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Hematological adaptations and detection of recombinant human erythropoietin combined with chronic hypoxia

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ABSTRACT

This study evaluated whether recombinant human erythropoietin (rhEpo) treatment combined with chronic hypoxia provided an additive erythropoietic response and whether the Athlete Biological Passport (ABP) sensitivity improved with hypoxia.

Two interventions were completed, each containing four weeks baseline, four weeks exposure at sea-level or 2,320m of altitude and four weeks follow-up. Participants were randomly assigned to 20 IU·kg bw⁻¹ rhEpo or placebo injections every second day for three weeks during the exposure period at sea-level (rhEpo n=25, placebo n=9) or at altitude (rhEpo n=12, placebo n=27). Venous blood was analyzed weekly.

Combining rhEpo and hypoxia induced larger changes compared with rhEpo or hypoxia alone for [Hb] (P<0.001, P>0.05, respectively), reticulocyte percentage (P<0.001) and OFF-hr score (P<0.01, P<0.001, respectively). The most pronounced effect was observed for reticulocyte percentage with up to ~35% (P<0.001) and ~45% (P<0.001) higher levels compared with rhEpo or hypoxia only, respectively. The ABP sensitivity for the combined treatment was 54 and 35 percentage points higher for [Hb] (P<0.05) and reticulocyte percentage (P<0.05), respectively, but similar for OFF-hr score, compared with rhEpo at sea-level. Across any time point, [Hb] and OFF-hr score combined identified 14 unique true-positive participants (56%) at sea-level and 12 unique true-positive participants (100%) at altitude. However, a concurrent reduction in specificity existed at altitude.

In conclusion, rhEpo treatment combined with hypoxic exposure provided an additive erythropoietic response compared to rhEpo or hypoxic exposure alone. Correspondingly, ABP was more sensitive to rhEpo at altitude than at sea-level, but a compromised specificity existed with hypoxic exposure.
INTRODUCTION

Many athletes sojourn at altitude to improve sea-level endurance exercise performance via hypoxia induced increases of hemoglobin concentration ([Hb]), reticulocyte percentage and total hemoglobin mass. Like hypoxic exposure, injections of recombinant human erythropoietin (rhEpo) augments erythropoiesis which lead to increased reticulocyte percentage, [Hb] and total hemoglobin mass.

However, the hematological response to combined altitude exposure and rhEpo injections remain unknown. If hypoxic exposure and rhEpo injections are combined, a simple additive effect must be expected as a dose-dependent relationship exist between the increase in endogenous erythropoietin concentration and the erythropoietic response. Identifying the response to altitude and rhEpo injections is particularly important for anti-doping purposes, as reports indicate that altitude exposure is utilized as a masking technique for atypical hematological fluctuations, e.g. induced by rhEpo.

The hematological module of the Athlete Biological Passport (ABP) is developed for indirect detection of rhEpo misuse. The ABP monitor athletes’ hematological fluctuations longitudinally in an adaptive model and calculates an upper and lower intra-individual threshold for [Hb], reticulocyte percentage, OFF-hr score ([Hb] – 60 × √reticulocyte percentage) and an Abnormal Blood Profile Score (ABPS). If a sample crosses a threshold it is considered an outlier, which initiate an expert panel blood profile review. The expert review is highly dependent on existing research illuminating likely and unlikely fluctuations. Indeed, the number of outliers has been investigated following different rhEpo treatment strategies with sensitivities ranging from 20-100% depending on the treatment dose and it is known that chronic altitude exposure constitute a confounding factor that may reduce the specificity of the passport. However, the impact of combined altitude exposure and rhEpo injections on the ABP parameters, number of outliers and the passport specificity is currently unknown.
In the present study we hypothesized that (1) intravenous injections of 20 IU·kg bw\(^{-1}\) epoetin alpha every second day for three weeks with simultaneous exposure to 2,320 m of altitude induce an additive erythropoietic response compared to altitude exposure alone and compared to sea level treatment with similar doses epoetin alpha in both males and females and (2) the fraction of true positive outliers by the ABP in participants treated with intravenous injections of 20 IU·kg bw\(^{-1}\) epoetin alpha every second day for three weeks is higher with simultaneous exposure to 2,320 m of altitude compared to sea level exposure, but a concurrent reduced specificity exist with altitude exposure.
METHODS

