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Reticulocytes in athletes: Longitudinal aspects and the influence of long- and short-term exercise

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Reticulocytes (Ret) are a key variable in the emerging concept of the athlete's biological passport and the longitudinal monitoring of biological parameters in the field of anti-doping. In this context, knowledge on the variability of Ret in athletes and the influence of exercise is necessary. The aim of the present study was to evaluate longitudinal variation in Ret and the influence of short- and long-term exercise.

Ret% in 793 samples of 238 athletes were determined and analyzed in different study parts for inter- and intra-individual variation and the impact of long- (competitive season) and short-term (all out) exercise.

Median Ret% was 0.9 (CI(0.5–99.5%) 0.4–2.7). Intra-individual variation for Ret% was 0.0118; inter-individual variation 0.0124. During periods of intensive exercise Ret% was slightly lower (mean −0.1%, p=0.048). After a short, all-out exercise bout, Ret% was increased (+0.5%, p=0.0028).

Athletes mostly display similar Ret% than the normal population; however, intra-individual variation in athletes is higher. During the competitive season of endurance athletes, Ret% is slightly decreased. After short bouts of intense exercise Ret% is increased. These data can be used for the interpretation of blood profiles in athletes.

Keywords: variability; blood; profile; athlete's biological passport; doping

Introduction

The longitudinal monitoring of biological variables is of increasing importance in the field of anti-doping. With the emerging concept of the athletes biological passport (ABP),[1] variations in certain biological markers are monitored and quantified. The results are used for disciplinary purposes by sporting federations, such as in a recent cases by the International Cycling Union, who filed anti-doping procedures against athletes based on variations in certain blood markers, such as reticulocyte percentage (Ret%). To evaluate such variations and to differentiate 'normal' from 'abnormal', appropriate reference collectives and knowledge on the natural variation in athletes of the variables used in the ABP are of utmost importance. For several, well-standardized markers such as haemoglobin concentration, extensive information on these topics exist, both for untrained individuals and for elite athletes.[2,3] In contrast, for Ret%, which is a key variable for the ABP in view of blood doping detection, the available data are limited. The currently available information on Ret% is based on cross sectional data of athlete reference collectives[4,5] or longitudinal investigations with smaller sample sizes.[6]

The aim of the present study was therefore to add data from a collective of endurance-trained athletes to strengthen the current body of literature in view of the natural intra- and inter-individual variation of Ret% in athletes. A further purpose was to get insight into the regulation of Ret levels of athletes with special emphasis on short- and long-term exercise-related changes to improve the interpretation of Ret data in the context of the ABP.

The results might be important for the evaluation of blood profiles of athletes using the ABP and might serve as further reference for scientific expertise in this area.

Subjects and Methods

The study was performed in four parts to investigate different aspects of Ret variability:

1. Cross-sectional analysis of Ret in athletes
2. Longitudinal variation of Ret in athletes
3. Seasonal or activity-related variation in Ret
4. Short-term exercise-associated variation of Ret in athletes

Ret data obtained on occasion of routine health checks from 238 Caucasian athletes (191 males, 47 females, age 23.2 ± 6.1 years (mean ± SD)) competing at national or international level in different endurance sports (main competitions during the summer months) were analyzed in retrospective. Only athletes without pathological findings in these health tests were considered. In total, 793 samples obtained over a period of 9 years from these subjects were included in this study. Eighty-two healthy sedentary Caucasian individuals (mainly medical students, 36 males, 46 females, age 24.2 ± 2 (mean ± SD), total amount of weekly physical activity less than 3 h for the last year) served as control group for part C of this investigation.

All subjects gave their informed written consent to participate in the study which was approved by the ethics committee of our university.

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1. *Cross-sectional analysis of Ret in athletes*

To analyze the distribution of Ret% in a population of endurance athletes, one random sample of each athlete was considered. In total, 238 samples were included in this part of the investigation. Two different technologies to measure Ret% were used (see 'Blood sampling and analysis').

2. *Longitudinal variation of Ret in athletes*

For the assessment of the longitudinal variation in Ret%, a minimum of 4 samples interspaced by at least 7 days between each sample were required per athlete. Fifty-seven athletes (46 males, 11 females) with 515 samples (4–26 per subject) fulfilled this criterion and were included in this part of the study.

