectable concentration was 4 ng/dl for serum TT, 0.06 IU/l for serum FSH, and 0.15 IU/l for serum LH. Intra-assay coefficient of variation was 5% for TT, 2.2% for FSH, and 1.6% for LH; and the respective inter-assay coefficients of variation were 6.7%, 5.7%, and 3.3%.

For each subject, the serum TT/LH ratio was calculated as Total testosterone (nmol per liter)/LH (international units per liter). The serum TT/LH ratio was used as an indicator of Leydig cell function [20].

SHBG (nmol/l) was measured by chemiluminescence using IMMULITE 2000 assays (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The intra-assay and the inter-assay coefficients of variation were 3.0%, 4.4%, respectively.

Calculated free testosterone (cFT) was determined using the formula suggested by Vermeulen et al. [21]. Bioavailable testosterone includes free plus weakly bound to albumin. Calculation was done with a fixed albumin concentration of 4.3 g/dl. Data of serum-cFT levels were expressed in pmol/l, whereas serum BAT levels were referred in nmo/l.

Erythropoietin was measured by chemiluminescence using IMMUNOLITE 2000 kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The results are expressed as IU/l. Within-assay and between-assay coefficients of variation were 5.8% and 6.3%, respectively. Minimal detectable concentration for erythropoietin was 1.0 mIU/ml.

Hemoglobin measurement

Hemoglobin concentration was measured in situ with a HemoCue system (Anglholm, Sweden). Measurements were performed by the same person both at sea level and at HA. The hemoglobin values were confirmed with hematocrit measurements through the microhematocrit method. The coefficient of determination (R^2) between hemoglobin measurement and hematocrit was 0.99 in Lima and in Cerro de Pasco. In this study, the hemoglobin values were used for analysis. Data were expressed as g/dl.

Serum nitric oxide (NO) determination

Nitric oxide levels were determined using the Griess reaction assay described by Sun et al. [22]. In brief, serum samples were deproteinized with zinc sulfate/NaOH, and then the concentrations of nitrate ($NO3^-$) and nitrite ($NO2^-$) were determined using the Griess reagent at room temperature and read in a spectrophotometer at 543 nm. Nitric oxide concentrations were expressed as mM/l.

Serum zinc measurement

Serum Zn levels were measured by atomic absorption spectrophotometry. Values were expressed as mg/ml.

Pulse oxygen saturation (SpO_2)

The arterial oxygen saturation was measured in the second left finger using a pulse oximeter Nellcor N-20 (Pleasanton, Ca, USA). This equipment also provides simultaneously the heart rate value. All the measurements were performed inside the laboratories in Lima and in Cerro de Pasco for the low- and the HA groups, respectively. Measurements in both locations were performed by the same person.

CMS score

All the participants completed a test for assessment of signs and symptoms of CMS. The test included seven signs/symptoms: 1) breathlessness and/or palpitations; 2) sleep disturbance; 3) presence of cyanosis, 4) dilatation of veins,

5) paresthesia, 6) headaches, and 7) tinnitus. A value of 0 was assigned to negative answers and values from 1, 2, and 3 to positive answers (mild, moderate, and severe, respectively). For the diagnosis of CMS, a value of three was added if the hemoglobin value was ≥21 g/dl in Cerro de Pasco [9].

Statistical analyses

Analyses were performed with STATA 10.0 (Stata Corp, College Station, TX). Data are expressed as mean ± standard deviation (SD). The homogeneity of variances has been determined with the Bartlett test. If homogeneous, analysis of variance (ANOVA) test or Student's *t*-test was used to determine differences among groups. If there were differences, then the mean comparisons between the two groups were determined using the Student's *t*-test.

The variables with no homogenous distribution were analyzed using the Kruskal–Wallis non-parametric test, or they were transformed. The comparisons between two medians were determined by using the Mann–Whitney *U*-test. Chi-square test was performed to determine differences between frequencies.

Multiple lineal regression analyses were also performed. We have assessed the risk for high hemoglobin and for signs and symptoms of CMS in relation with main independent variables as serum androgen activity, nitric oxide, and Zn levels after controlling for confounders as chronologic age, pulse oxygen saturation, and BMI. For the multivariable analysis, a value of $P < 0.05$ was considered as statistically significant.