Thirty-nine regularly physical active, low-altitude residents and non-smoking healthy males (n=23, 27±7 yrs, 76±9 kg, 183±8 cm and a VO_{2\text{max}} of 60±8 ml·min·kg⁻¹) and females (n=16, 26±4 yrs, 63±8 kg, 171±8 cm and a VO_{2\text{max}} of 47±5 ml·min·kg⁻¹) gave written informed consent to participate in a randomized, single-blinded, placebo-controlled study approved by the ethics committee of Copenhagen, Denmark (H-17036662), registered on www.clinicaltrials.gov (NCT04227665) and performed in accordance with the Declaration of Helsinki. No participant had been exposed to altitude higher than 1000 m for >1 month or donated blood for >3 months before the study and were instructed to refrain from such during the study.

Design

The study included two interventions, each containing four weeks baseline, a four-week exposure at either sea level or 2,320m of altitude (Centro de Alto Rendimiento, Sierra Nevada) and four weeks of follow up (Figure 1). At least 2 months separated the interventions. Participants traveled to ~700 m of altitude for 5-7 h twice during altitude exposure and biked occasionally to 1,800 m following immediate ascent to 2,320 m as well as biked or hiked to 3400m. Participants were randomly assigned to rhEpo (Epoetin alpha, Eprex, Janssen-Cilag, Birkerød, Denmark) injections of 20 IU·kg bw⁻¹ (n=25 at sea level, 13 males and 12 females; n=12 at altitude, 7 males and 5 females) or saline (n=9 at sea level, 6 males and 3 females; n=27 at altitude, 16 males and 11 females) given intravenously every second day for three weeks with a total of 11 injections. Treatment started at day 1 and day 3 of the sea level and altitude exposure period, respectively. Participants wore a blindfold during injections to maintain blinding. A small rhEpo treatment dose was applied to mimic a likely real-life treatment regime.
Participants were instructed to ingest ~250 mg ferric sulfate (Tardyferon, Pierre Fabre Pharma GmbH, Freiburg, Germany) every second day from the third baseline week until completion of follow up measurements in both interventions.

**Sampling**

Blood was collected weekly from an antecubital vein in EDTA vacutainers (BD Vacutainer®: K2EDTA (K2) CE cat no 368856, Becton Dickinson, NJ, USA) according to the World Anti-Doping Agency (WADA) guidelines during both interventions and collections were separated by at least 5 days. Briefly, ≥10 min of seated rest and <30 s of tourniquet application was applied before collection and ≥15 min of sample homogenization on a roller mixer preceded a duplicate complete blood count and reticulocyte analysis on a Sysmex XN-450 hematology analyzer (Sysmex, Kobe, Japan). The first result of the duplicate analysis was the primary outcome if the variance between the analyses were acceptable according to WADA guidelines. If not, the sample was reanalyzed according to WADA guidelines until acceptable. Physical activity was not allowed for 2 h prior to blood sampling. Instrument maintenance, including timely analysis of internal quality controls were performed according to WADA guidelines.

**Athlete Biological Passport**

The Anti-Doping Administration & Management System (ADAMS) training platform developed by WADA was used to examine the sensitivity and specificity of the hematological module of the ABP by creating individual passport profiles. The adaptive model was applied on each exposure period (sea level or altitude) separately, i.e. one participants’ four baseline samples from the respective intervention was introduced along with one specific sample (e.g. the second week of altitude exposure) to evaluate whether the specific sample triggered an outlier, i.e. crossed a calculated threshold. Thresholds were calculated for [Hb], reticulocyte percentage, OFF-hr score and the ABPS.
Since the ABP guidelines does not define an outlier for the ABPS biomarker to trigger an atypical passport finding, the ABPS marker was only presented for potential future interest, but not discussed further within the present study.

