CHAPTER FIVE

Reticulocytes in Sports Medicine: An Update

Giovanni Lombardi*,[1], Alessandra Colombini*, Patrizia Lanteri*, Giuseppe Banfi*,†

*I.R.C.C.S. Istituto Ortopedico Galeazzi, Milano, Italy
†School of Medicine, University of Milano, Milano, Italy
[1]Corresponding author: e-mail address: giovanni.lombardi@grupposandonato.it

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Abstract

Reticulocytes are young red blood cells which develop from erythroblasts and circulate in the bloodstream for about 1–4 days before maturing into erythrocytes. With the introduction of reticulocyte count in equations and statistical models for detecting suspected blood doping, its application to sports medicine has attracted growing interest in reticulocyte behavior during training and competition seasons in athletes and experimental blood doping treatment in healthy volunteers. An update on recent publications is therefore needed to improve the interpretation of reticulocyte analysis and its variability in sportsmen.

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Reticulocyte count constitutes a robust parameter during the preanalytical phase, but cell stability can be assured only if blood samples are kept at constantly cold temperatures (4 °C) and test results will differ depending on the blood analyzer system used. Marked intraindividual variability is the principal finding to be evaluated when exercise-induced changes are observed or illicit procedures suspected. Furthermore, reticulocyte variability is greater than that of other hematological parameters such as hemoglobin or hematocrit.

Ideally, any variation should be interpreted against long-term time series for the individual athlete: values obtained from large athlete cohorts ought to be used only for extrapolating outliers that deserve further examination. Reticulocyte distribution in athletes is similar to that found in the general population, and a gender effect in some sports disciplines or selected athlete groups may be seen. Reticulocyte variability is strongly influenced by seasonal factors linked to training and competition schedules and by the type of sports discipline. Published experimental data have confirmed the high sensitivity of reticulocyte analysis in identifying abnormal bone marrow stimulation by either erythropoietin administration or blood withdrawal and reinfusion.

1. INTRODUCTION

Reticulocytes (Ret), immature precursors of erythrocytes, contain nucleic acid (RNA) residues, are larger in volume than mature red blood cells (RBCs) but have a lower hemoglobin (Hb) concentration. Produced by the red bone marrow from mature erythroblasts that undergo enucleation, they still retain RNA residues. After a brief period in the bone marrow, they are released into the bloodstream where they remain for up to 4 days until they mature into erythrocytes. Their lifetime is much shorter than that of mature RBCs, which have a mean lifetime of 120 days. While circulating in the bloodstream, Ret undergo several rapid changes as they mature: membrane remodeling, volume change, and extrusion of organelles [1].

The reference method for estimating Ret count is the manual count through a microscope (100 ×) of a blood smear after supravital dying (1:1 with EDTA-anticoagulated whole blood) with new methylene blue (basic blue 24, color index 52030). All enucleated RBCs with two blue-stained subparticles are counted as Ret and related to 1000 erythrocytes in the same field. This technique is highly operator dependent, however.

Automatic counting methods rely on staining the particles with classical dyes (new methylene blue, brilliant cresyl blue, or similar) or with fluorophores (acridine orange) which bind the RNA residues, making them recognizable and numerable. Automation combines simultaneous enumeration of stained particles and recording of particle size with the advantage of high precision,

since a high number of events are collected for each count and better throughput. The main drawback to automated counting is that there are no universally recognized calibrators that would allow alignment between systems utilizing different technologies. Currently, two standards have been approved: the standard of the International Committee for Standardization in Haematology involves manual microscopic reference performed by two pairs of clinical pathologists on two different films of the same sample and the U.S. National Committee for Clinical Laboratory Standard (H44-A, revised in 2004 as H44-A2) for evaluating automated analyzer systems [2].

Ret evaluation in sportsmen is important because of its usefulness in monitoring athletes' health, as, for example, in detecting sports anemia. Professional athletes are particularly prone to developing this condition in which Hb loss is accompanied by an increase in Ret count. Sport-induced anemia is associated with various causes, but erythrocyte destruction due to intravascular hemolysis is the major one [3,4] responsible for continuous stimulation of bone marrow which can be monitored by Ret count.

Sports anemia is evidenced by marked changes in blood cell count and iron-associated parameters during periods of intense training or, even more accentuated, at the start of the training season [5], and it is frequently encountered in endurance athletes. It is a transient condition, with only 8% of elite athletes presenting frank anemia (Hb concentration below reference limits: 135 g/L in males and 120 g/L in females). Training induces an increase in plasma volume which, through hormonal (e.g., Epo) and osmotic responses, stimulates erythropoiesis, inducing augmentation of the red cell mass [5], including RBC and immature erythroid forms (e.g., Ret) [6]. While the plasma volume may rise by over 20%, the increase in red cell mass will range between 10% and 18%, with a final relative decrease in hematocrit. The advantages of these changes are that plasma expansion decreases blood viscosity and improves blood flow in large vessels, while the greater deformability of newly formed erythrocytes increases capillary flow [5].

It is unlikely, however, that such exercise-induced hematological variations will be exclusively due to hemodilution [4,7]. For instance, microcytosis in endurance athletes is secondary to reactive reticulocytosis due to intravascular hemolysis. Hemolysis may result from the breakdown of erythrocytes in exercising muscles (swimmers, weightlifters, rowers) or from the impact with the ground (runners) [4,7].

Within this context, it is useful for sports physicians and clinical pathologists to understand the effect various conditions (type of sport and training, competition schedules, altitude) can have on Ret values.

Ret measurement in sports medicine attracted attention after Ret count began being applied for antidoping purposes [2]. Several authors expressed doubt about whether the medical community was being adequately informed on Ret behavior in athletes and provided with mounting scientific evidence that would make Ret measurement a reference point in the detection of blood doping. To this end, a review of the literature on Ret in sports medicine was published to give an overview of data culled from the few studies that investigated the behavior of immature RBC in athletes [2].

In a recent review, Jelkmann and Lundby reported data on blood doping and its detection. Specifically regarding the administration of erythropoietin-stimulating agents and related variation in RBC parameters, Jelkmann and Lundby had reported in their study the effect of rhEpo administration in healthy subjects on the Ret values to highlight the physiological response to hormone stimulation. In detail, bolus rhEpo injections (150 U or 300 U/kg body weight) increase the immature reticulocyte fraction (IRF) starting 36 h after a single dose of rhEpo, peak after 3–4 days, and normalize within 7 days. rhEpo transiently increases the lifespan of circulating Ret from a baseline value of 1.7–3.4 days and increases Ret values twofold by inducing increased Ret release from the bone marrow and prolonging the maturation time of circulating Ret. After frequent weekly injections for 14 days and a concomitant doubling in reticulocyte percentage (Ret%), Ret% returned to basal levels even with weekly rhEpo injections and continuously high [Hb], suggesting a decreased sensitivity to prolonged rhEpo treatment [8].