Results

Men of the HA group without EE had lower BMI, SpO_2 ($P < 0.01$) and serum LH levels than men from the low-altitude group ($P < 0.05$). In addition, higher hemoglobin concentration ($P < 0.01$), SHBG ($P < 0.01$), and erythropoietin levels ($P < 0.01$), and high score for signs/symptoms of CMS ($P < 0.01$) were observed in the HA group without EE than in the group at LA. No differences were observed in chronologic age, heart rate, serum FSH, TT, cFT, bioavailable testosterone, TT/LH, Zn, and nitric oxide levels ($P > 0.05$) (Table 1).

Men with EE had higher hemoglobin concentration ($P < 0.01$), serum TT ($P < 0.05$), cFT ($P < 0.05$), BAT ($P < 0.05$), TT/LH ratio ($P < 0.05$), SHBG ($P < 0.01$), erythropoietin ($P < 0.01$), Zn levels ($P < 0.01$), and high score for signs/symptoms of CMS ($P < 0.01$) than in men at LA. Also, lower values of BMI ($P < 0.05$) and SpO_2 ($P < 0.01$) were observed in men with EE compared to those at LA. No differences were observed in chronologic age, heart rate, and serum LH, serum FSH, and nitric oxide levels ($P > 0.05$) (Table 1).

When men with and without EE at HA were compared, the score for signs/symptoms of CMS ($P < 0.01$), hemoglobin ($P < 0.01$), TT ($P < 0.05$), cFT ($P < 0.01$), BAT ($P < 0.01$) and serum Zn levels ($P < 0.01$) were higher in subjects with EE than in those without EE. SpO_2 and TT/LH ratio were lower in men with EE than in those without EE ($P < 0.05$). Chronologic age, BMI, heart rate, LH, FSH, SHBG, erythropoietin and nitric oxide levels did not differ between these groups ($P > 0.05$) (Table 1).

Table 2 shows the frequency of each sign/symptom of CMS in each study group at LA and at HA. Both groups of men from HA showed higher rates for almost all signs/symptoms of CMS than the men at LA. In men at LA, no cases of cyanosis were observed. Men with EE showed a higher percentage of breathing/palpitations, cyanosis, and headaches at the level of $P < 0.05$ than those men at HA without EE. The other signs/symptoms of CMS did not differ between men with and without EE ($P > 0.05$).

Only one man (3.3%) at LA showed six to seven signs per symptoms compared to six men without EE at HA (20.7%, $P < 0.05$) and 17 men with EE at HA (51.5%; $P < 0.001$). In HA groups, men with EE showed a significant higher presence of more than five symptoms ($P < 0.01$) compared with those without EE (Data not shown).

To determine contribution of SpO_2, serum Zn, nitric oxide, and testosterone (TT, cFT, or BAT) levels on hemoglobin levels, an adjusted multiple regression model was built. Table 3 shows the model that included BAT as an independent variable. Models with TT and cFT show the same results (Data not shown). Analysis showed an association between increased amounts of Zn ($P = 0.007$), increased levels of BAT ($P = 0.002$), and reduced SpO_2 values ($P = 0.0001$) were associated with high hemoglobin levels. These data are consistent with a model in which testosterone and Zn act via independent mechanisms to increase hemoglobin. Table 4 shows a similar analysis to determine risk for signs/symptoms of CMS. Hemoglobin was positively related with signs/symptoms of CMS; however, both Zn ($P = 0.01$) and nitric oxide ($P = 0.03$) were associated with reduced risk for signs/symptoms of CMS.

Discussion

In Peru, approximately 30% of the population live at an altitude above 2,000 m [23]. The population in the Peruvian Andes has been residing there for more than 10,000 years, whereas the resident population in the Himalayas has been

Table 1 Physiologic parameters and hormone levels in men living at low altitude and at high altitude (4,340 m) without excessive erythrocytosis (Hb < 21 g/dl) and with excessive erythrocytosis (Hb ≥ 21 g/dl)