Sensitivity was calculated for each time point as the fraction of rhEpo treated participants exceeding the upper or lower calculated threshold for a passport biomarker with a specificity of 99% or exceeding a probability of 99.9% for an atypical longitudinal profile. Specificity was calculated as the fraction of outliers exceeding the upper or lower calculated threshold in samples collected at baselines for all participants in both interventions as well as during the sea level exposure and follow up period in placebo treated participants. Potential false-positive results within the baseline samples were investigated by introducing four baseline samples from one intervention and comparing each baseline value with the thresholds calculated at the fourth baseline sample to reduce the likelihood of false-positives caused by insufficient adaptation of the initial thresholds. To investigate the impact of altitude exposure on the specificity, specificity was also calculated for samples collected exclusively from placebo treated participants during altitude exposure and the follow up period.

The sensitivity and specificity for [Hb], OFF-hr score, reticulocyte percentage and ABPS were investigated independently and in combination, including the combination of [Hb] and OFF-hr score as well as [Hb], OFF-hr score and reticulocyte percentage. The purpose was to determine whether the biomarkers have an additive effect, as some individuals may cross the thresholds for [Hb] while others cross for OFF-hr score. Furthermore, adding the reticulocyte percentage is previously demonstrated to improve sensitivity of the passport.\textsuperscript{13}

### Statistics

SPSS was used for statistical analyses (IBM SPSS Statistics, version 25.0.0). A mixed linear model for repeated measurements was used.\textsuperscript{19} To answer the hypotheses the following analyses were
completed: (1) ‘rhEpo’ (n=12) vs. ‘placebo’ (n=27) during the 12-week altitude intervention to
determine whether an additive erythropoietic response is induced by rhEpo injections at altitude; (2)
‘altitude’ (n=12) vs ‘sea-level’ (n=25) during both 12-week interventions in the rhEpo group only to
determine whether rhEpo injections and hypoxic exposure induce an additive erythropoietic response
compared to rhEpo injections at sea level; (3) ‘rhEpo’ (n=25) vs. ‘placebo’ (n=9) during the 12-week
sea-level intervention to determine the physiological response to rhEpo injections at sea level. Fixed
factors were ‘time’, ‘treatment’ (rhEpo or placebo) and the interaction ‘time × treatment’ (analysis 1
and 3) or time, intervention (altitude or sea level) and time × intervention (analysis 2). Participant ID
was used to identify repeated measures and to define a random factor.

In addition, a generalized linear mixed model using time and biomarker as fixed factors was used to
investigate whether biomarkers of the ABP was more sensitive at altitude than at sea level while time
× biomarker was added subsequently as fixed factor to determine any temporal effects. Finally, to
investigate whether an effect of sex was present, all analyses were repeated with inclusion of sex as
a fixed factor including incorporation of sex in the interactions (e.g. time × treat × sex). Significant
main effects for any interaction was followed by a Sidak-adjusted pairwise comparison.

Due to the unpaired group design, the relative differences of hematological variables between groups
were calculated by the delta method, i.e. log transforming data followed by calculation of the mean
within each group and then transforming the means back to a linear scale. The ratio between back
transformed means provided the relative difference between groups. The 95% confidence intervals
were calculated as the difference between means on the log scale ± 1.96 × the standard error of the
difference followed by back transformation to a linear scale. Results are expressed as mean [lower
limit; upper limit] with lower and upper limit being the 95% confidence interval. The level of
significance was set at P<0.05.
RESULTS

Additive effect of rhEpo and hypoxia compared to hypoxia only (Fig. 2)

During the 12-week altitude intervention a time × treatment interaction existed for [Hb] (P<0.05), reticulocyte percentage (P<0.001) and OFF-hr score (P<0.001). The pairwise analysis revealed no specific time points at which [Hb] was different between the rhEpo and placebo group. In contrast, the reticulocyte percentage was 46% [27;67], 39% [19;63] and 26% [8;46] higher (P < 0.001) in the rhEpo group in the second, third and fourth week of altitude exposure, respectively. Lastly, the OFF-hr score was reduced (P<0.05) in the rhEpo group (-5% [-21;14]) in the second week of altitude exposure, compared with placebo.