The deviation from the individual mean for each sample was calculated for each athlete who had more than one sample; 680 measures from 125 athletes (2 to 26 samples per subject) were considered for this part of the investigation. In comparison to Part 1, inter-individual variations have been removed.

3. *Seasonal or activity-related variation in Ret*

A subset of Ret data obtained from 53 athletes (40 male, 13 female) and data from 82 subjects of the control group were investigated in view of differences related to a competitive season. One sample from each individual of the two groups was obtained at two different time points in a sports season. One sample was taken during the competition period (1 May until 20 September), where training and competition load is usually high; another sample was drawn during the resting period (1 October until 31 December), where training load is reduced in athletes. Both samples of each individual were analyzed using the same analyzer. Therefore, in addition to the lack of inter-individual variations, any potential inter-instrument bias on the studied issue (seasonal variation) has been excluded in this part.

4. *Short-term exercise-associated variation of Ret in athletes*

In 23 Caucasian endurance athletes (19 male, 4 female, aged 18–56 years) who were not part of the initial 238 athletes, blood was sampled immediately before and within 10 min of the termination of a standardized incremental exercise test until exhaustion on a treadmill or cycling ergometer (the tests were conducted as part of routine performance assessment, data not demonstrated). The test duration ranged between 30 and 45 min. In this part, the lack of any inter-individual, inter-instrument, and long-term variations of Ret% allows the study of a potential short-term effect of the confounding factor 'exercise'.

Blood sampling and analysis

Blood samples were obtained through venipuncture of an antecubital vein from the athlete in a sitting position. Blood was drawn into an EDTA-container (Sarstedt S-Monovette 2.7 ml, Sarstedt AG, Nuernbrecht, Germany). A tourniquet was used with tourniquet time never exceeding 30 s. After sampling, the blood was stored at 4 °C and processed according to standard laboratory procedures. The analysis took place within 3 h.

For Parts 1 and 2 of the study, the blood samples were analyzed on two different analyzers (Bayer H3, Bayer Diagnostics, Leverkusen, Germany) and Sysmex XE2100 (Sysmex Europe GmbH, Norderstedt, Germany), as the study period extended over several years and different analyzers were in use in our laboratory. It has to be pointed out that for a longitudinal follow-up of blood variables under the WADA guidelines,[7] all analysis should be conducted by the same type of analyzer. As no correction for analyzer difference was applied, our data of Parts 1 and 2 therefore includes an additional source of variation related to different measurement technologies.

All samples of Parts 3 and 4 of the study were analyzed on the same automated haematology analyzer (Sysmex XE2100, Sysmex Europe GmbH, Norderstedt, Germany) according to the current laboratory standards. For all parts of the study, each sample was measured once.

The instruments used in this study were submitted to regular internal and independent external quality controls; our laboratory is DIN EN ISO 9001-2000 certified. The analytical coefficient of variation (CV_a)(%) during the period of the study for Ret ranged between 4.2 and 5.2% (based on different levels of quality control solutions). For this investigation, only reticulocyte percentage (Ret%) is reported.

Since the goal of this study is a better understanding of the Ret% variations in athletes in various conditions rather than analytical issues of anti-doping, we have not replicated the stricter protocols used in the network of anti-doping laboratories for the ABP.[7]

Statistical analysis

Non-parametric descriptive statistics (median, quantiles) were used to present the data (in order to account for the non-normal distribution of Ret%) unless otherwise specified.

For longitudinal data assessment (Part 1), the most commonly used measures of dispersion in anti-doping studies were calculated: intraindividual (CV_{intra} (%), considering all samples of an athlete) and interindividual (CV_{inter} (%), considering one random sample per athlete) coefficient of variation, individual range and the within- and between-subject variance. The latter were estimated after square root transformation using a modelling approach with the athlete as a fixed variable. In order to determine the significance of the difference between two measurements, the critical difference at the 95% level of probability as suggested by Fraser[8] was calculated as follows: $CD(95\%) = 1.96 \times (\text{analytical variation } (CV_a)(\%)^2 + \text{within subject variation}(CV_{intra})(\%)^2)^{1/2}$.

