Plasma soluble erythropoietin receptor is decreased during sleep in Andean highlanders with Chronic Mountain Sickness

Running Head: Plasma soluble Erythropoietin receptor during sleep in CMS

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Abstract

Excessive erythrocytosis (EE) is the main sign of Chronic Mountain Sickness (CMS), a highly prevalent syndrome in Andean highlanders. Low pulse O₂ saturation (SpO₂) during sleep and serum androgens have been suggested to contribute to EE in CMS patients. However, whether these factors have a significant impact on the erythropoietin (Epo) system leading to EE is still unclear. We have recently shown that morning soluble Epo receptor (sEpoR), an endogenous Epo antagonist, is decreased in CMS patients suggesting increased Epo availability (increased Epo/sEpoR). The present study aimed to characterize the nocturnal concentration profile of sEpoR and Epo, and their relationship with SpO₂, Hct and serum testosterone in HH and CMS patients. Epo and sEpoR concentrations were evaluated every 4h (6pm-6am) and night-time SpO₂ was continuously monitored (10pm-6am) in thirty-nine male participants (CMS, n=23; HH, n=16) aged 21-65yrs, from Cerro de Pasco, Perú (4340m). CMS patients showed higher serum Epo concentrations throughout the night and lower sEpoR from 10pm-6am. Consequently, Epo/sEpoR was significantly higher in the CMS group at every time-point. Mean sleep-time SpO₂ was lower in CMS patients compared to HH, while the percentage of sleep-time spent with SpO₂<80% was higher. Multiple regression analysis showed mean sleep-time SpO₂ and Epo/sEpoR as significant predictors of hematocrit corrected for potential confounders (age, body mass index and testosterone). Testosterone levels were neither associated to Hct, nor to erythropoietic factors. In conclusion, our results
show sustained erythropoietic stimulus driven by the Epo system in CMS patients, further enhanced by a continuous exposure to accentuated nocturnal hypoxemia.

New and Noteworthy

Andean highlanders suffering from Chronic Mountain Sickness (CMS) show consistently lower levels of plasma soluble erythropoietin (Epo) receptor (sEpoR) and higher Epo-to-EpoR ratios (Epo/sEpoR) during sleep compared to their healthy counterparts. This indicates higher blood Epo availability in CMS patients and continuous nocturnal erythropoietic stimulus. Additionally, morning Epo/sEpoR and mean sleep-time SpO₂ are independent main predictors of Hct. These findings support the role of the Epo system in the development of excessive erythrocytosis in CMS.

Key Words: Soluble erythropoietin receptor, Chronic Mountain Sickness, Excessive erythrocytosis, Sleep, Andes
Introduction

Chronic Mountain Sickness (CMS) is a highly prevalent syndrome among Andean highlanders. The prevalence of this condition increases with age and altitude (16, 27, 35, 40), and affects 16-20% of the population living permanently above 4000m, particularly males and post-menopausal women (19, 25, 27). CMS is characterized by excessive erythrocytosis (EE, Hb concentration ≥21.3g/dL in males and ≥18.6g/dL in females) and it is frequently accompanied by exaggerated hypoxemia (17, 20, 25, 26). The associated symptoms are headaches, dizziness, breathlessness, tinnitus, palpitations, sleep disturbances, mental and physical fatigue, and confusion (17, 20). Pulmonary hypertension is also commonly associated with the condition (21, 28, 30, 37), and people affected by CMS often suffer from vascular dysfunction and heart failure in early adulthood (17, 24, 33, 38), possibly due to increased blood viscosity (15).

Prolonged hypoxia exposure represents the underlying stimulus for EE and CMS (20, 23, 43); however, the fundamental pathophysiological mechanism is still debatable. It is not clear why only some individuals chronically exposed to high-altitude (HA) develop this syndrome.