Recent studies on Ret in athletes have looked into the many different changes that may arise in connection with the preanalytical phase, confounding factors, the effect of short-term and long-term exercise, and the use of illicit substances and procedures. While adding new data, these studies also mentioned several confounding factors which need to be correctly interpreted to better understand Ret behavior in sportsmen. Therefore, an update of the previous review of the scientific literature has become necessary to improve the correct use of Ret data in sports medicine and in antidoping testing. More recently, Ret analysis has acquired a major role in the assessment of adaptation to altitude by athletes exposed to recurrent living and training conditions at different altitudes to enhance their performance.

As the number of studies is limited, especially as concerns certain aspects discussed here, some conclusions were drawn from the few data currently available in the literature.

With a view to provide an update on Ret behavior in athletes, and building on our previous review published in 2008 [2], we searched the PubMed

database (www.pubmed.gov), a service of the U.S. National Library of Medicine, by entering the term "reticulocytes" matched with the terms "athletes" and "sport" as keywords. Studies published since 2007 and involving healthy subjects, physically active subjects, and sportsmen were retrieved and evaluated, along with selected articles cited in the references of the papers included in the analysis.

2. PREANALYTICAL PHASE

2.1. Blood drawing

Accuracy and rigorous standardization of blood collection techniques are needed to avoid spurious changes in hematological parameters and of Ret in particular [9]. According to a study on 36 athletes, the use of different sampling techniques (cannula or syringe) had no significant impact on Ret% measurement when carried out over a day, during different days of a year with equal timing of sampling days to preclude the effect of climatic extremes, nor did the environmental temperature modify the Ret% values [10].

2.2. Diurnal variation

Ret count shows a physiological circadian rhythm with an acrophase occurring at 01:00 a.m. (95% confidence interval between 07:48 p.m. and 04:28 a.m.), reflecting the diurnal variations in serum Epo that peaks at 01:00 a.m. These variations in circulating Ret constitute 37% of the total variability encountered over a day. The intraindividual daily range is 130%, with the highest value expressed as a percent of the lowest [11,12].

The coefficient of variation (CV) of diurnal changes in erythrocytes, Hb, and Ht was analyzed in 96 healthy subjects grouped by gender and assayed in three different hospitals (two equipped with a Coulter STKS® and one with a Technicon H*3®, later Bayer H*3®). The values for erythrocytes, Hb, and Ht were very similar (4.8×10^{12}/L, 142 g/L, and 42%; and CV% at 3.5%, 3%, and 3.7%, respectively). In contrast, the Ret value was 20% higher on average in a population of 64.8×10^{9}/L. The explanation for the high variability in diurnal Ret compared to the low variability in erythrocyte values was the limited lifespan of immature particles [13].

The sleep/wake cycle is responsible for synchronizing the body's biological clock, and resetting it can take up to 42 days. After intercontinental flights across time zones, athletes involved in competitions worldwide often experience circadian rhythm disruption [11,12]. The variation in Ret%

values was studied in a group 36 athletes: endurance ($n=20$) and nonendurance ($n=16$) [10]. Baseline samples drawn at 08.00 a.m. had a mean Ret% of 0.99 (standard error 0.10), as measured on a Sysmex XE2100 system. Ret% was measured every hour from 08:00 a.m. to 07:00 p.m. Unlike Hb, which differed significantly over time, Ret% showed no changes remarkable enough to be considered in the preanalytical phase. These data confirm previous reports in athletes [2].

In 23 male professional cyclists participating in a 5-day road race, Ret% showed no specific fluctuations in blood samples drawn every morning prior to the start of the race and at the end of exercise [14]. Similarly, in 13 physically active males and 3 healthy physically active females (all nonprofessional athletes), Ret% was stable from 08:00 a.m. to 04:00 p.m. [15]. The circadian rhythm of Ret is therefore mainly due to modifications in their release during the night.

2.3. Biological variability

The biological variability of a laboratory parameter refers to the variability of cyclical biological rhythms and fluctuations beyond a homeostatic value [2]. The amplitude of the fluctuation, which is independent of the preanalytical phase, corresponds to intraindividual variability, expressed as a coefficient of variation (CV_i). The variability of a homeostatic value among a group of different individuals corresponds to interindividual variability (CV_g). The total variability (V_t) is the sum of analytical variability (V_a) and biological variability (V_b). In general, V_a is quite low in automatic hematology analyzers ($<3\%$); thus, V_t is highly dependent on V_b [2].

Previous studies, however, observed a higher V_a as measured by different analyzer systems. Ashenden reported three different values for V_a with regard to Ret: 9.1%, 7.6%, and 5.9% for low, medium, and high Ret%, respectively [16]. Other V_a values were 11.8% [17], 11.0%, and 9.1% [18] for Ret%; 9.0% [19] and 5.7% [20] for Ret count.

The classical analytical goals for laboratory parameters calculate $V_a = 1/2V_b$. Hematology parameters have low V_a but also low V_b, and analytical goals are certainly reached for such parameters as Hb, RBC, and derived parameters. Ret, however, has a higher V_b than either erythrocytes or Hb but one similar to leukocytes. Accordingly, analytical goals need to be carefully evaluated method by method [2].

Intraindividual variability was found to be greater than interindividual variability in both genders among top-level ski athletes [21]. In a large athlete

cohort [22], however, the interindividual CV% (28%) was calculated by taking one random sample per athlete and resulted higher than the intra-individual CV% (21%) (range, 5.4–38.8%), calculated considering all the samples of an athlete. The within- and between-subject variances were estimated after square root transformation using a modeling approach with the athlete as a fixed variable. The within-subject variance for Ret in 793 samples obtained from 238 athletes was 0.0118, the between-subject variance was 0.0124 [22].

The critical difference (CD) refers to the value indicating that a difference between two consecutive results in the same subject is statistically significant and is therefore unlikely attributable to casual oscillation of values. In general, a more than 95% difference in results, moving either up or down, that will probably indicate a true change in a subject is described as $CD^{95} = 2.77 \times (CV_a{}^2 + CV_i{}^2)^{1/2}$. The factor of 2.77 is equal to $\sqrt{2}$ times the z score for the difference. In other terms, CD identifies whether an external factor (training, therapy, or other) somehow altered the result of the parameter and whether the alteration depends or not on instrumental or biological variability.

CD was calculated as 42%, slightly higher than the 36% previously reported [2], owing to different intraindividual CV (21.1% in the former and 5.8% in the latter). The authors remarked that the intraindividual CV calculated for endurance athletes was higher than that described for the general population, possibly because of a specific setting of the hematological analyzer system and because of different hematological systems used in the study [22].