Matched pairs analysis was used to investigate seasonal- (competitive season *vs* off-season; Part 3) and exercise-related differences (pre-post exercise; Part 4)). Bland-Altman plots were used to illustrate the data.[9] p values smaller than 0.05 were considered to indicate a statistical significant difference.

Results

The results are displayed in Table 1 and Figures 1–3. No significant gender differences were found in our data sets (results not shown), therefore, no further gender differentiation in the analysis and the results was made.

Cross-sectional analysis of Ret% in athletes/Longitudinal variation of Ret% in athletes

Table 1 and Figure 1 illustrate the distribution and the derived measures of dispersion for Ret% in endurance athletes.

Seasonal or activity-related variation in Ret%

Athletes showed lower Ret% during the competitive season ($p=0.048$). The average decrease was 0.1% (95%Confidence

Table 1. Cross-sectional and longitudinal analysis of Ret% in endurance athletes. Where applicable, data are represented as median (min-max)

Study part	Distribution of reticulocytes (%)	n	
	100% Quantile (max)		2.8
	99.5% Quantile		2.7
	75% Quantile		1.1
A	50% Quantile (Median)	238	0.9
	25% Quantile		0.7
	0.5% Quantile		0.4
	0% Quantile (min)		0.4
	Measures of dispersion		
	CV_{inter} (%)		28.0
	CV_{intra} (%)		21.1 (5.4–38.8)
	Individual range (%)		0.5 (0.1–1.4)
B		57	
	Within subject variance (sqrt)		0.0118
	Between subject variance (sqrt)		0.0124
	Critical difference (%)		42.6

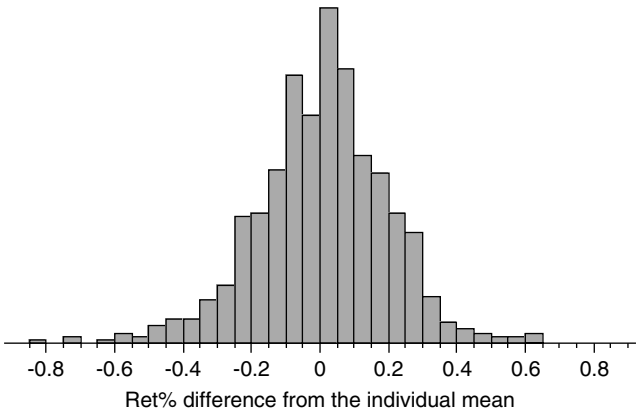

Figure 1. Distribution of the difference from the individual mean (normalized at '0') for 680 Ret% samples of 125 athletes (2–26 samples per athlete).

interval [0.04–0.2]). This effect was not visible in untrained control subjects (Figure 2). Untrained controls subjects showed lower Ret% values than athletes.

Short-term exercise-associated variation of Ret% in athletes

Ret% increased significantly after short term exhaustive exercise (p=0.0028) (Figure 3). The average increase was found to be 0.05% (95% Confidence interval [0.02–0.09]).

Discussion

Cross-sectional analysis of Ret in athletes

The results for Ret% of our cross-sectional analysis (Table 1) confirm previous findings made in the normal population and athletes with the average Ret% ranging around 1%.[10,11] The minor difference to our results (0.9%) might be due to the fact that we investigated mainly endurance athletes. Other authors have reported lower Ret% in this group compared to non-endurance athletes.[12] The reason for this phenomenon is still unclear; a potential mechanism is discussed below. Furthermore, we used the median as the statistical measure to account for the non-normal distribution of Ret%, which might result in slightly lower readings compared to the 'mean'. Another point is the fact that different types of analyzers were used in comparable studies which might have impacted Ret%.[13,14] In Parts 1 and 2 of our investigation, not all samples were analyzed on the same analyzer although WADA aims at harmonizing the analytical approach and minimizing this issue by using the same type of analyzer and a strict quality control system for all participating laboratories.[7] However, from a statistical perspective, the between-subject variance for Ret% (square root transformed) (Table 1) determined in our data is similar to the one found by Sottas *et al*.[15] which is used in the current model of the ABP, despite the use of two different analyzers in our investigation. Considering these points, we are therefore confident that our data are valid and might therefore serve as reference for the evaluation of Ret% in endurance athletes.