Additive effect of rhEpo and hypoxia compared to rhEpo only (Fig. 2)

When the rhEpo groups in the altitude and sea level intervention were compared, a time × intervention interaction existed for [Hb] (P<0.001), reticulocyte percentage (P<0.001) and OFF-hr score (P<0.01).

Specifically, the [Hb] was reduced (P<0.01) in the altitude group (-3.2% [-9.0;3.0]) at the second baseline but 3.7% [-3.4;11.3], 5.5% [-1.0;12.5] and 5.6% [0.1;11.5] higher at the first (P<0.01), third (P<0.001) and fourth (P<0.001) week of altitude exposure, respectively, compared with sea level. Likewise, [Hb] of the altitude group was 5.6% [0.1;11.5], 2.7% [-1.9;7.5] and 5.3% [0.6;10.3] higher in the first (P<0.05) and third (P<0.001) week of follow up, respectively. The reticulocyte percentage was 32% [14;53], 31% [13;51] and 36% [12;65] higher (P<0.001) in the altitude group at the second, third and fourth week of altitude exposure, respectively, compared to sea level. Finally, the OFF-hr score was reduced (P<0.05) in the altitude group (-12% [-29;8]) in the second exposure week and 8% [-2;18], 9% [-2;21] and 8% [-5;23] higher in the second (P<0.05), third (P<0.05) and fourth (P<0.05) week of follow up, respectively, compared with sea level.

Erythropoietic effect of rhEpo treatment at sea level (Fig. 2)
During the 12-week sea level intervention a time × treat interaction existed for [Hb] (P<0.05), reticulocyte percentage (P<0.001) and OFF-hr score (P<0.001).

Following pairwise comparison, there were no specific time points at which [Hb] was different between the rhEpo and placebo group. However, the reticulocyte percentage was 29% [0;65], 69% [38;106], 46% [26;70] and 39% [10;74] higher in the rhEpo group at the first (P<0.05), second (P<0.001), third (P<0.001) and fourth (P<0.01) week of exposure, respectively, compared to placebo. In contrast, the reticulocyte percentage was reduced (P<0.05) in the rhEpo group (-23% [-38;-6]) at the second week of follow up compared with placebo. Lastly, the OFF-hr score was lower (P<0.01) at the second week of exposure in the rhEpo group (-20% [-30;-7]), but 18% [3;35] higher (P<0.05) in the first follow up week, compared with placebo.

The individual response for [Hb], OFF-hr score and reticulocyte percentage during both interventions are available in the supplemental figure 1 and 2.

**Athlete Biological Passport**

A main effect of biomarker existed (P < 0.001) for the sensitivity of the ABP. Specifically, [Hb] (P < 0.05), reticulocyte percentage (P < 0.05) and ABPS (P < 0.01) were more sensitive in the altitude intervention compared with sea level, whereas the OFF-hr score provided a similar sensitivity. Additionally, when [Hb] and OFF-hr score was combined, i.e. how many participants exceeded the thresholds for either biomarker, a higher (P < 0.01) sensitivity was evident in the altitude intervention compared with sea level. Finally, combining [Hb], OFF-hr and reticulocyte percentage also provided a higher (P < 0.001) sensitivity in the altitude intervention. However, no main effect of time × biomarker existed.