Physiologic parameters and hormone levels	Low altitude ($n = 30$)	High altitude without excessive erythrocytosis ($n = 29$)	High altitude with excessive erythrocytosis ($n = 33$)
Age (years)	43.2 ± 6.9	42.5 ± 7.7	44.7 ± 9.3
BMI (Kg/m^2)	26.0 ± 3.1	24.0 ± 2.4[a]	24.6 ± 2.6
Hb (g/dl)	14.9 ± 1.0	18.6 ± 1.5[a]	22.6 ± 1.4[a,b]
SpO2 (%)	97.6 ± 1.8	88.1 ± 3.0[a]	85.8 ± 3.6[a,b]
Heart rate (beats/min)	74.9 ± 10.2	71.6 ± 10.6	74.5 ± 10.1
Serum LH (IU/l)	3.5 ± 1.5	2.8 ± 0.9[c]	3.0 ± 1.6
Serum FSH (IU/l)	2.89 ± 2.7	3.3 ± 1.8	3.4 ± 1.5
Serum TT (nmol/l)*	14.7 ± 4.0	14.9 ± 4.2	17.2 ± 4.9[a,d]
Serum cFT (pmol/l)	355.8 ± 79.4	314.7 ± 104.4	413.5 ± 128.2[b,c]
Serum BAT (nmol/l)	8.3 ± 1.9	7.6 ± 2.5	9.4 ± 3.0[b]
Serum TT/LH ratio	5.2 ± 3.2	5.6 ± 5.7	7.7 ± 5.0[c]
SHBG (nmol/l)	22.5 ± 6.3	31.9 ± 9.5[a]	29.5 ± 9.0[a]
Serum Epo (IU/l)	10.8 ± 3.5	21.8 ± 8.7[a]	26.0 ± 12.9[a]
Serum Zinc (mg/l)*	2.7 ± 1.0	2.8 ± 2.2	4.2 ± 3.8[a,b]
Nitric oxide (μM/l)	50.7 ± 11.7	54.3 ± 20.1	54.4 ± 19.7
CMS score	1.1 ± 1.9	4.3 ± 2.5[a]	10.1 ± 4.0[a,b]

Data are mean ± SD. *CMS* chronic mountain sickness; CMS score: score of signs and symptoms of CMS and of high hemoglobin levels. *BMI* body mass index, *Hb* hemoglobin, *LH* luteinizing hormone, *FSH* follicle-stimulating hormone, *SpO2* pulse oxygen saturation; *serum TT* serum total testosterone, *serum cFT* serum-calculated free testosterone, *serum BAT* serum bioavailable testosterone, *serum SHBG* serum sex hormone-binding globulin, *serum Epo* serum erythropoietin

* The logarithmic and square root transformations are used for serums TT and Zn, respectively

[a] $P < 0.01$ and [b] $P < 0.01$ versus values at low and high altitudes without excessive erythrocytosis, respectively. [c] $P < 0.05$ and [d] $P < 0.05$ versus values at low and high altitudes without excessive erythrocytosis, respectively

Table 2 Rates of signs/symptoms of CMS in men living at low altitude (LA), at high altitude (HA) with excessive erythrocytosis—EE (Hb ≥ 21 g/dl), and at high altitude without excessive erythrocytosis (Hb < 21 g/dl)

Signs/symptoms of CMS	LA ($n = 30$)	HA without EE ($n = 29$)	HA with EE ($n = 33$)
Breathing/palpitation	3 (13.3%)	11 (37.9%)[c]	23 (69.7%)[a,d]
Insomnia	1 (3.3%)	10 (34.5%)[a]	14 (42.4%)[a]
Cyanosis	0 (0.0%)	19 (65.5%)[a]	29 (87.9%)[a,d]
Venous dilatation	2 (6.7%)	18 (62.1%)[a]	23 (69.7%)[a]
Paresthesia	5 (16.7%)	15 (51.7%)[a]	23 (69.7%)[a]
Headache	7 (23.3%)	11 (37.9%)	23 (69.7%)[a,d]
Tinnitus	3 (10.0%)	10 (34.5%)[b]	13 (39.4%)[a]

Data are expressed as the number of men with signs/symptoms and percentage (between parenthesis, %). [a] $P < 0.01$ and [b] $P < 0.01$ versus rates in men at low altitude (LA) and at high altitude (HA) without excessive erythrocytosis (EE), respectively. [c] $P < 0.05$ and [d] $P < 0.05$ versus rates in men at LA and at HA without EE, respectively

there for more than 25,000 years. Associated with this fact, hemoglobin levels in the Andean population have been found to be higher than those observed in the population of the Himalayas living at the same altitudes [14, 24]. This suggests that Peruvian Andean residents are less adapted to HA than people residing in the Tibet.