The intraindividual CV calculated on 500 samples from 43 subjects [10] was $8.37 \pm 1.64\%$ in endurance athletes, $8.08 \pm 2.27\%$ in nonendurance athletes, and $7.4 \pm 1.12\%$ in nonathletes. These values suggest an enhanced stimulation of bone marrow in the athletes, although the differences among the groups were not significant. By comparison, the coefficient of variation of Hb in the same individuals was $2.65 \pm 0.67\%$, $3.0 \pm 0.79\%$, and $2.36 \pm 0.42\%$, respectively [10].

High interindividual variability among elite athletes was reported in a large group of speed skaters [23], especially on precompetition screening when very high values for some athletes ($> 3.0\%$) were measured. In the total of 11,600 samples, Ret% $> 2.4\%$ was found in 271 (2.3%) samples collected from 116 skaters, 64 of which had only one single value $> 2.4\%$, whereas 52 had more than one value above threshold. In one of the two athletes with consistently elevated Ret%, the increased Ret count was due to Howell Jolly bodies in the

RBC mimicking Ret and due to a blood disease. In the remaining 50 athletes, two to three consecutive values >2.4% were recorded, arousing suspicion of abnormal bone marrow production [23].

In conclusion:

- the data confirm the robustness of the Ret parameter during the preanalytical phase
- Ret intraindividual variability is higher in athletes than in nonathletes
- Ret has a high interindividual variability also in homogeneous athlete populations
- Ret variations should be interpreted against long-term time series in the individual athlete; values obtained from large athlete cohorts should only be used for extrapolating outliers which deserve further examination

3. KINETICS OF RET PRODUCTION IN HUMANS

RBCs are produced in the bone marrow; their formation (i.e., erythropoiesis) involves a vast variety and number of cells at different stages of maturation, starting with the first stem cell progeny committed to erythroid differentiation and ending with the mature circulating RBC [24]. Under normal conditions, the rate of RBC production is such that the red cell mass in the body is regulated and constant. Erythropoiesis can be divided into various stages: commitment of pluripotent stem cell progeny into erythroid differentiation, erythropoietin-independent or early phase erythropoiesis, and erythropoietin-dependent or late phase erythropoiesis.

It takes approximately from 12 to 15 days for a cell at the burst-forming unit-erythroid (BFU-E) stage to mature into an erythroblast. Within 6–8 days, a BFU-E proliferates and differentiates into a colony-forming unit-erythroid, which needs another 5–7 days to proliferate and develop into basophilic erythroblasts [24].

The least mature recognizable erythrocyte precursor cell is known as the pronormoblast or proerythroblast. Cells characteristic of subsequent stages of maturation are termed normoblasts or erythroblasts. The various stages of maturation, in order of increasing maturity, are known as pronormoblasts, basophilic normoblasts, polychromatophilic normoblasts, and orthochromatic normoblasts [24].

Pronormoblasts are round cells (diameter, 25 μm) with a thick nucleus and locally condensed chromatin, clear nucleoli, and a strong basophilic cytoplasm. Basophilic normoblasts are smaller (diameter, 15 μm) and contain a nucleus with "wheel-shaped" condensed chromatin, no evidence of

nucleoli, and deep basophilic cytoplasm. In polychromatophilic normoblasts, the picnotic nucleus is condensed, dark, and near the cellular membrane; the cytoplasm is not totally basophilic, but there are pinkish areas depending on the start of Hb deposition. These features are present also in orthochromatic normoblasts, and the nucleus is extruded at the end of cellular maturation.

After the nucleus has been extruded, the cell is referred to as a Ret, an immature RBC larger in volume than erythrocytes by about 24%, with a lower Hb concentration (about 17%) and a similar Hb (pg) content [25]. Ret lifetime is much shorter than that of erythrocytes (1–4 vs. 120 days).

4. REPORTED RET VALUES IN ATHLETES

Recent studies have confirmed that Ret is not normally distributed (Table 5.1) [10,21,23]. The data were recorded in athletes before the competition season. The differences in Ret values could have been due to different training workloads. Of note is that nearly all studies reported Ret values with mean ± standard deviation (SD) even though, because of their well-known nonparametric distribution, they should be given as median and range values instead.

Ret values confirmed those obtained with different methods and previously reported for athletes from various sports disciplines [2]. It should be remarked that the results are similar for different groups of athletes (endurance and anaerobic athletes), when presented as median and percentiles (25th–75th) [10,21]. Conversely, different results were recorded in athletes practicing the same discipline (triathlon) probably due to different workloads [10,26]. Elite speed skaters showed values clearly higher than those measured in athletes practicing other disciplines: a specific cluster could be recognized, but the particular methodology could also be cited to explain this difference [23,31,32].

Ret values are not influenced by body mass index (BMI; weight in kg divided by the square of height in cm) of athletes. In a study on a heterogeneous group of male professional athletes ($n = 126$) practicing different sports and characterized by a wide range in BMI (22–28 kg/m^2), no correlation was found. BMI partially influenced Hb and erythrocyte values, reinforcing the robustness of Ret as a parameter for evaluating the hematological status in athletes [33].

A thorny problem is whether the relationship between Ret and Hb values is consistent. From an analysis of studies investigating seasonal variability of hematological parameters in athletes, it appears that variations in

Table 5.1 Ret values in athletes (either national or international level)

Sport discipline	No. of athletes, gender, and age (years, range, or mean ± SD)	Level of physical activity	Ret%	Instrument	Reference
Triathlon	7 M, 32.6 ± 2.9	Professional	1.06 ± 0.3[a]	Pentra 120 Horiba	[26]
Biathlon	83 M, 18–39	Professional	1.0 ± 0.3[a]	Sysmex R500	[27]
Endurance (cycling, triathlon)	20 M, 30.3 ± 7.1	Professional	0.76 ± 0.2[a]	Sysmex XE2100	[10]
Nonendurance (ball disciplines)	16 M, 25.5 ± 5	Professional	0.94 ± 0.4[a]		
Nonathletics	7 M, 25.6 ± 1.57	Sedentary	1.03 ± 0.3		
Endurance	238 (191 M, 47 F), 23.2 ± 6	Professional	0.9[b] 0.7–1.1[c] 0.4–2.7[d]	Bayer H3 and Sysmex XE2100	[22]
Alpine ski	18 (10 M, 26–33; 8 F, 24–28)	Professional	1.04[b] 0.77–1.28[c] 0.59–1.99[e]	Abbott Cell Dyn 3700 and Sapphire	[21]
Cycling	8 M, 19–26	Professional	1.3 ± 0.6[a,f]	Sysmex XE2100	[28]
Field hockey	17 F, 24.8 ± 3.0 17 M, 24.2 ± 2.9	Professional	1.17 ± 0.39[a] 1.06 ± 0.34[a]	Sysmex XT2000i	[29]
Aerobic disciplines (track and field, swimming, tennis, dance, biathlon, cross-country skiing, biathlon, cycling, triathlon)	165 M, 22.1 ± 4.95 70 F, 21.4 ± 3.88	N.D.	0.93 ± 0.26[a] 1.17 ± 0.81[a]	Siemens Advia 120	[30]
Anaerobic disciplines (ski	163 M, 22.9 ± 4.06	N.D.	0.93 ± 0.35[a] 1.11 ± 0.49[a]	Siemens Advia 120	[30]