Across any time point, the passport identified 3 (12%), 11 (44%), 14 (56%) and 6 (24%) of the 25 participants treated with rhEpo at sea level with an outlier for [Hb], OFF-hr score, reticulocyte
percentage and ABPS, respectively. At altitude, the passport identified 11 (92%), 10 (83%), 12
(100%) and 9 (75%) of the 12 participants treated with rhEpo with an outlier for [Hb], OFF-hr score,
reticulocyte percentage and ABPS, respectively. For [Hb] and OFF-hr score combined, the passport
identified 14 unique participants (56%) at sea level and 12 unique participants (100%) at altitude
across any time point.

Investigating the specific time points revealed that during the sea level intervention, the highest
sensitivity at a single time point was 4% (1/25 participants), 24% (6/25 participants), 40% (10/25
participants) and 12% (3/25 participants) for [Hb], OFF-hr score, reticulocyte percentage and ABPS,
respectively, while all markers had a nadir of 0%. When combining [Hb] and OFF-hr score, the
sensitivity peaked at 28% (7/25 participants) for a single time point, which was 44% (11/25
participants) when the reticulocyte percentage was added as well.

During the altitude intervention, the highest sensitivity at a single time point was 58% (7/12
participants), 50% (6/12 participants), 75% (9/12 participants) and 42% (5/12 participants) for [Hb],
OFF-hr score, reticulocyte percentage and ABPS, respectively. All markers had a nadir of 0%, expect
ABPS which detected at least 8% (1/12 participants) at any time point. Combining [Hb] and OFF-hr
score elicited a peak sensitivity of 58% (7/12 participants) and adding the reticulocyte percentage
provided a peak sensitivity of 83% (10/12 participants). The time at which each biomarker obtained
the highest sensitivity alone or combined is illustrated in figure 3.

Table 1 reports the number of false-positive outliers, number of participants with false-positive
outliers and the calculated specificity of the ABP. There was no effect of sex for the changes in [Hb],
reticulocyte percentage, OFF-hr score or for sensitivity of the ABP.
DISCUSSION

The main finding of the present study was that four weeks of chronic hypoxic exposure at 2,320m simultaneously with three weeks of 20 IU·kg bw$^{-1}$ rhEpo injections provided an additive erythropoietic response compared to chronic hypoxia or rhEpo injections at sea level alone. Additionally, the fraction of true positive outliers by the ABP was higher in participants treated with rhEpo at altitude compared with rhEpo treatment at sea level, but the specificity was compromised with altitude exposure.

Additive erythropoietic effect of hypoxia and rhEpo

Altitude exposure$^{16}$ and rhEpo treatment$^{11,20}$ increase erythropoiesis independently. However, here we demonstrate that the two treatments provided an additive erythropoietic response when combined. The most pronounced effect was observed for reticulocyte percentage with ~30-45% higher levels in the combined treatment group during the final three weeks of the exposure period compared with only altitude exposure or rhEpo treatment. The additive erythropoietic effect of a combined treatment is further supported by studies demonstrating that injections of rhEpo 2-3 times per week in even higher doses (30-50 IU·kg bw$^{-1}$) than the present study only increase reticulocyte percentage ~0.6-0.8 percentage points,$^{11,20}$ which appear inferior to the reticulocyte percentage peak within the combined treatment group of ~1.1 percentage points compared with baseline. Additionally, chronic hypoxic exposure at 1800-2760 m only increase reticulocyte percentage ~0.2-0.4 percentage points$^{21-23}$.

Following altitude exposure$^{24}$ or rhEpo treatment,$^{11,12}$ the reticulocyte percentage is expected to decrease below baseline levels. Indeed, a lower reticulocyte percentage was observed for the rhEpo group at sea level at the second follow up week compared to placebo (Figure 2). A recent meta-analysis indicate that a higher amount of hypoxic exposure is associated with a larger decrease in
reticulocyte percentage upon descent from altitude, which is likely a consequence of an increased red cell volume expansion with increasing hypoxic exposure. However, despite the higher red cell production as indicated by the higher reticulocyte percentage in the combined treatment group, the reticulocyte percentage decreased to a similar level as in the altitude placebo or sea level rhEpo groups with nadirs of 0.7 ± 0.2% (combined), 0.8 ± 0.3% (altitude only) and 0.8 ± 0.2% (rhEpo only). The explanation for the similar nadirs remain unknown.