It is possible that ethnic admixture produced after the Spanish conquest in the sixteenth century may have interfered in the process of adaptation to HA [23]. For this reason, it was possible to find two populations at Peruvian HA—one more adapted and the second less adapted to live at HAs. For instance, in Cerro de Pasco (4,340 m of altitude) located at the Peruvian Central Andes, we have observed two populations distinguished by different hemoglobin levels: One with hemoglobin levels over 21 g/dl and diagnosed as EE [3] and the second, with hemoglobin levels between 16 and 21 g/dl considered as normal hemoglobin for this altitude [14]. In both cases, hemoglobin levels were higher than those observed at LA, as confirmed in the present study.

Table 3 Adjusted multiple regression models' analyses to determine the association between hemoglobin levels and oxygen saturation (SpO_2), logarithm of zinc, nitric oxide, and bioavailable testosterone values in men living at high altitude (4,340 m)

Hemoglobin levels	Coefficient of regression	SEM	*P* Value	Confidence interval (at 95%)	
SpO_2	−0.47	0.04	0.0001	−0.54	−0.39
Logarithm of zinc	2.84	1.02	0.007	0.81	4.86
Logarithm of nitric oxide	−1.03	1.74	0.554	−4.50	2.43
Bioavailable testosterone	0.27	0.08	0.002	0.10	0.43

Chronologic age, BMI, and heart rate have been controlled in the model

Table 4 Adjusted multiple regression models' analyses to determine the association between signs/symptoms of CMS and logarithm of zinc and nitric oxide, hemoglobin, and bioavailable testosterone values in men living at high altitude (4,340 m above sea level)

Signs/symptoms of CMS	Coefficient of regression	SEM	*P* Value	Confidence interval (at 95%)	
Hemoglobin	0.63	0.16	0.0001	0.31	0.95
Zinc logarithm	−4.05	1.56	0.01	−7.15	−0.96
Bioavailable testosterone	0.25	0.16	0.12	−0.07	0.57
Nitric oxide	−7.35	3.27	0.03	−13.91	−0.80

Chronologic age, pulse oxygen saturation, BMI, and heart rate were controlled in the model. To perform the present analyses, the values related to Hb levels were no considered in the dependent variable (CMS symptoms)

Erythropoiesis is hormonally regulated by the glycoprotein hormone erythropoietin [10]. Subjects acutely exposed to HA show an increase in serum erythropoietin levels; meanwhile, erythropoietin was found to be reduced when native men at HA descend to sea level [25].

Although, erythropoietin levels were higher at HA, subjects with EE did not show a further significant increase when compared with men without EE at HA. As noticed, the participation of erythropoietin in EE at HA among mountain dwellers is not yet conclusive [24, 26]. In fact, there was no correlation between erythropoietin levels in Andean natives with both, EE and CMS and those without these conditions [26]. Also, no association was found between genes involved in hypoxia sensing and erythropoiesis and extreme erythropoietic response in Andean subjects with CMS [27].

It was suggested that erythropoietin was responsible for increasing hemoglobin levels at HAs but it was not associated with the exaggerated erythropoiesis observed in subjects with CMS [14]. This suggests that another hormone or factor was associated with EE at HAs. Testosterone decreases ventilation [12] and increases erythropoiesis [13], and both effects may be associated with EE. In fact, TT, cFT, and BAT were higher in the group with EE at HA than in the group without EE and with men at LA. This suggests that hemoglobin values ranging from 16 to 21 g/dl at HA were increased as an effect of increased erythropoietin levels, whereas hemoglobin values from 21 g/dl to higher seems to be associated with increased total and free testosterone levels.

An increase in TT/LH ratio, a marker of Leydig cell reserve [20], observed in men at HA suggests a higher functionality of the Leydig cells at HA than at sea level. From in vitro studies, it is known that during hypoxia, there is an increase of human chorionic gonadotropin-induced proliferation of Leydig cells, and testosterone release [28]. This may explain the increase in TT/LH ratio observed in men at HA. In addition, the group without EE at HA had a reduction in serum LH levels with respect to values observed in men at LA. This may avoid further erythropoiesis in this group.