Table 5.1 Ret values in athletes (either national or international level)—cont'd

Sport discipline	No. of athletes, gender, and age (years, range, or mean ± SD)	Level of physical activity	Ret%	Instrument	Reference
jumping, track and field, swimming, kayaking, alpine skiing)	120 F, 19.9 ± 4.51				
Mixed disciplines (track and field, swimming, handball, alpine skiing, volleyball, rowing, mountain bike)	186 M, 22.4 ± 4.57 169 F, 20.4 ± 4.50	N.D.	0.99 ± 0.31[a] 1.07 ± 0.38[a]	Siemens Advia 120	[30]
Speed skating	972 M, age not specified 680 F, age not specified	Professional	1.36 ± 0.43[a] 1.39 ± 0.47[a]	Siemens Advia 120	[23]

Advia is manufactured by Siemens (Tarrytown, NY, USA), Sysmex XE 2100 and Sysmex R500 by Sysmex (Kobe, Japan), LH 750 by Beckman Coulter (Hialeah, FL, USA), Pentra 120 by Horiba ABX (Montpellier, France).
[a]Mean ± standard deviation.
[b]Median.
[c]25th–75th percentile.
[d]0.5th–99.5th percentile.
[e]5th–95th percentile.
[f]Data extrapolated from a figure.

Ret and Hb are unrelated [34]. Moreover, preanalytical factors were found to influence Hb but not Ret [10], and the values of Ret% were identical in groups of biathletes in spite of significant differences in Hb concentration [27]. Therefore, these inconsistencies should be taken into account when interpreting Ret changes in athletes.

4.1. Gender effect

Gender could be a source of variation in Ret distribution. Higher Ret values were described for female Alpine ski athletes during four consecutive seasons [21]. The differences between genders were confirmed in all seasons; however, the trends of Ret changes within each season and between consecutive seasons ran parallel in both genders.

Measurement of Ret% stability was assessed over the course of four competitive seasons in 10 male and 7 female elite triathletes [6] who underwent strenuous and prolonged physical exercise. Differences in Ret% between genders and a high between-subject variability were observed. Within-gender analysis revealed that although the values remained stable in the males (no period or season-related effect), differences between periods within seasons were noted in the females.

In contrast, no gender-related influence on Ret distribution was observed in endurance athletes [22,23]. The observation of no significant differences between male and female speed skaters in Ret values repeatedly measured since 2000 is intriguing [23].

Specific studies on professional female athletes are needed, because the gender effect described in one study may have been due to a casual distribution of values within a relatively small group of individuals. In a large group ($n = 873$) of athletes practicing different sports disciplines [30], the Ret values were higher for the females, but no specific statistical analysis was applied. Also, Ret% was higher among females in aerobic, anaerobic, and mixed disciplines, but the differences among the three groups were not significant. Conversely, Ret parameters, as measured on a Siemens Advia system, showed significant differences between athletes from different sports disciplines: the mean reticulocyte volume (MCVr) and the hemoglobin content of reticulocytes (CHr) were higher for both male and female athletes engaging in aerobic disciplines than in those practicing anaerobic or mixed disciplines.

In conclusion:

- in general, Ret values in athletes are similar to those found in the general population; however, evaluation needs to take into account the period of the training season; a gender effect may be observed in some sports disciplines or selected groups of athletes. Gender-specific Ret fluctuations in response to physical activity need to be elucidated
- Ret values are influenced by seasonal variations linked to training and competitions and by the type of sports discipline
- comparability across studies is hampered by Ret variability in the phase of a season when blood samples are drawn and by different counting methods

5. STABILITY

Stability refers to the capability of a sample material to retain the initial property of a measured constituent for a period of time, within specified limits, when the sample is stored under defined conditions. Instability is

defined as an absolute difference, quotient, or percentage deviation from results obtained from measurement at time 0 and after a given period of time [35].

Blood parameter stability in modern cytometers, which can also automatically release Ret%, has been studied in healthy individuals, physically active subjects not athletes [19,36,37], and athletes (Table 5.2) [19,37,38].

According to one study, a parameter was considered stable when its average change was less than one CV (%) of the assessed method, allowing a 5% risk of error [36]. In two other studies [19,38], Student's *t* test was used to compare initial values with those measured at various time points. Another study used random variation of the intercepts between subjects and within subjects, and the curve parameters were tested to discover interactions with ambient temperature. The significance of the interaction of the fitted parameters corresponding with storage temperature gave models that were compared by the use of likelihood statistics to define parameter stability [37].

The results produced by an Advia system after 24 h at 4 °C did not agree with those described in a previous paper [39] where instability was found.

Ret was found less stable than Hb or RBC in all studies. Cell maturation into RBC could be a source of relative instability, as would the much smaller number of Ret than RBC and the difficulty with different methods (absorbance, fluorescence, impedance) to detect them. Depending on the method and instrument used, markedly different results are seen; for example, Ret results obtained with a Siemens Advia differed from those released by a Sysmex analyzer.

Analyzer accuracy relies on correct calibration and quality control and is essential to assure expected stability results. Protocol standardization, and statistical analysis in particular, for studying hematological parameter stability is key to reducing variability in data interpretation. If different statistical methods are applied to define parameter stability and acceptability, then complete comparability across studies will ultimately be limited. Specific studies on the stability of hematological parameters between 24 and 48 h are acknowledged for validating the 36-h limit and its possible extension to 48 h. Robinson *et al.* [38] recommended extending it to even 72 h on the basis of the good stability they found.

In conclusion:

- Ret is less stable than Hb
- Ret stability depends on the counting method applied
- storage at cold temperatures (ideally 4 °C) is essential to guarantee the stability of Ret values
- specific studies on Ret stability between 24 and 48 h are acknowledged by using a standardized protocol

Table 5.2 Ret stability with different analyzer systems

Instrument[a] and storage temperature	4 h[b]	6 h	8 h	10 h	24 h	30 h	48 h	72 h	Reference
Siemens Advia									
RT	Stable	–	–	Stable	Unstable	–	Unstable	Unstable	[36]
RT	–	Stable	–	–	Unstable	Unstable	Unstable	–	[37]
4 °C	Stable	–	–	Stable	Stable	–	Stable	Stable	[36]
4 °C	–	Stable	–	–	Stable	Stable	Stable	–	[37]
Sysmex									
RT	Stable	–	–	Stable	Stable	–	Stable	Unstable	[36]
RT	Stable	–	Stable	–	Unstable	–	Unstable	Unstable	[19]
4 °C	Stable	–	–	Stable	Stable	–	Stable	Unstable	[36]
4 °C	Stable	–	Stable	–	Stable	–	Stable	Stable	[19]
4 °C	–	–	–	–	Stable	–	Stable	Stable	[38]
Coulter LH 750									
RT	Stable	–	–	Stable	Stable	–	Stable	Unstable	[36]
4 °C	Stable	–	–	Stable	Stable	–	Stable	Stable	[36]

Ret% was usually given except in one study [36] which reported the absolute count.