The [Hb] was also different between the combined treatment and placebo at altitude, but the analysis did not reveal a specific time point for the difference. However, applying the simplest possible comparison, i.e. an unpaired t-test, on changes from the mean of all baselines to the final week of exposure, as this is where [Hb] is expected to peak with altitude exposure or rhEpo treatment, indicated a higher (P<0.001) change in [Hb] by the combined treatment compared with altitude alone. The peak of [Hb] within the combined treatment group was approximately 1.6 g·dL⁻¹ higher than baseline, which is comparable to increases of ~1.3-1.4 g·dL⁻¹ when higher (30-50 IU·kg bw⁻¹) rhEpo doses are injected 2-3 times per week. In addition, the applied altitude dose is expected to increase [Hb] by only ~1 g·dL⁻¹, which is in accordance with the present study observing a 0.9 g·dL⁻¹ higher [Hb] during altitude compared with baseline in the altitude placebo group. These results further support that an additive response of the combined treatment exist.

Changes in OFF-hr score is dependent on changes in [Hb] and reticulocyte percentage. Accordingly, the OFF-hr score decreased at the second exposure week in the combined treatment group compared with altitude or rhEpo alone due to a higher reticulocyte percentage and similar [Hb]. However, despite a higher reticulocyte percentage of the combined treatment group in the remaining exposure period, no difference in OFF-hr existed for any subsequent time point when compared to altitude only. These results indicate that a meaningful difference existed for [Hb] between the combined and
altitude only group, as an increase in [Hb] will minimize the effect of an increased reticulocyte percentage on the OFF-hr score.

**Athlete Biological Passport**

The present study demonstrated that the ABP was more sensitive to rhEpo treatment at altitude than at sea level for all variables but the OFF-hr score. Albeit the analysis did not identify a specific time point for the differences in ABP biomarker sensitivity, a 54 and 35 percentage point numerically higher sensitivity was present for [Hb] and reticulocyte percentage (Figure 3), respectively, which is likely due to the confounding effect of hypoxic exposure on the hematological response.

The peak in sensitivity occurred at the expected time points. For instance, [Hb] is expected to peak in the final week of altitude exposure\(^{16,26}\) and the first week after rhEpo treatment\(^{11,27}\), which in the present study corresponds to a peak in sensitivity at 58% (7 out of 12 participants) at altitude. However, it is difficult to interpret the optimal timing at sea level as the sensitivity only reached 4% (1 out of 25 participants). In addition, the reticulocyte percentage is expected to peak 1-2 weeks into rhEpo treatment\(^{11,20}\) or altitude exposure\(^{21}\), at which the highest sensitivities of 67-75% (8-9 out of 12 participants) and 24-40% (6-10 out of 25 participants) were observed at altitude and sea level, respectively. Finally, the OFF-hr score is designed to be sensitive in periods with decelerated erythropoiesis\(^{30}\), as evident by the highest sensitivity of 50% (6 out of 12 participants) observed in the third week after altitude. However, in accordance with previous findings\(^{13}\), the OFF-hr score is also sensitive in early periods of accelerated erythropoiesis where the reticulocyte percentage is high but the effect has not translated into [Hb] changes yet, as indicated by the sensitivity of 50% (6 out of 12 participants) in the second exposure week at altitude.