One of the problems was different BMI values among men at LA and the two HA groups. BMI may affect androgen and SHBG status. In fact, BMI higher than 30 kg/m^2 was associated with lower SHBG [29] and low cFT [30]. Men at HA had lower BMI than men at LA. This could be associated with higher SHBG. Then, higher SHBG observed at HA could be a consequence of lower BMI. In fact, when this confounding variable was controlled in the multivariable analysis, it was observed that SHBG was increased as an effect of age. For this reason, in further analysis, chronologic age and BMI were controlled.

Data suggest that high serum testosterone levels could not be adaptive to life at HAs. This is supported with the finding that healthy adult HA-native Aymara men tested at 3,600 m have lower average morning salivary testosterone concentrations and lower Hb concentrations than urban men at the same altitude [31]. Rural Aymaras have longer residence at HA and are considered more adapted to living at HA than urban population [23].

Recently, the importance of the hypoxia-inducible factor (HIF) in regulating adaptive processes by hypoxia has been established [32]. Emerging evidence supports a role for HIF in regulating systemic responses to hypoxia across the principal organ systems responsible for oxygen delivery to cells, encompassing erythropoiesis as well as pulmonary, cardiac, and vascular functions [33]. HIF-1alpha was noticeably induced by testosterone in prostate cells [34] and this association could also be observed during exposure to hypoxia at HA.

Score for CMS is a good test to diagnose this pathology at HA [9]. Some signs included in the CMS test as cyanosis are not observed in men residing at LA; however, others, such as headache, were common at LA with a similar prevalence than in men without EE at HA. The distinctive signs and symptoms that discriminate rates between men with and without EE at HA were breathing/palpitations, cyanosis and headache.

The prevalence of these signs/symptoms was higher in the group with EE than in those without EE. The fact that insomnia, venous dilation, paresthesia and tinnitus were also increased in a similar prevalence in the HA group with and without EE suggests that not all men in the group without EE can be diagnosed as CMS in the future or that, these four variables are not good markers of CMS.

It is of interest to note that serum Zn levels were increased in the group with EE at HA compared with those from men without EE at HA and to those at LA. Serum Zn concentrations have been observed to be correlated with hemoglobin in men under different conditions [35, 36]. In our study, men with EE showed higher serum Zn levels than those without EE at HA. Furthermore, levels of serum Zn were inversely associated with functionality of Leydig cells measured by the TT/LH ratio. The source of Zn in the group with EE is still uncertain. However, previous studies showed that another two divalent cations, cobalt [37] and lead [14], were also increased and associated with EE in Cerro de Pasco, the same place this study was performed.

The enzyme delta-aminolevulinic acid dehydratase (delta-ALAD) is a zinc metalloenzyme inhibition by lead of which is the first and the most sensitive indicator of lead exposure and the decreased activity of which has been implicated in the pathogenesis of lead poisoning [38]. Carriers of the ALAD2 allele had higher blood lead levels than those who were ALAD1 homozygous and higher hemoglobin and lower Zn protoporphyrin [39]. Zn protects d-ALAD from oxygen inactivation [40]. It is probable that elevated Zn levels may increase d-ALAD activity at HA. Further studies will be necessary to determine polymorphism for ALAD in these populations at HA.

Our results demonstrated that serum Zn levels were inversely correlated with score of signs and symptoms of CMS. This study may suggest that a subject at HA with EE may be protected from CMS if serum Zn levels are higher, but this needs to be investigated in a cohort study.

HA residents with EE had higher levels of oxidative stress compared to HA residents without EE [41]. Zn is also an antioxidant and anti-inflammatory agent. If the CMS group is oxidatively stressed and generate increased inflammatory cytokines, then perhaps the effect of Zn may be as an anti-inflammatory agent.

Zn may also act on HIF activation [42]. Our study also showed that increased serum nitric oxide levels were also associated with low scores for signs and symptoms of CMS. Recently, it has been demonstrated that nitric oxide and Zn are endogenous signaling molecules acting in response to environmental stress [43, 44].

Limitations of the study were the lack of evaluation of platelet counts, complete blood counts, iron status, and inflammation markers in the samples studied. Other limitations include the cross-sectional design because data show associations or correlations but not causality. Another limitation is that nutrition has not been evaluated. A different diet between sea level and HA groups could perfectly explain the higher nitric oxide and Zn levels in HA groups. Further studies will be required to determine the role of Zn and nitric oxide on CMS.