When comparing our cross-sectional dispersion data (CV_{inter}) with reference measures of dispersion for the normal population used in quality control for Ret assessment, it is of note that reference values in this context are not readily available for Ret%, but only for Ret count (Ret count). Ret% is usually calculated from Ret count and Red blood cell count (RBC) (Ret%=Ret count (10^3/microliter) $\times$100/RBC (10^3/microliter)). For obvious reasons, the data for Ret% and Ret count cannot fully be compared because Ret% variability depends in part on RBC variation which is usually relatively low (~3%).[2,3] It is therefore of note that the CV_{inter} of Ret% in our study (28%) is comparable to the CV_{inter} reported for Ret count in the reference literature (between 28% and 33%[3,16]).

Longitudinal variation of Ret in athletes

Figure 1 provides an impression of the distribution of values around an individual mean. Longitudinal data for Ret% in athletes are only available from very few studies. Different measures of dispersion to describe the variation are used in these investigations, which allow only limited comparison. The within-subject variance determined using the same approach as our research (square-root transformation, analysis of variance)[17,15] yielded similar results, despite the fact that two different analyzers were used for this part of our research. In our study, the within-subject variance was found to be 0.0118 (Table 1), in the investigation of Ashenden *et al*. 0.0103, and in the data published by Sottas 0.0121, if we assume independence between the Ret% and haemoglobin.

It is to be debated which measure of dispersion is the most appropriate for a longitudinal follow-up, as many authors and quality control publications use simple coefficients of variation (CV_{intra}) to describe the within-subject disparity. However, CV(%) has the limitation of a certain dependence on scaling, as variation as a percentage will not be the same at every level of the measure (i.e. 10% variation is not the same at 1.0 Ret% and at 2.0 Ret%). Considering the CV_{intra} as a simple measure, our data (CV_{intra}: 21.1%) is slightly higher than measures from a comparable study with athletes (16.4%,[18]). To the best of our knowledge, no other CV_{intra} values have been published for Ret% in athletes.

The critical difference (the change in a result making it significantly different from a previous result) of 42% for CD(95%) found in our data is slightly larger than the one of 36% reported by Banfi,[11] mainly due to the fact that the CV_{intra} used by Banfi in the

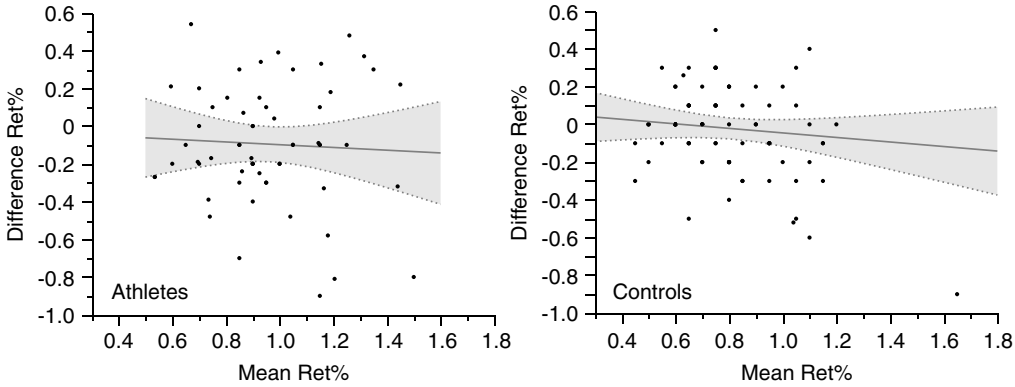

Figure 2. Bland Altman plot[9] of the difference in Ret% during the competitive season and a period of reduced training in 53 endurance athletes (left panel). Eighty-two untrained control subjects (right panel) were examined at the same time points for comparison. The full line represents the fitted change in the mean from the off-season to the competitive season, the dotted lines delimit the 95% confidence interval.