Sleep-disordered breathing leading to accentuated hypoxemia has been proposed as a possible factor triggering EE. However, studies of sleep abnormalities at HA show discordant results. Spicuzza and co-workers (36) showed no differences in the number and duration of apneas or hypopneas between patients with EE and healthy controls, while Julian and colleagues (10) have reported that young male
Residents with EE show a higher frequency of apneas (central and obstructive) and hypopneas during REM sleep, and a greater apnea-hypopnea index. Despite controversy on the occurrence of sleep abnormalities, both studies agree on the presence of lower nocturnal pulse O2 saturation (SpO2) in subjects with EE compared to their healthy counterparts, which is consistent with other studies of nocturnal SpO2 in HA polycythemic patients (8, 14, 31).

Also, whether accentuated hypoxemia during sleep has a significant impact on circulating erythropoietin (Epo) concentration that could explain the occurrence of EE in CMS patients is still controversial. Despite greater night-time hypoxemia, most studies have shown similar morning Epo concentration in patients with EE compared to healthy highlanders (HH) (6, 10, 18, 39). In contrast, Bernardi and co-workers (1) reported in a small number of highlanders of Cerro de Pasco (4340m) that the hormone is slightly but consistently higher in CMS patients during day and night compared to healthy controls. However, high variability in serum Epo between subjects with EE suggests other mechanisms involved in the development of an excessive erythropoietic response. The soluble Epo receptor (sEpoR), an endogenous Epo antagonist (22, 29), has been suggested to play a role as a physiological regulator of erythropoiesis (11, 39). We have recently shown that EE is strongly associated to low circulating morning sEpoR values and therefore to high Epo-to-sEpoR ratios (Epo/sEpoR), leading to elevated plasma Epo availability, and thereby a stronger stimulus for erythropoiesis (39). However, it is still unknown if differences in sEpoR concentration during sleep, and its relationship with Epo and SpO2 are associated with EE.
In addition, other hormonal factors, such as testosterone, have been proposed as possible contributors to the development of EE and CMS (6). Elevated morning serum testosterone has been shown to be associated to EE in Andean highlanders (6, 7). However, whether serum testosterone contributes to the Epo system on the excessive production of erythrocytes in CMS is yet to be evaluated.

The aim of the present study was to characterize the concentration profile of sEpoR and serum Epo during night-time, and their relationship with SpO₂ and hematocrit (Hct) in CMS patients and HH. Additionally, we aimed to assess the association between testosterone concentration and Epo and sEpoR at early morning.
Methods

The study was approved by the Institutional Ethics Committee (CIE) of Universidad Peruana Cayetano Heredia (Lima, Peru). Participants provided written, informed consent.

Study population. Thirty-nine male participants (CMS, n=23; HH, n=16) in the age range from 21 to 65 years, from Cerro de Pasco, Perú at 4340m were studied. Exclusion criteria were the presence of pulmonary, cardiovascular or renal disease, recent phlebotomy, journeys to lower altitude for more than 7 days during the previous 6 months, and employment in mining activities.

A sample size of 36 was calculated based on Epo measurements from a previous study in the same population (32). This erythropoietic marker was employed since it is the study parameter known to show the largest variability. Taking into account a potential 20% loss of participants due mainly to missing data or extreme values, we evaluated 45 individuals. Finally, 39 highlanders with complete data were included in the analysis.

Study procedures. Participants underwent a clinical examination and answered a general health and a CMS Score questionnaire (17). CMS was confirmed in participants with Hct>63% (Hb>21g/dL) and a CMS Score ≥6 according to international consensus (17). Hct was determined by microcentrifugation using a small blood sample obtained from a puncture on the fingertip. Night-time SpO₂ was continuously monitored from 10pm to 6am through a wrist pulse oximeter (WristOx₂ Model 3150, Nonin, Plymouth, MN). Blood samples for Epo and sEpoR
determination were taken every 4h from 6pm to 6am. Specific sandwich enzyme immunoassay kits were used for serum Epo (DRG International Inc., Springfield, NJ) and sEpoR (USCN Life Science Inc., Houston, TX) concentration quantification. The standard sample storage and analysis procedure described by the manufacturer was followed for each kit running each sample in duplicate. Serum from the 6am sample was also used for the assessment of serum iron, ferritin, transferrin, free and total testosterone which were analyzed at Medlab clinical laboratories (ISO 9001:2000), Lima, Perú.