The findings in the present study shift attention from the traditional focus on pulmonary and hematologic systems to hormone factors contributing to adaptation to living at HA.

In conclusion, high serum testosterone and Zn levels were associated with excessive erythrocytosis whereas high hemoglobin levels were associated with signs/symptoms of CMS. Serum Zn and nitric oxide levels correlated with reduced risk of CMS signs and symptoms.

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References

1. M.C. Monge, Chronic mountain sickness. Physiol. Rev. **23**, 166–184 (1943)
2. G. Xing, C. Qualls, L. Huicho, M. Rivera-Ch, T. Stobdan, M. Slessarev, E. Prisman, S. Ito, H. Wu, A. Norboo, D. Dolma, M. Kunzang, T. Norboo, J.L. Gamboa, V.E. Claydon, J. Fisher, G. Zenebe, A. Gebremedhin, R. Hainsworth, A. Verma, O. Appenzeller, Adaptation and mal-adaptation to ambient hypoxia; Andean, Ethiopian and Himalayan patterns. PLoS ONE **3**, e2342 (2008)

3. J.T. Reeves, F. Leon-Velarde, Chronic mountain sickness: recent studies of the relationship between hemoglobin concentration and oxygen transport. High Alt. Med. Biol. **5**, 147–155 (2004)
4. D. Peñaloza, J. Arias-Stella, The heart and pulmonary circulation at high altitudes: healthy highlanders and chronic mountain sickness. Circulation **115**, 1132–1146 (2007)
5. M.A. Pasha, J.H. Newman, High-altitude disorders: pulmonary hypertension: pulmonary vascular disease: the global perspective. Chest **137**, 13S–19S (2010)
6. C.C. Monge, A. Arregui, F. León-Velarde, Pathophysiology and epidemiology of chronic mountain sickness. Int. J. Sports Med **13**, S79–S81 (1992)
7. F. León-Velarde, M.A. Ramos, J.A. Hernandez, De Idiaquez, D., Munoz, L.S., Gaffo, A., Cordova, S., Durand, D., Monge, C.: The role of menopause in the development of chronic mountain sickness. Am. J. Physiol. **272**, R90–R94 (1997)
8. K. Okuyima, R. Sakamoto, Y. Kimura, M. Ishine, Y. Kosaka, T. Wada, C. Wada, M. Naktsuka, Y. Ishimoto, M. Hirosaki, Y. Kasahara, A. Konno, W. Chen, M. Fujisawa, K. Otsuka, M. Nakashima, H. Wang, Q. Dai, A. Yang, H. Qiao, H. Gao, Z. Li, Y. Zhang, R.L. Ge, K. Matsubayashi, Comprehensive geriatric assessment of elderly highlanders in Qinghai, China II: the association of polycythemia with lifestyle-related diseases among the three ethnicities. Geriatr. Gerontol. Int. **9**, 342–351 (2009)
9. F. León-Velarde, M. Maggiorini, J.T. Reeves, A. Aldashev, I. Asmus, L. Bernardi, R.L. Ge, P. Hackett, T. Kobayashi, L.G. Moore, D. Penaloza, J.P. Richalet, R. Roach, T. Wu, E. Vargas, G. Zubieta-Castillo, G. Zubieta-Calleja, Consensus statement on chronic and subacute high altitude diseases. High Alt. Med. Biol. **6**, 147–157 (2005)
10. T.D. Richmond, M. Chohan, D.L. Barberm, Turning cells red: signal transduction mediated by erythropoietin. Trends Cell Biol. **15**, 146–155 (2005)
11. M. Zitsmann, Effects of testosterone replacement and its pharmacogenetics on physical performance and metabolism. Asian J. Androl. **10**, 364–372 (2008)
12. R. Favier, H. Spielvogel, E. Caceres, A. Rodriguez, B. Sempore, J. Pequignot, Differential effects of ventilatory stimulation by sex hormones and almitrine on hypoxic erythrocytosis. Pflugers Arch **434**, 97–103 (1996)
13. A.D. Coviello, B. Kaplan, K.M. Lakshman, T. Chen, A.B. Singh, S. Bhasin, Effects of graded doses of testosterone on erythropoiesis in healthy young and older men. J. Clin. Endocrinol. Metab. **93**, 914–919 (2008)
14. G.F. Gonzales, M. Gasco, V. Tapia, C. Gonzales-Castañeda, High serum testosterone levels are associated with excessive erythrocytosis of chronic mountain sickness in men. Am. J. Physiol. Endocrinol. Metab. **296**, E1319–E1325 (2009)
15. S. Banudevi, P. Elumalai, R. Arunkumar, K. Senthilkumar, D.N. Gunadharini, G. Sharmilla, J. Arunakaran, Chemoprotective effect of zinc on prostate carcinogenesis induced by *N*-methyl-*N*-nitrosourea and testosterone in adult male Sprague-Dawley rats. J. Cancer Res. Clin. Oncol. **137**, 677–686 (2010)
16. A.K. Baltaci, K. Ozyurek, R. Mogulkoc, E. Kurtoglu, E. Oztekin, A. Kul, Effects of zinc deficiency and supplementation on some hematologic parameters of rats performing acute swimming exercise. Acta Physiol. Hung. **90**, 125–132 (2003)