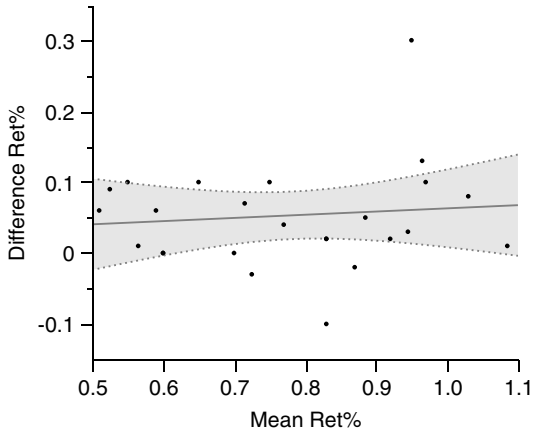

Figure 3. Bland Altman plot[9] of the differences in Ret% before and immediately after an exercise test until volitional exhaustion in 23 endurance-trained athletes. The full line represents the fitted change in the mean from before to after exercise; the dotted lines delimit the 95% confidence interval.

calculation was very low (5.8%). For the normal population, a value of 35% was reported,[16] calculation of the CD from published data of Ret count from other authors results in readings of 24% for CD(95%).[3]

Compared to Ret count variation data from the untrained population reported in reference publications from the area of laboratory medicine (11%[3,16]), CV_{intra} in athletes seems to be slightly higher. The reason for this fact is not clear and might be found in the distinct regulation of the haematologic system of endurance athletes which is discussed below. Analytical issues (not the same type of analyzer was used for all samples) have certainly to be considered in the interpretation of our results.

Seasonal or activity-related variation in Ret

The fact that athletes display significantly lower Ret% during the competitive season observed in our study (Figure 2) has been reported before.[12,18] Our data confirm this finding for a large group of endurance athletes. In the control group of untrained individuals, this feature was not present (Figure 2B). The reason for this observation is still under debate. It seems unlikely that reduced erythropoiesis is the reason for this phenomenon, as it is well known that athletes have higher blood volumes than untrained individuals.[19] Purely pre-analytical causes such as exercise-induced plasma volume shifts are not likely, either, as Ret% is calculated from Ret count and RBC, which are both affected to the same extent by plasma volume shifts. The decrease in Ret% during the competitive season is nevertheless limited, with an average difference of 0.1%. It has to be noted that the control group presented significantly lower Ret% readings throughout the study period compared to the group of athletes: this can be attributed to the different distribution of use of the different analyzers between groups (in the control group, mainly the Sysmex XE 2100 was used). This fact however does not influence the investigated pattern, as both samples of each athlete were always analyzed with the same analyzer. Furthermore, a purely analytical issue is improbable as the phenomenon has been observed in various other studies using different types of analyzers. A possible physiological explanation is discussed below.

In the interpretation of the results for Parts 1–3 of this study, it has to be pointed out that Ret% were analyzed independent of Hb. In fact, variations in Ret% could, in theory, be the result of changes in Hb. However, all athletes included in our study were found healthy in the routine examinations for this study and did not report any blood loss or blood donation in timely vicinity of the study period. Our collective is therefore very similar to what will likely be encountered in athletes in the field of doping control.

Short-term exercise-associated variation of Ret in athletes

Ret% increased significantly after short-term exhaustive exercise (Figure 3). Although small in magnitude (average 0.05%) when compared to the overall variability in Ret% and therefore probably irrelevant from a practical point of view, this phenomenon is visible in nearly all subjects. Other authors have described this observation using different exercise protocols.[20] For the increase in Ret%, exercise intensity seems to be a key factor, as no increase was observed in investigations that monitored Ret% over a training day in endurance athletes mainly training at moderate intensities,[21] during a cycling stage race[22] or after other types of endurance exercise of moderate intensity. As for the low Ret% observed in endurance athletes, it seems unlikely that plasma volume shifts (in this case: haemoconcentration) are causing the phenomenon, as such shifts would affect both Ret count and RBC (and therefore result in unchanged Ret%). Furthermore, Morici *et al*[23] have shown that the increase in Ret% after exercise is

mainly due to an increase of immature, young Ret from the bone marrow, which confirms the observation as a true phenomenon.