**Statistical analysis.** Differences between groups for the general characteristics of the study participants were obtained through unpaired t-tests, when data met requirements of normality and homocedasticity, or Wilcoxon rank sum tests if otherwise. Generalized estimating equations (GEE) were used to evaluate the effect of time, group (CMS or HH), and their interaction on Epo, sEpoR, the Epo/sEpoR ratio and SpO₂ profiles, adjusted for potential confounders (age, BMI and testosterone concentration). In addition, differences between time-points within each group, and between groups at each time-point were estimated by the use of post-regression analysis. Finally, correlation analysis was performed to evaluate bivariate associations, and multiple linear regressions were used to assess the association between Hct and SpO₂ and markers of the Epo system (Epo, sEpoR, and Epo/sEpoR ratio) in the presence of potential confounders. Only multiple regressions that met requirements of linearity, normality and homocedasticity were included, and variables with no linearity were categorized. A significance level of 0.05 was used.
**Study variables.** CMS Score was determined in agreement with the 2005 Consensus Statement on chronic and subacute HA diseases (17). Awake SpO₂ corresponds to the value obtained from clinical examination which took place around 6pm while the person was at rest, and calculated as the mean of 2 measurements taken 2 minutes apart. Basal pre-sleep SpO₂ measurements at 10pm were retrieved from the continuous night-time monitoring, readings from 5 minutes after the device had been placed and the patient was in bed were used, and a mean value was calculated using two readings 2 minutes apart. Mean values for sleep-time SpO₂ from 2am and 6am used for the bivariate correlations were calculated in the same manner. Mean sleep-time SpO₂ was calculated using measurements collected every 30 seconds throughout the time the patient was asleep. Continuous night-time readings were also used to calculate mean SpO₂ for every hour of sleep and for the sampling period. The percentage of sleep-time spent with SpO₂ below 80% was calculated using hours of sleep spent below this value in relation to total sleep time (TST). The cut-off point was chosen in accordance with previous literature where SpO₂ below 80% has been described as a threshold for the stimulation of Epo production (2, 36). For multiple regression analysis, BMI was dicotomized into normal and overweight using a cut-off value of 25kg/m² in agreement with WHO guidelines (41), and age was categorized using 45 years as a general cut-off value for the onset of increased morbility (4, 25, 27).
Results

**General characteristics.** Mean age and BMI were similar between study groups. As previously reported (20), CMS Score and Hct were significantly higher in CMS patients compared to HH, while awake (6pm) SpO₂ was significantly lower. No differences were found in serum iron, ferritin, transferrin concentration or transferrin saturation between groups (Table 1).

**SpO₂.** Basal pre-sleep (10pm) SpO₂ and SpO₂ throughout the night were consistently lower in CMS patients compared to HH, including the sampling period (Figure 1). Moreover, CMS patients showed significantly lower mean sleep-time SpO₂ (p<0.01). The percentage of TST spent with SpO₂ below 80% was significantly greater in CMS patients compared to HH (Figure 2). In additional correlation analysis, awake SpO₂ (6pm) as well as sleep-time measurements from 2am, 6am and mean sleep-time SpO₂ showed significant negative associations with Hct (rho= -0.59, p<0.01; rho= -0.44, p<0.01; rho= -0.42, p<0.01 and rho= -0.56, p<0.001, respectively); and awake (6pm) and 2am SpO₂ with the CMS score (rho= -0.55, p<0.001; rho= -0.47, p<0.01, respectively). Mean sleep-time SpO₂ showed a tendency for a correlation with 6am (awake) Epo (rho= -0.31, p=0.05).