Notably, the peak sensitivity was numerically 16 and 25 percentage points higher when adding the reticulocyte percentage to the [Hb] and OFF-hr score at sea-level and altitude, respectively. Although
the specificity was below 99% at altitude, all three variables maintained a specificity >99% at sea
level, which provide further evidence that implementing the reticulocyte percentage as a stand-alone
marker for detection may improve the passport.13

Despite the higher sensitivity in the altitude intervention, a concurrent reduced specificity existed
(Table 1), which is in line with previous studies.14,15,31 The reduced specificity appear to be related to
expected physiological adaptations to hypoxia,21,25,26 i.e. an initial increase in reticulocyte percentage
followed by an increased [Hb] in the final week of exposure, which is followed by a reduced
reticulocyte percentage causing the OFF-hr to increase (Table 2). The current tool applied to avoid
false-positive outcomes in anti-doping is the blood profile review by experts. The experts must
determine whether the fluctuations are due to blood manipulation, which highlight the importance of
knowing the natural fluctuations during hypoxic exposure with and without rhEpo treatment. The
hematological response was augmented in the combined treatment group, but the direction of the
changes appear similar to hypoxic exposure only. Thus, the experts will have to rely on the magnitude
of the changes. Additionally, there does not appear to be a direct relationship between the decreased
specificity and the increased sensitivity at altitude, as the sensitivity peaks numerically 2-3 weeks
into the altitude exposure (Figure 3D), whereas 3-5 false-positive results exist at all time points during
and following altitude exposure, expect for the first exposure week (Table 2).

In an attempt to account for the confounding factor of altitude exposure and distinguish between the
placebo and rhEpo group at altitude on an individual level with >99 % specificity, a pooled baseline
was calculated for each individual in the altitude intervention (i.e. mean of all baseline sample
collected for an individual). Differences from the pooled baseline to all samples collected during the
exposure and follow period was calculated for each individual, and the mean ± 2.58 standard
deviations (corresponding to >99% specificity) of the changes was calculated for all the placebo
subjects. In total, 2 (17%), 4 (34%) and 7 (58%) of the 12 rhEpo treated subjects exceeded the
calculated mean ± 2.58 standard deviations across all time points for [Hb], OFF-hr score and reticulocyte percentage, respectively. However, each variable was also exceeded by 2 (7%) of the 27 subjects in the placebo group. In order to have no placebo subjects exceeding the calculated threshold, a mean ± 3.3 standard deviations was needed. This threshold was exceeded by 1 (8%), 1 (8%), and 4 (33%) of the 12 rhEpo treated subjects across all time points for [Hb], OFF-hr score and reticulocyte percentage, respectively.

Importantly, it must be emphasized that the present protocol provide a high probability of direct detection of rhEpo misuse. Even smaller doses are detectable for 18-24 h in >50% of the participants, albeit this has not been investigated with concurrent hypoxic exposure.

Finally, it should be noted that the group sizes differed, with the smallest group of nine being the sea-level placebo treatment group. However, as significant interactions of time × treatment was evident for all the hematological analyses, the required power was reached. In addition, we did not control for the menstrual cycle which may affect reticulocyte percentage, but as we did not observe any effect of sex on the hematological changes it does not appear to constitute a limitation.

CONCLUSION

The present study demonstrates that rhEpo treatment combined with hypoxic exposure provide an additive erythropoietic response compared to rhEpo treatment or hypoxic exposure alone. In addition, the Athlete Biological Passport is more sensitive to rhEpo treatment at altitude than at sea level, but a concurrent compromised specificity exist with altitude exposure.

ACKNOWLEDGEMENTS

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excellent assistance during the studies carried out at altitude. The study was funded by the World Anti-Doping Agency.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.
REFERENCES


Table 1. Obtained specificity for hemoglobin concentration ([Hb]), OFF-hr score, reticulocyte percentage and Abnormal Blood Profile Score (ABPS) as well as [Hb] and OFF-hr score combined and [Hb], OFF-hr score and reticulocyte percentage combined (i.e. how many false-positive existed for at least one biomarker threshold). Specificity was calculated as the number of outliers in “no altitude exposure” samples, which include samples collected from all participants in the two baseline periods and from the placebo treated participants during the sea level exposure and follow up period. Specificity was also calculated for "altitude exposure" samples, which include samples collected from participants treated with placebo during altitude exposure and the follow up period.