17. A.S. Prasad, C.S. Mantzoros, F.W. Beck, J.W. Hess, G.J. Brewer, Zinc status and serum testosterone levels of healthy adults. Nutrition **12**, 344–348 (1996)
18. O. Kaya, K. Gokdemir, M. Kilic, A.K. Baltaci, Zinc supplementation in rats subjected to acute swimming exercise: its effect on testosterone levels and relation with lactate. Neuro. Endocrinol. Lett. **27**, 267–270 (2006)
19. S.C. Erzurum, S. Ghosh, A.J. Janocha, W. Xu, S. Bauer, N.S. Bryan, J. Tejero, C. Hemann, R. Hille, D.J. Stuehr, M. Feelisch, C.M. Beall, Higher blood flow and circulating NO products offset high-altitude hypoxia among Tibetans. Proc. Natl. Acad. Sci. USA **104**, 17593–17598 (2007)
20. J.Y. Li, X.Y. Li, M. Li, G.K. Zhang, F.L. Ma, Z.M. Liu, N.Y. Zhang, P. Meng, Decline of serum levels of free testosterone in aging healthy Chinese men. Aging Male **8**, 203–206 (2005)
21. A. Vermeulen, L. Verdonck, J.M. Kaufman, A critical evaluation of simple methods for the estimation of free testosterone in serum. J. Clin. Endocrinol. Metab. **84**, 3666–3672 (1999)
22. J. Sun, X. Zhang, M. Broderick, H. Fein, Measurement of nitric oxide production in biological systems by using Griess reaction assay. Sensors **3**, 276–284 (2003)
23. G.F. Gonzales, Peruvian contributions to the study on human reproduction at altitude: from the chronicles of the Spanish conquest to the present. Respir. Physiol. Neurobiol. **158**, 172–179 (2007)
24. T.Y. Wu, Chronic mountain sickness on the Qinghai-Tibetan plateau. Chin. Med. J. **118**, 161–168 (2005)
25. L. Rice, W. Ruiz, T. Driscoll, C.E. Whitley, R. Tapia, D.L. Hachey, G.F. Gonzales, C.P. Alfrey, Neocytolysis on descent from altitude: a newly recognized mechanism for the control of red cell mass. Ann. Intern. Med. **134**, 652–656 (2001)
26. F. León-Velarde, C. Monge, A. Vidal, M. Carcagno, M. Criscuolo, C.E. Bozzini, Serum immunoreactive erythropoietin in high altitude natives with and without excessive erythrocytosis. Exp. Hematol. **19**, 257–260 (1991)
27. O.M. Mejía, J.T. Prchal, F. León-Velarde, A. Hurtado, D.W. Stockton, Genetic association analysis of chronic mountain sickness in an Andean high-altitude population. Haematologica **90**, 13–19 (2005)
28. G.S. Hwang, S.W. Wang, W.M. Tseng, C.H. Yu, P.S. Wang, Effect of hypoxia on the release of vascular endothelial growth factor and testosterone in mouse TM3 Leydig cells. Am. J. Physiol. Endocrinol. Metab. **292**, E1763–E1769 (2007)
29. N.P. Goncharov, G.V. Katsya, N.A. Chagina, L.J. Gooren, Testosterone and obesity in men under the age of 40 years. Andrologia **41**, 76–83 (2009)
30. B.B. Yeap, O.P. Almeida, Z. Hyde, P.E. Norman, S.A. Chubb, K. Jamrozik, L. Flicker, In men older than 70 years, total testosterone remains stable while free testosterone declines with age. The health in men study. Eur. J. Endocrinol. **156**, 585–594 (2007)
31. C.M. Beall, C.M. Worthman, J. Stallings, K.P. Strohl, G.M. Brittenham, M. Barragan, Salivary testosterone concentration of Aymara men native to 3,600 m. Ann. Hum. Biol. **19**, 67–78 (1992)
32. H.G. Li, Y.M. Ren, S.C. Guo, L. Cheng, D.P. Wang, J. Yang, Z.J. Chang, X.Q. Zhao, The protein level of hypoxia-inducible factor-1alpha is increased in the plateau pika (Ochotona curzoniae) inhabiting high altitudes. J. Exp. Zool. **311**, 134–141 (2009)
33. T.G. Smith, P.A. Robbins, P.J. Ratcliffe, The human side of hypoxia-inducible factor. Br. J. Haematol. **141**, 325–334 (2008)
34. S.H. Li, J.H. Ryu, S.E. Park, Y.S. Cho, J.W. Park, W.J. Lee, Y.S. Chun, Vitamin C supplementation prevents testosterone-induced hyperplasia of rat prostate by down-regulating HIF-1alpha. J. Nutr. Biochem. **21**, 801–808 (2010)
35. M. Idei, K. Miyake, Y. Horiuchi, Y. Tabe, N. Miyake, N. Ikeda, T. Miida, Serum zinc concentration decreases with age and is associated with anemia in middle-aged and elderly people. Rinsho Byori **58**, 205–210 (2010). (in Japanese)
36. D. Mafra, L. Cuppari, D.I. Fávaro, S.M. Cozzolino, Zinc levels after iron supplementation in patients with chronic kidney disease. J. Ren. Nutr. **14**, 164–169 (2004)
37. J.A. Jefferson, E. Escudero, M.E. Hurtado, J. Pando, R. Tapia, E.R. Swenson, J. Prchal, G.F. Schreiner, R.B. Schoene, A. Hurtado, R.J. Johnson, Excessive erythrocytosis, chronic mountain sickness, and serum cobalt levels. Lancet **359**, 407–408 (2002)