**Epo, sEpoR, and Epo/sEpoR ratio.** Epo concentration, sEpoR and the Epo/sEpoR ratio were significantly different between groups throughout the measurements, except for the receptor at 6pm (Figure 3). Comparisons between time-points within each group showed a marked increase in the receptor from 6pm...
to 10pm in the HH group (p=0.011), while values decreased significantly between these time-points in the CMS group (p=0.046). Accordingly, a significant interaction was found between time and group effects for the receptor (Figure 3B). Also, in the HH group, the Epo/sEpoR ratio showed significantly higher values at 10pm and 2am compared to 6pm (p=0.002 and p=0.041, respectively) (Figure 3C). Finally, no differences were found in Epo between time-points within each group (Figure 3A).

**Testosterone.** Morning total testosterone and free testosterone concentrations were not significantly different between the CMS and HH groups neither in the study subjects (Figure 4), nor in a larger sample evaluated for this parameter (n: HH=47 and CMS=31). Also, no significant correlations were observed between total or free testosterone and morning Epo, sEpoR or the Epo/sEpoR ratio (data not shown).

**Regression.** Multiple regression analysis showed mean sleep-time SpO\(_2\) and the Epo/sEpoR ratio as significant predictors of Hct corrected for potential confounders (Table 2). Similar results were obtained when regressions included Epo or the sEpoR as predictors (data not shown).
Discussion

This is the first study to report the temporal profile of plasma sEpoR during sleep in CMS patients and HH, and its relationship with circulating Epo and SpO₂.

The results from night-time measurements of Epo, the receptor and the Epo/sEpoR ratio from the present study complement our previous findings on the association of low morning sEpoR with EE (39). Here we show that besides lower morning plasma sEpoR, night-time concentrations are also significantly lower in CMS patients compared to HH. Additionally, differentiated time-dependent variations in sEpoR were observed in the HH group and CMS patients group. Results show that, while in HH the receptor increases from 6pm to 10pm, and remains elevated through the night, it decreases in CMS patients and remains low until morning. The behavior of the receptor suggests the presence of rhythmicity in its secretion, being different between groups, although measurements throughout the entire day are needed to evaluate the possibility of a circadian rhythm in HH and possible alterations in CMS patients. Similar differences between groups were observed for the Epo/sEpoR ratio. In congruence with our previous work (39), in the present study the Epo/sEpoR ratio showed higher values in CMS patients in the morning and throughout the night compared to HH, indicating a constant increased availability of Epo from 6pm to 6am and therefore a continuous erythropoietic stimulus in these patients.

Different studies have shown that SpO₂ during daytime is lower in patients with EE compared to HH, and that this difference is maintained throughout the night (8, 13,
In agreement, we found lower daytime and night-time SpO2 in CMS patients compared to HH, and a similar drop in SpO2 could be observed in both groups during sleep. However, despite similar absolute nocturnal desaturation in HH and CMS patients, the latter begin the night with lower values compared to their healthy counterparts, so that the drop in SpO2 during sleep is enough to reach values below 80%, a threshold known to trigger the erythropoietic response (2). Spicuzza and colleagues (36) showed that patients with CMS spent 38% of the night with SpO2 values between 76 and 80%, while healthy controls spent most of the night above 81%. Accordingly, we found that the percentage of TST spent with SpO2 values below 80% in CMS patients is nearly twice the values observed in HH, and also a strong negative association between mean sleep-time SpO2 and Hct was observed. Thus, it would be expected that a greater exposure time to accentuated hypoxemia further stimulates the Epo system and contributes to the development of EE. However, most studies have reported similar morning serum Epo concentration in HH and patients with EE (6, 18, 39). Nevertheless, since these studies used measurements from a single time-point, the occurrence of variations in the hormone levels throughout 24h were not considered. In this sense, studies with repeated measurements provide more precise comparisons than cross-sectional studies, yielding more reliable results. Additionally, longitudinal studies shed light into potential differences between groups at several times of the day and into diurnal or nocturnal variations in the hormone, and in the Epo system as whole, that might contribute to the development of EE.
Although there is some disagreement between reports of variations of Epo during 24h (9, 12, 34), some studies reported a circadian rhythm for the hormone, both in SL inhabitants (3, 5, 12, 42) and HA healthy natives (1), consistently showing a nadir in the morning and a zenith in the afternoon. To evaluate diurnal and nocturnal variations of Epo, Bernadi and coworkers (1) studied a small number of HA dwellers from Cerro de Pasco with and without EE by taking measurements every 4 hours beginning at 8am. The study showed that circadian rhythm was disrupted in subjects with EE, with no variations between day-time and night-time Epo concentration, as opposed to the 40-60% variation observed in HH. At every time-point, Epo was higher in patients with EE compared to HH, and there was neither morning nadir nor distinguishable zenith, although great variability between subjects was observed. In agreement with Bernardi et al, we found higher Epo concentration throughout the measurements in CMS patients compared to HH, although the highest variability in both groups could be observed in the morning. Also, we found no signs of rhythmicity in Epo secretion neither in CMS patients, nor in HH, probably due to lack of daytime measurements since the peak and the nadir of Epo concentration have been reported around 4-8pm and 8-12am respectively (12, 42), outside our sampling period.