Physiological regulation of Ret% in endurance athletes – a speculation on possible mechanisms

In our data and other investigations, two phenomena in Ret% have repeatedly been described. The significantly and durably lower Ret% in endurance-trained athletes during periods of intense training and competition[24,12,6] and the short-term increase in Ret% after short, strenuous exercise bouts.[11,20] Together with the well-known fact that athletes have an increased red cell mass and higher blood volumes compared to untrained individuals,[19] this constellation presents as an obvious paradox: it would be more understandable that durably decreased Ret% would lead to decreased red cell mass. A possible explanation for these observations might therefore be that after exercise-related mobilization of young red cells from the bone marrow through increased blood flow,[25] exercise accelerates the maturation process of Ret. In fact, Ret usually develop into mature red cells within 1–3 days. There is evidence from different studies, that strenuous exercise mobilizes haematopoietic growth factors[23,23] and growth hormone[26,27,28] that might influence the maturation process of the young red cells. By these means, the larger number of red cells that are mobilised from the bone marrow after acute exercise would maturate to red cells faster, thus leading to a decreased Ret% in the peripheral blood and an increased red cell mass. This is supported by data from Banfi[12] and Morici[23] who showed that during periods of intense training, mainly the immature reticulocyte fraction (IRF) are increased. Interestingly, this process seems to occur independent of erythropoietin (EPO), the major erythropoietic growth factor, as in several studies, no clear exercise-associated EPO increase was found and no correlation in the EPO level and the post exercise Ret% increase could be established.[23,20] It highlights the fact that EPO might not be the sole regulator of red cell mass. The exercise-associated mobilization of growth factors influencing cell differentiation might also be the reason for another of our observations, i.e. the slightly larger intra-individual variability in Ret% of athletes compared to the normal population. It is conceivable that in athletes, red cell production and maturation is influenced by the described growth factor mobilization at different degrees depending on the intensity and volume of training, thus leading to a larger variation in Ret% compared to the normal population. Unfortunately, there are no studies on the maturation time of reticulocytes in athletes that could support our theory. Further work is necessary to elucidate this problem.

Considerations for anti-doping

Ret% plays an important role in the indirect detection of blood doping and is one of the key variables included in the ABP. Ret% reacts sensibly to blood manipulations such as EPO administration or blood transfusion. In addition, the main advantage of Ret% compared to other measures used in this context, such as haemoglobin concentration or haematocrit is the fact that Ret% is independent of exercise associated plasma volume shifts.[29] Plasma volume shifts are major confounders in the evaluation of doping-related variations in all concentration-based measures and might blur doping-related changes. In this context, endurance athletes are known to present distinct features and adaptations in their haematological system compared to other athletes and the normal population. Our data, however, demonstrates that most characteristics of Ret% described for the normal population do apply for endurance athletes as well. Several minor influences, such as a slightly larger intra-individual variation, a mild post exercise Ret% increase and a lower Ret% during competitive periods have to be considered when evaluating Ret% in the ABP. It has to be pointed out that the post-exercise increase is covered by blood sampling guidelines (no blood test within 2 h after exercise) and the algorithms included in the ABP already take into account athlete derived within subject variances, which cover the above-mentioned features in large parts.

Nevertheless, analytical and pre-analytical differences to the current ABP guidelines need to be highlighted in the interpretation of our results: In the longitudinal part of the current study, two different analyzers were used, thus introducing a 'between analyzer' variability, which was not accounted for in the calculations and might introduce an additional bias.[13] Furthermore, the strict pre-analytical standards for ABP blood sampling were not respected in full extent in the present investigation.[7] Therefore, the variability presented here takes into account multiple sources of variations (inter-individual, inter-instrument, inter-day, etc.) and certainly represents a 'worst case' scenario in the context of anti-doping. Variations of Ret% observed in the field that are larger than the data presented in this study are therefore very likely abnormal.

Perspective

In this study, we demonstrated that Ret% in athletes mainly presents with values similar to the normal population. Intra-individual variations in Ret% in athletes are slightly higher; inter-individual variations are similar to values reported for the normal population. During periods of intense exercise (competitive season of endurance athletes), Ret% is slightly decreased. After short bouts of intense exercise, however, Ret% was found to be increased. It is speculated that an exercise-induced release of growth factors accelerating the maturation process of Ret in endurance athletes might be the cause for this observation. The presented information might be used in the interpretation of blood data from the ABP in the fight against doping.

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