[a]Advia is manufactured by Siemens (Tarrytown, NY, USA), Sysmex XE 2100 (21) and Sysmex XT-2000i (22,24) by Sysmex (Kobe, Japan), LH 750 by Beckman Coulter (Hialeah, FL, USA).

[b]The time elapsed from basal analysis is expressed as number of hours (e.g., 4 h: 4 h after baseline analysis).

6. COMPARISON OF RET VALUES BETWEEN ATHLETES AND SEDENTARY PEOPLE

Differences were found neither between Ret values for 20 male endurance and 16 male nonendurance athletes and 7 sedentary controls [10] nor between a group of nonprofessional female athletes ($n=70$) who practiced volleyball, soccer, martial arts, skiing, and cycling and a control group ($n=121$) ($1.20\pm0.42\%$ and $1.20\pm0.38\%$, respectively) [40]. Ret% was significantly lower in a group of male endurance athletes ($n=20$) than in male controls ($n=7$) (0.76 ± 0.2 vs. 1.03 ± 0.3), but there was no difference between male nonendurance athletes ($n=16$) and the same control group (0.94 ± 0.4 vs. 1.03 ± 0.3) [10].

A difference was found between a group of endurance athletes ($n=53$) observed during a competitive season and a period of reduced training and untrained subjects ($n=82$) [22]; noteworthy is that the difference (reduction in Ret%) was observed when the athletes were in intense training. When measurements taken during short bouts of intense exercise were analyzed, however, an increase in Ret% was found. These findings confirm that the increase in Ret% is more prominent at the start of the training, this also being the period when athletes need to be monitored for sports anemia [5].

In conclusion:

- there are no apparent differences between athletes and sedentary people when athletes are at rest
- differences eventually emerge for small groups of athletes during the competitive season, and controls
- endurance athletes could have higher or lower values than the general population, depending on the phase of training
- gender-specific fluctuations in Ret distribution are evident in specific groups of athletes and in comparison with sedentary controls, but more studies are needed to better clarify this point

7. EFFECTS OF EXERCISE ON RET

Variations in Ret distribution induced by physical activity need to be considered differentially depending on the level of physical activity (professional or recreational athletes or sedentary people) and on the length of the study period to distinguish acute response from chronic and established changes. To this end, it could be helpful to examine short-term and

long-term training-induced effects separately, the latter being more useful to explain the Ret distribution in professional athletes.

Studies investigating the acute effects of exercise in short-term training may involve subjects tested immediately after a single bout of exercise or a single competition or a brief training session. Long-term effects, on the other hand, refer to the result of a phase within a season or an entire season or even consecutive seasons and are studied to evaluate physiologically established changes. Both short- and long-term exercise-induced Ret changes depend on the kind of sports practiced and environmental conditions in which training is performed (e.g., altitude).

7.1. Short-term effects

Ret% increased after short-term exhaustive exercise as demonstrated in 23 Caucasian endurance athletes (19 males, 4 females; age range, 18–56 years) [22]. Blood samples were drawn immediately before and within 10 min after the end of a standardized incremental test until exhaustion on a treadmill or cycling ergometer (duration, 30–45 min). The increase in Ret was low (mean, 0.05%) but evident in nearly all subjects [22]. Exercise intensity is a key factor for determining Ret changes: no increase was observed by the same authors over a day in 36 male athletes, 20 endurance (cycling, triathlon), and 16 nonendurance (ball disciplines) mainly training at moderate intensity [10].

No difference in Ret% was found between prerace and postrace values measured in 132 male and 112 female speed skaters ($1.32 \pm 0.30\%$ and $1.34 \pm 0.53\%$ in males and $1.38 \pm 0.39\%$ and $1.38 \pm 0.38\%$ in females, respectively) [23]. The Ret% values recorded by the International Skating Union since 2000 (972 male and 980 female athletes) showed similar mean values and distribution of Ret% in out-of-competition, postcompetition, and postcompetition phases. Of note is that some values exceeded the SD (2.5–4.2%). More outliers were seen on precompetition than post-competition screening; the lowest number of outliers was reported for the out-of-competition phase [23].

In professional triathletes out-of-competition observed over a period of 63 days, a significant decrease in Ret% was found only at the end of the study (between day 49 and days 56 and 63), while no change in MCVr was noted [26].

7.2. Long-term effects

Training and competition workloads will inevitably influence longitudinal hematological data in athletes. Few studies to date have investigated hematological variations in long-term time series or over an entire competitive

season. A specific review of the literature on this topic summarized that some hematological parameters can be influenced by long-term training and competition periods [34]. Depending on the sports discipline, Hb was found to decline from 3% to 8% during the competition season, while Ret% rose between 5% and 21%. A decrease in Ret after long periods of training and competition was observed, but its variation was not necessarily associated with that of Hb [34].

In their review article, the authors also compared the differences in Ret-related parameters between athletes practicing different sports at different levels and controls as measured on different automated systems. Generally, no variations in all Ret-related parameters (CHr, RetHb, and MCVr) were found between the athletes and the controls [34], except for MCVr in young females (age range, 14–18 years) where the athletes (volleyball, soccer, martial arts, skiing, cycling) had higher MCVr values before the start of the season (athletes 108.12 ± 3.17; controls 103.81 ± 2.58; $p < 0.001$) [40].

Unlike qualitative variations in hematological parameters, which are largely independent of the sports discipline, quantitatively they are dependent on the sports discipline.

In 28 top-level male cyclists of a Danish cycling team, the hematological values were repeatedly controlled over 1 year (from December 2006 to November 2007) by drawing 374 blood samples. The Ret values remained fairly stable over the entire season (0.8–1.1%), but those recorded in July and September were lower than the baseline values [41].