Some potential limitations can be identified in our study. First, subjects had to be awaken during the night for sample drawing procedure. To overcome this shortcoming we reduced the sampling period to a minimum, as well as disturbances caused by sound or light. Additionally this period was not included in the calculation of night-time hypoxemia-related variables. Second, it was not
possible to evaluate a circadian rhythm of Epo and the receptor, since no complete day-time measurements were obtained. However, our results of night-time values of the Epo system variables shed light into the behavior of sEpoR and set a precedent for the study of circadian rhythmicity of the Epo system as a whole.

The findings of the present investigation suggest that higher Epo concentration during the night might play a role in maintaining the erythropoietic stimulus responsible for EE. However, high between-subject variability in Epo concentration, especially in the CMS group, suggests that there are other mechanisms promoting the excessive erythropoietic response. Our findings of higher Epo/sEpoR ratio in CMS patients compared to HH at every time-point, in contrast with controversial results on day-time Epo, indicates that the erythropoietic drive in CMS might be better explained in terms of Epo availability (Epo/sEpoR ratio) rather than Epo concentration alone. Additionally, we show that morning Epo/sEpoR ratio and mean sleep-time SpO2 are independent predictors of Hct, and that the ratio is a better predictor compared to serum Epo.

Our results also indicate that testosterone is neither a significant predictor of Hct, nor a relationship was observed between the hormone and the presence of CMS, in contrast with results from previous studies by Gonzales and collaborators (6, 7). Also, no significant association was found between total or free serum testosterone concentration and Epo, sEpoR or the Epo/sEpoR neither in the bivariate analysis nor the multiple regressions including mean sleep-time SpO2, age and BMI.
In conclusion, the present study shows that the sEpoR is consistently lower in CMS patients during night-time, leading to an increased Epo/sEpoR ratio, which suggests a continuous elevated Epo availability and consequently a stronger stimulus for erythropoiesis, without a significant contribution from serum androgens. These, together with our previous findings, suggest that CMS patients show a sustained erythropoietic stimulus driven by the Epo system, further enhanced by the continuous exposure to accentuated hypoxemia during sleep.
Acknowledgements

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Grants

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7. Gonzales GF, Tapia V, Gasco M, Rubio J, and Gonzales-Castaneda C. High serum zinc and serum testosterone levels were associated with excessive erythrocytosis in men at high altitudes. Endocrine 40: 472-480, 2011.