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<td>ABPS</td>
<td>4</td>
<td>3</td>
<td>98.1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>[Hb]+OFF-hr</td>
<td>3</td>
<td>1</td>
<td>98.6</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>[Hb]+OFF-hr+reticulocyte percentage</td>
<td>3</td>
<td>1</td>
<td>98.6</td>
<td>14</td>
</tr>
<tr>
<td>Women</td>
<td>[Hb]</td>
<td>1</td>
<td>2</td>
<td>99.3</td>
<td>8</td>
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<tr>
<td>n = 16</td>
<td>OFF-hr</td>
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<td>0</td>
<td>100</td>
<td>13</td>
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<td>Reticulocyte percentage</td>
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<td>2</td>
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<td>9</td>
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<tr>
<td>altitude exposure = 87 samples</td>
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</tr>
<tr>
<td></td>
<td>[Hb]+OFF-hr</td>
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<td>2</td>
<td>99.3</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>[Hb]+OFF-hr+reticulocyte percentage</td>
<td>3</td>
<td>4</td>
<td>97.9</td>
<td>20</td>
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</table>
Table 2. Description of the time points and the direction for crossing of the calculated threshold in the Athlete Biological Passport for [Hb], reticulocyte percentage and OFF-hr score in samples collected from participants treated with placebo during altitude exposure and the follow up period.

<table>
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<tr>
<th>week</th>
<th>High [Hb]</th>
<th>Low [Hb]</th>
<th>High reticulocyte percentage</th>
<th>Low reticulocyte percentage</th>
<th>High OFF-hr</th>
<th>Low OFF-hr</th>
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</table>
FIGURE LEGENDS

Figure 1. Illustration of the study design. Arrows indicate the weekly venous blood collection and the hatched area indicate the treatment period with intravenous injections of 20 IU·kg bw\(^{-1}\) epoetin alpha or placebo every second day for three weeks.
Figure 2. Changes in hemoglobin concentration ([Hb]), reticulocyte percentage and OFF-hr score before, during and after treatment with recombinant human erythropoietin (rhEpo) or placebo in the sea level (SL) and altitude (AL) intervention. The hatched bar indicate the rhEpo or placebo treatment period. Values are means and errors bare indicate one standard deviation. Symbols at the dotted line indicate the statistical main effects. Statistical significant differences: *, **, *** P < 0.05, 0.01 and 0.001, respectively, for comparison between AL rhEpo and AL placebo. #, ##, ### P < 0.05, 0.01 and 0.001, respectively, for comparison between AL rhEpo and SL rhEpo. §, §§, §§§ P < 0.05, 0.01, 0.001, respectively, for comparison between SL rhEpo and SL placebo.
Figure 3. Obtained number of outliers by the Athlete Biological Passport for hemoglobin concentration ([Hb]), OFF-hr score, reticulocyte percentage and Abnormal Blood Profile Score (ABPS) in the sea level (panel A) and altitude (panel C) intervention for participants treated with recombinant human erythropoietin. In addition, the number of outliers for [Hb] and OFF-hr score combined as well as [Hb], OFF-hr score and reticulocyte percentage combined, i.e. how many participants exceeded at least one biomarker threshold, is presented for the sea level (panel B) and altitude (panel D) intervention. The dotted line indicates the number of participants included and the hatched bar indicates the treatment period. Statistical significant differences: #, ##, ### P < 0.05, 0.01 and 0.001, respectively, for comparison between altitude and sea level.
SUPPLEMENTAL MATERIAL

Supplemental figure 1. Individual changes in hemoglobin concentration ([Hb]), reticulocyte percentage and OFF-hr score before, during and after treatment with recombinant human erythropoietin (rhEpo) or placebo in the sea level intervention.

**SEA LEVEL**
Supplemental figure 2. Individual changes in hemoglobin concentration ([Hb]), reticulocyte percentage and OFF-hr score before, during and after treatment with recombinant human erythropoietin (rhEpo) or placebo in the altitude intervention.