In a large-scale survey of elite cross-country skiers, 3961 samples were collected from 440 female and 3120 samples from 634 male athletes between 2001 and 2007 and analyzed on Sysmex instrumentation [42]. The Hb and Ret data were given as the mean of each season versus the values for 1997–1999. The seasonal variation in Hb concentration was 148–157 g/L in the males and 134–142 g/L in the females during the testing period; the variation in Ret values was 0.8–1.3% and 0.9–1.3% for the males and the females, respectively. There was a decrease in mean Hb concentration between 2001–2002 and 2002–2003, followed by a subsequent increase in the next season, but the values were always lower than those observed in the 1997–1999 period, likely due to more accurate and frequent anti-doping controls. The Ret values decreased in both genders: in the males the values started from 1.3%, plateaued at 1.1% during three seasons (2002–2005), and then declined to 0.9% and 0.8% in the last two seasons, respectively; in the females, Ret decreased from 1.3% to 1.1% over the first three seasons, except for a peak in the fourth year of observation (1.2%), and then declined again to 1.0% and 0.9% in the last two, respectively. In this

study, Hb and Ret levels were measured only once a season by calculating the mean of Hb and Ret. It should be remarked, however, that the differences between seasons were particularly evident for Hb and that there was no correlation between Hb and Ret values, that is, a similar response as in the present study. Both values, however, were related to altitude as expected, with higher values recorded when blood drawings were taken above 600 or 1200 m above the sea level [42].

In a survey conducted over four consecutive competitive seasons and involving 18 top-level Italian Alpine skiers (10 males and 8 females), differences were noted among seasons [21]. A significant variation in Hb values, but not in Ret%, was observed for both genders within each competitive season; for instance, a consistent difference in Ret was observed only between the first and the second blood drawing, that is, between the basal value before the start of training and competitions and the value at midseason when the training workload was highest [21]. This finding is consistent with that found in 238 athletes, where a mean decrease of 0.1% was found during periods of intensive exercise [22].

Analysis of variance for ranked Hb concentration and Ret and IRF% was applied to values obtained from Alpine skiers to determine possible differences over seasons. Unlike Ret%, Hb values changed significantly across competitive seasons for both genders. Comparison between consecutive seasons (e.g., 2005–2006 vs. 2006–2007) showed significant differences for both parameters. The differences between seasons were greater than the within-season differences for Ret [21].

Banfi and Del Fabbro [43] reported that during an entire training and competitive season, Ret% fluctuations differ among sports disciplines involving different training volumes and stimuli. Their study involved 63 male professional athletes primarily engaged in aerobic sports and sharing several common characteristics: an extended competitive season, a period of heavy training before the competition season, high intensity, and frequency of competitions. The athletes were 13 rugby players from the Italian National Team, 12 alpine skiers from the Italian National Team, 19 professional cyclists from a ProTour team, and 19 football players from a First Division National Italian championships team. The athletes were observed for the length of the entire season: before the start of the training period (precompetitive phase), at the beginning, in the middle, and at the end of the competitive season.

Ret counts always fell within the reference limits and were consistently stable during the competition season. In the rugby players and the skiers, the

Ret decrease during the season paralleled the decrease in Hb, whereas no such variation was observed in either the cyclists or the soccer players [43].

The behavior of Hb, Ret, and IRF across the competitive seasons ran parallel in the females but differed in the males. Of note is that changes in Ret do not follow variations in Hb over the course of a season, as demonstrated by the lack of a correlation between the two parameters. This should be interpreted, however, considering the time intervals between the blood drawings over the season: before the start of training (May), at the end of training and before the start of competitions (October) and before the World Championships of the Olympic Games, and toward the end of international competitions (January). A different relationship between Hb and Ret may be seen with more frequent blood drawing [21].

Finally, Ret% and [Hb] were assessed over the course of four competitive seasons in elite triathletes from the Spanish National Team (10 males and 7 females) [6] to investigate the stability of the two parameters in sportsmen undergoing high training loads and in a combination of different sports disciplines. A total of 228 samples (2005–2009) were obtained during specific periods throughout each year: start of the season, precompetitive period, first half, and end of the competition period. Analysis of variance for ranked [Hb] and Ret showed significant differences between genders and interindividual variability. No difference in [Hb] for the entire study population emerged, whereas Ret% varied between seasons, between different periods over the 4 years of observation, and between the different periods within a single season. Ret% fluctuations were observed in the females between the periods within the seasons, but not in the males, confirming the gender-related differences in Ret behavior [6].

7.3. Differences among sports disciplines

There is significant difference between endurance and nonendurance athletes [10]. The MCVr and CHr parameters, which are relatively independent of plasma volume expansion, were higher in aerobic than in anaerobic or mixed aerobic–anaerobic athletes, in both males and females alike, indicating an accelerated erythropoiesis [30].

7.4. Effect of altitude

Exposure to natural or artificial hypoxia to stimulate Hb production and release of RBC from bone marrow is a common practice in sports medicine. An effect of altitude on Ret was recently demonstrated in 13 elite cyclists

monitored for a 5-week period, which included a 3-week training camp [44]. Eight athletes were exposed to 3 weeks of natural altitude living at 2760 m and training at 1000–3000 m in the surrounding mountains for 2–6 h/day, where they rode at >1800 m for the majority of time. Five athletes trained at sea level were the controls. Baseline Ret%, as measured by a Siemens Advia 120, was 0.9 ± 0.2 for the altitude-exposed athletes and 1.6 ± 0.3 for the control group. Ret% increased slightly during altitude training and peaked after 12 days, with an increase of $20.4 \pm 25.3\%$ over basal values. The interindividual variability was very high, but this finding was also evident in the group which trained at sea level. Five days were sufficient to reach a higher concentration in the altitude-exposed group than in the controls. The values measured at 20 and 32 days were very similar to baseline. After return to sea level, the mean Ret% was lower than baseline in the altitude-exposed group. The Ret% changes were identical to those revealed by transferrin-soluble receptor (sTfR), proving the stimulating effect altitude has on bone marrow and Hb production [44]. The correlation between sTfR and Ret% was confirmed in studies where top-level Alpine skiers (20 males and 14 females) were followed for an entire season [44], in cyclists [27] and in male, but not female, field hockey players [29].

A manifest altitude effect on Hb and Ret% was demonstrated in a study on 160 male and 117 female elite speed skaters whose values were measured at low altitude (<750 m) and moderate altitude (1425 m). Hb concentration was found to be slightly higher in both the males and females when they lived at 1425 m (mean increase 2.3 g/L in males, $p<0.05$, and 4.8 g/L in females, $p<0.01$) than at <750 m. Ret% was significantly ($p<0.01$) higher in samples drawn at altitude in both genders ($1.31 \pm 0.42\%$ at <750 m and $1.55 \pm 0.41\%$ at altitude in males, and $1.34 \pm 0.45\%$ and $1.61 \pm 0.41\%$ in females, respectively). It should be underscored, however, that the elevated Ret values were due to acute altitude exposure at the time of blood withdrawal, because many of the athletes had already sojourned at altitude for a certain period before testing [22].