38. K.H. Astrin, D.F. Bishop, J.G. Wetmur, B. Kaul, B. Davidow, R.J. Desnick, Delta-Aminolevulinic acid dehydratase isozymes and lead toxicity. Ann. N.Y. Acad. Sci. **514**, 23–29 (1987)
39. F. Scinicariello, H.E. Murray, D.B. Moffett, H.G. Abadin, M.J. Sexton, B.A. Fowler, Lead and delta-aminolevulinic acid dehydratase polymorphism: where does it lead? A meta-analysis. Environ. Health Perspect. **115**, 35–41 (2007)
40. A.S. Prasad, Antioxidant effect of zinc in human. Free Radic. Biol. Med. **37**, 1182–1190 (2004)
41. J.A. Jefferson, J. Simoni, E. Escudero, M.E. Hurtado, E.R. Swenson, D.E. Wesson, G.F. Schreiner, R.B. Schoene, R.J. Johnson, A. Hurtado, Increased oxidative stress following acute and chronic high altitude exposure. High Alt. Med. Biol. **5**, 61–69 (2004)
42. I. Kim, C.H. Kim, G.H. Seo, H.S. Kim, J. Lee, D.G. Kim, Y.S. Ahn, Inhibitory effect of zinc on hypoxic HIF-1 activation in astrocytes. Neuroreport **19**, 1063–1066 (2008)
43. M. McMahon, D.J. Lamont, K.A. Beattie, J.D. Hayes, Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals. Proc. Natl. Acad. Sci. USA **107**, 18838–188343 (2010)
44. A. Tomat, R. Elesgaray, V. Zago, H. Fasoli, A. Fellet, A.M. Balaszczuk, L. Schreier, M.A. Costa, C. Arranz, Exposure to zinc deficiency in fetal and postnatal life determines nitric oxide system activity and arterial blood pressure levels in adult rats. Br. J. Nutr. **104**, 382–389 (2010)