**Figure Legends**

**Figure 1.** Time-course of nocturnal pulse oxygen saturation (SpO\(_2\)) in patients with Chronic Mountain Sickness (●, n=23) and healthy highlanders (○, n=16). The figure shows SpO\(_2\) while the patients were awake in bed and for each hour of sleep. The period highlighted in grey corresponds to the sampling time for which patients were awakened. Values are expressed as means ± SE. *p<0.05, **p<0.01

**Figure 2.** Percentage of total sleep time (TST) spent with pulse oxygen saturation (SpO\(_2\)) below 80% in patients with Chronic Mountain Sickness (CMS, n=23) and healthy highlanders (HH, n=16). The figure shows a scatter of individual values for each group. Horizontal lines represent means ± SE. *p<0.05

**Figure 3.** Time course of erythropoietin (Epo), serum soluble Epo receptor (sEpoR) and the Epo-to-sEpoR (Epo/sEpoR) ratio in patients with Chronic Mountain Sickness (●, n=23) and healthy highlanders (○, n=16). The figure shows Epo (A) and sEpoR (B) concentrations, and the Epo/sEpoR ratio (C) of each group at every time-point. Arrows on parallel x-axis point each at specific time points on Epo, sEpoR, and Epo/sEpoR measurements and correspondent time-points at 10pm (pre-sleep) and 2am (4hrs of sleep) from Figure 1. Values are expressed as means ± SE. *p<0.05, **p<0.01, ***p<0.001

**Figure 4.** Serum total testosterone (TT) and free testosterone (FT) concentration in patients with Chronic Mountain Sickness (CMS, n=23) and healthy highlanders (HH, n=16). Values are expressed as means ± SE.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Table 1. General characteristics of study participants

<table>
<thead>
<tr>
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<th>Healthy Highlanders</th>
<th>CMS Patients</th>
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<tr>
<td>Age, yrs</td>
<td>40.1 ± 2.83</td>
<td>44.26 ± 2.72</td>
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<td>BMI, kg/m²</td>
<td>25.4 ± 0.70</td>
<td>26.07 ± 0.73</td>
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<td>SpO₂, %</td>
<td>86.9 ± 1.02</td>
<td>82.33 ± 1.17*</td>
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<tr>
<td>Hct, %</td>
<td>52.7 ± 0.70</td>
<td>69.82 ± 1.13***</td>
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<tr>
<td>CMS Score</td>
<td>2.4 ± 0.60</td>
<td>8.52 ± 0.78***</td>
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<tr>
<td>Serum iron, µg/dL</td>
<td>115.6 ± 16.54</td>
<td>106.03 ± 10.01</td>
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<td>Serum ferritin, ng/mL</td>
<td>102.8 ± 20.61</td>
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<td>Transferrin, mg/dL</td>
<td>257.7 ± 7.01</td>
<td>267.00 ± 12.28</td>
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<tr>
<td>Transferrin saturation, %</td>
<td>45.9 ± 5.92</td>
<td>42.52 ± 4.74</td>
</tr>
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Values are expressed as means ± SE; healthy highlanders (n=16), Chronic Mountain Sickness (CMS) patients (n=23). BMI: body mass index, SpO₂: pulse O₂ saturation. *p<0.05, ***p<0.001
Table 2. *Multiple linear regression models for prediction of hematocrit*

### A.

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<th>SE</th>
<th>p</th>
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### B.

<table>
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<th>SE</th>
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<td>Epo/sEpoR</td>
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<td>Total testosterone</td>
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<td>0.708</td>
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A. Multiple linear regression for prediction of hematocrit including mean sleep-time SpO₂, Epo/sEpoR ratio, being over 45 years of age, having over 25 kg/m² of BMI, and free testosterone as independent variables. B. Multiple linear regression for prediction of hematocrit including mean sleep-time SpO₂, Epo/sEpoR ratio, being over 45 years of age, having overweight (BMI>25 kg/m²), and total testosterone as independent variables. SpO₂: pulse $O_2$ saturation, BMI: Body Mass Index. β: regression coefficient, SE: standard error, $R^2$: coefficient of determination.