Exposure of eight elite male track endurance cyclists (age range, 19–26 years) to an altitude of 1905 m for a 3-week period led to a significant increase in Ret% immediately after return from altitude training, followed by a decrease above baseline at 9 and 16 days after return from altitude training [28]. The authors indicated the relevance of IRF measured on a Sysmex XE2100, which showed a marked increase after altitude training, followed by a decrease after 9 days and another increase after 16 days. The apparent discrepancy between Ret% and IRF at this time can be explained by

erythropoietic suppression leading to a reduced production in immature Ret accompanied by selective removal of early Ret circulating at day 9 after return from altitude. The subsequent regeneration of immature Ret seen at day 16 is consistent with recovery if erythropoiesis to sea level is steady state [28].

In a study involving seven male triathletes for a 63-day period, training workload was progressively increased from day 1 to day 21; four subjects stayed at 1850 m altitude from day 21 to day 28, whereas three subjects stayed at lowlands (150 m). Ret was not affected by exposure to altitude, since, as reported by the authors, spending a similar length of time in these hypoxic conditions is insufficient to stimulate bone marrow to produce RBCs [26].

In conclusion:

- depending on its intensity, acute exercise can modify Ret count
- training and competitions influence Ret values during a season; Ret decrease can be seen during the more intense phase of the season, but this effect is not always evident
- the differences between consecutive seasons are greater than within-season differences for Ret in the same group of athletes; the differences are gender specific
- average Ret values decrease in the past decade in elite athletes
- Ret changes during a season are not always parallel to changes in Hb
- qualitative variations in Ret are mainly independent of the sports discipline, but quantitatively dependent on the sports discipline
- Ret is influenced by exposure to altitude, level of altitude, and especially the duration of exposure to hypoxia are crucial for determining Ret changes

8. DOPING

Ret is a crucial parameter for suspecting and discovering the illicit use of substances and/or procedures which can enhance oxygen transfer to muscles. The use of Ret for detecting suspected recombinant erythropoietin (rHuEpo) abuse has been widely described. In the past years, the reappraisal and resurgence of blood transfusions by cheating athletes led to studies on Ret for detecting suspected blood withdrawal and reinfusion [45–47].

A study on the use of different rHuEpo treatment schemes involved 24 healthy male volunteers divided into three groups of eight subjects each [48]. Two groups received rHuEpo injections (65 ± 5 U/kg) for a 4-week period

(2 weeks "boosting" phase plus 2 weeks "maintenance"). During the boosting phase, the subjects were injected with the hormone every second day (4 injections/week) and during the maintenance period, they had one injection per week. The injection period was followed by a 3-week washout period. The third group received rHuEpo (60 ± 4 U/kg) for 10 weeks, with 3 weeks of boosting (the frequency of injections was the same as for the other two groups), followed by 7 weeks of maintenance, with one injection per week and 1 week of washout. Blood samples were taken before, during, and after rHuEpo injection 12 times until day 49 from the start of treatment in the first group, 18 times until day 46 in the second, and 19 times until day 71 in the third. Analyses were performed using Sysmex instruments. Ret increased in all treated subjects during the boosting period; the increase was more pronounced in the two groups treated with 2 weeks of boosting and 2 weeks of maintenance. Ret values were higher than the highest cutoff (2.4%) in 8 out of 24 subjects during the boosting period, 4 from the groups treated for 2 weeks, and 4 from the group treated for 3 weeks. During the maintenance period, only one subject, treated for 2 weeks, had Ret $> 2.4\%$. No subjects had Ret $< 0.2\%$ during the entire observation period. Ret was the most sensitive parameter for discovering the rHuEpo treatment, because Ret values were higher than the threshold (2.4%) in 8 out of 24 subjects, while Hb was outside its limit (170 g/L) in 7 out of 24 subjects, and OFFhr was higher than 133 in 3 subjects during the washout period. An individual athlete's injection regime is a critical factor in successful hematological screening for substance abuse. Ret values and increases are fundamental for detecting suspected forced stimulation of bone marrow [48].

The sensitivity of Ret increase after rHuEpo administration was also demonstrated in eight male volunteers injected subcutaneously (dose, 5000 U) every second day. Blood parameters were measured on a Sysmex R3000 1 week before the first injection, and at days 8 and 16 during the treatment period. Ret % was significantly higher than baseline at days 8 and 16. The Ret% trend was parallel to the increase in Hb. Interestingly, the Ret trend ran opposite that of haptoglobin values which decreased from 1.12 ± 0.18 to 0.96 ± 0.15, and then to 0.85 ± 0.14 g/L. The study identified four haptoglobin isoforms, together with two isoforms of transferrin, and a mixture of hemopexin and albumin, which significantly decreased at day 16 after rHuEpo administration. Out of the 97 proteins evaluated, these molecules could be used in novel approach to identify rHuEpo abuse [49].

Another rHuEpo administration regimen is with the use of microdoses to maintain high concentrations of hematological parameters that can

escape detection by antidoping methodologies, namely the ABP. After administration of rHuEpo at a dose of 10 U/kg for 4 weeks in four subjects and the same quantity twice a week in six subjects but for a period of 2 weeks, Ret% tended to increase at the beginning of treatment, but were found unchanged at the end of treatment in comparison with baseline values [50].

Blood manipulation can also be performed by expanding and diluting plasma to mask increased enhancement of Hb and erythrocytes. In a study on the testing of desmopressin-induced hemodilution in eight physically active males [51], the hematological parameters were measured on a Sysmex XT2000i analyzer. After desmopressin treatment, a significant decrease in Ht, Hb, and OFFhr was found. The mean Ret value before and after desmopressin treatment was $0.8 \pm 0.2\%$ and $0.6 \pm 0.2\%$, respectively; the mean Ret posttreatment value after the ingestion of water was $0.7 \pm 0.2\%$. Desmopressin abuse is an effective method for diluting blood. Ret, owing to its measurement as a percentage, is less sensitive than Hb and other chemistry parameters. No effect on Ret% was reported in a study on 36 athletes accurately monitored for fluid intake [10].

Ret behavior during blood manipulation by withdrawal and reinfusion (autotransfusion) has been recently investigated in studies involving adults or athletes to define variations in hematological parameters when high quantities of blood are drawn, stored, and then reinfused in healthy subjects. The aim of these studies obviously differs from the bulk of the scientific literature where the focus is on transfusion as therapeutic treatment. Although the use of different regimens make comparison across studies difficult, the schemes followed the practice schedules usually reported by rare convicted athletes and so can be assumed to reflect the real world of blood manipulation. Furthermore, the trend of hematological parameters is the same, irrespective of the administration scheme used.

In a study involving seven male triathletes for a 63-day period [26], from day 1 to day 21, the training workload was progressively increased up to a regular weekly training of 5 h. From day 21 to day 28, four subjects remained at 1850 m altitude and three at lowlands (150 m). On day 30, a phlebotomy was performed, blood samples were drawn, and 250 mL of concentrated RBC and 200 mL of plasma for each subject were stored at 4 °C. The subsequent 20-day period between days 30 and 50, during which a physiological response to blood withdrawal was expected, was termed "biological return" by the authors. On day 51, blood was reinfused, and the period from days 52 to 63 was defined as the postinfusion phase.

No significant differences between consecutive measurements of Ret and MRV were found; the final values at 5 and 12 days after reinfusion differed significantly from the value measured after blood withdrawal. The authors stated that some absolute variations in hematological parameters in a period of 15 days could be used for detecting suspected blood manipulation: 6% for Ht%, 4% for Hb, and 20% for OFFhr. These values, however, are lower than possible changes induced by training and competitions [34]. Moreover, this approach was also criticized [19] because of the short period of exposure to altitude and not having taken into account the analytical interassay variability of the analyzer [52].

Ret remains constant during blood withdrawal. Its decrease during recovery and after reinfusion is more informative. However, if the amount of blood withdrawn is high, that is, three blood bags over a period of 2 weeks or two blood bags in 1 week, Ret will increase due to the natural stimulation of bone marrow to release new erythrocytes. This increase is followed by a decrease, with a normalization of values during the recovery period, and then an additional decrease after reinfusion which inhibits bone marrow activity. For this experiment [46], 24 healthy, recreationally active male subjects were assigned to four groups. Eight subjects had one 450-mL bag of blood withdrawn on three different occasions, with an interval of 7 days between the first and the second withdrawal, and 6 days between the second and third withdrawal; four subjects composing the control group did not undergo blood manipulation. The RBC units were frozen for 72 ± 5 days at -80 °C, then thawed and deglycerolized. The thawed RBCs were stored at 4 °C for 1.5 ± 1.1 days in a saline solution, then heated to 41 °C and reinfused. In eight other subjects, two bags of blood were withdrawn together, followed by a third bag 7 days later. The blood was fractionated and the RBC units stored at 4 °C. All three bags were reinfused 26 ± 3 days later. There was a control group composed of four subjects for each group that underwent blood withdrawal. Hematological parameters were measured in the blood stored at -80 °C from the treated subjects and their respective controls at baseline, at 1, 4, 7, 8, 11, 13, 14, 18, 21, 28, 35, 42, and 81 days (the day of reinfusion) after the first phlebotomy, and then at 1, 3, 7, 14, 21, and 28 days after reinfusion. The same parameters were measured in the blood stored at 4 °C from the treated subjects and their respective controls twice at baseline, at 1, 5, 8, 15, 22, 27, and 28 days (the day of reinfusion) after the first phlebotomy and then at 1, 3, 7, 14, 21, and 28 days after reinfusion. The blood parameters were measured on a Siemens Advia 120 system.

In the subjects from whom three bags were withdrawn on three different occasions, the average Ret maximum was 2.2% at 3 weeks after the first phlebotomy; in those subjects from whom three bags were withdrawn on two occasions, a Ret peak of 2.6% was observed 8 days after withdrawal of the first two bags. In both groups, the average Ret was $>2\%$ for at least 1 week, and all subjects had Ret $>2\%$ on at least one measurement. Ret decreased after reinfusion, with the nadir (0.6%) recorded 1–2 weeks later; only three subjects had Ret $<0.3\%$. Ret was sensitive to the withdrawal phase, from day 1 after the first bag was drawn until 1 week after the third bag was drawn, while Hb and OFFhr were insensitive. During the reinfusion phase, from infusion until 4 weeks later, Hb was slightly more sensitive. In the subjects from whom three bags were drawn on three different occasions, the limits of 145.7 and 182.9 were exceeded in 35.4% and 18.8% of all samples during the 4 weeks following reinfusion. In the group from which three bags were drawn on two occasions, the limits were exceeded in 19.6% and 4.3% of all measurements. The number of samples with values outside the limits is high as compared to the naturally occurring high level of this ratio found in a large cohort of endurance athletes (0.8% and 0.2%, respectively) [46].

Ret changes after blood removal and reinfusion are key indicators for detecting blood manipulation through autotransfusion and can be determined by using the OFFhr parameter in the ABP program, as demonstrated in 11 subjects submitted to blood removal and reinfusion during one season (January–October) in comparison with 11 nontreated subjects. The blood samples were taken during the treatment period by a researcher masked to treatment allocation. The ABP program revealed the differences between the groups [53].

A hybrid parameter, termed Hbmr, derived from the equation $4.51 \times \ln(\text{Hbmass}) - \sqrt{\text{Ret\%}}$, was proposed for detecting blood transfusions [45]. The marker showed high sensitivity in detecting the highest dosage (three bags) of transfused blood. Hbmr also had high sensitivity, equal to that of OFFhr, during the reinfusion phase. The authors advocated the use of Hbmass and derived parameters for detecting acute variations in blood homeostasis caused by autotransfusion, but the technique cannot be proposed for routine use due to technical difficulties and potential legal implications [54].

The 36-h limit between blood drawing and analysis recommended by ABP scientists appears reasonable to assure analytical quality, as long as samples are transported at 4 °C and accompanied by certification that this temperature has been maintained during the transport chain.

In conclusion:

- Ret is a valid parameter for detecting suspected blood doping

- Ret is a specific and sensitive parameter for detecting suspected rHuEpo abuse
- Ret is a sensitive parameter for detecting suspected blood manipulation by transfusion during the recovery phase after withdrawal and after reinfusion
- Ret changes during blood manipulation by transfusion depend on the quantity of blood drawn and reinfused and the time of testing

9. CONCLUSIONS

The reliability of the Ret parameter in clinical pathology has been translated into sports medicine as a useful tool for the early detection of sport-induced anemia. More recently, Ret has attracted growing interest in sports medicine owing to its use as a parameter in antidoping settings starting under the Australian protocol. Most of the studies reviewed here were conducted for corroborating and validating the reliability of Ret use in suspected blood manipulation. Although Ret behavior in elite athletes has been widely described, Ret changes during and after rHuEpo administration are not yet fully understood; especially in instances of exposure to low doses of the hormone, Ret changes are less evident and putatively indistinguishable from physiological variations induced by high-level training.

The sensitivity of Ret as marker of fraudulent bone marrow stimulation with rHuEpo suggests its usefulness, together with Hb, for testing athletes during and within competitions. What is important, however, is to consider the variables that reportedly influence circulating Ret levels. The high biological variability and differences among sports disciplines require the interpretation of Ret values against long-term time series in the individual athlete. The gender effect and the influence of seasonal variations during training and competitions deserve further specific studies. The data obtained from athletes over a season could strengthen the use of Ret to identify abnormal bone marrow stimulation, in the conditions for which the relative studies have confirmed its high sensitivity.

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