

Reticulocytes in Sports Medicine

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Abstract

Reticulocytes are the transitional cells from erythroblasts to mature erythrocytes. Reticulocytes are present in blood for a period of 1–4 days and can be recognized by staining with supravital dyes, such as new methylene blue, or fluorescent markers, which couple residual nucleic acid molecules, a hallmark of the immature forms of erythrocytes. Although reticulocytes could be counted through a microscope (there is a standard of International Committee for Standardisation in Haematology for manual counting), this method is reported to be time consuming, inaccurate and imprecise. The integration of the reticulocyte count in automated haematology systems allowed the widespread use of these parameters, although the lack of calibration material and different markers, technologies and software used in automated systems could engender discrepancies among data obtained from different analytical systems.

The importance of reticulocytes in sports medicine derives from their sensitivity, the highest among haematology parameters, in identifying the bone marrow stimulation, especially when recombinant human erythropoietin is fraudulently used. Automated systems are also able to supply information on volume, density and the haemoglobin content of reticulocytes.

Some of the related parameters are also used in algorithms for identifying abnormal stimulation of bone marrow as reticulocytes haematocrit. The pre-analytical variability of reticulocytes (transportation, storage, biological variability) should be taken into account in sports medicine also. Reticulocytes remain stable for almost 24 hours at 4°C from blood drawing, they are affected by transportation, and biological variability is not high in general. It could be remarked, however, that the intra-individual variability is high when compared with other haematological parameters such as haemoglobin and haematocrit. The intervals of data reported in athletes are very similar to reference intervals characterizing the general population.

The reticulocyte count shows some modifications after training and during the competition season. The variability induced by exercise cannot be overlooked since the so-called haematological passport, a personal athlete's document in which haemoglobin and other parameters are registered, may be introduced by sports federations.

Exposure to naturally high altitude and 'living high-training low' programmes determined contentious results on reticulocytes. Simulated high altitude induced by intermittent hypobaric hypoxia does not modify reticulocytes, despite an increase in erythropoietin serum concentration. The variability among athletes competing in different sport disciplines is apparently limited. The knowledge of the behaviour of reticulocytes in training and competitions is crucial for defining their role in an antidoping control context. It is important for sport physicians and clinical pathologists to know the reticulocyte variability in the general population and in athletes, the pre-analytical warnings, the different methodologies for counting reticulocytes and the derived parameters automatically available, and, finally, the possible influence of training, competitions, type of sport and altitude.

Reticulocytes are immature erythrocytes (red blood cells [RBC]) containing nucleic acid (RNA) residues. Reticulocytes have a larger volume than RBC, and a lower haemoglobin (Hb) concentration, but the Hb content is identical.^[1] RBC have a mean lifetime of 120 days, but reticulocytes are present in the bloodstream for a period of 1–4 days until complete maturation and are almost devoid of erythropoietin (Epo) receptors.^[2]

The role of reticulocyte measurement in sports medicine has become important ever since counts of immature erythrocytes started being used for antidoping purposes.

The introduction of methods based on the measurement of various haematological parameters for identifying the possible causes of suspected blood doping was surprisingly not supported by many studies of behaviour of reticulocytes in athletes,

with the exception of studies^[3,4] that proposed the mathematical models now used in the antidoping context. The relevance of reticulocyte counts and related parameters in this field merits further research; an outline of the current knowledge regarding reticulocytes and related parameters in sport is illustrated in the present article.

The evaluation of reticulocytes in sports medicine is very important, not only for identifying the possible surreptitious use of banned substances to stimulate bone marrow production, but also for monitoring athletes. The risk of sports anaemia is high in professional athletes. The Hb loss, accompanied by a corresponding reticulocyte increase, has various sources, but RBC destruction due to intravascular haemolysis is the major cause.^[5,6] The stimulation of bone marrow in athletes is continuous and reticulocyte count could be a useful parameter for monitoring the physiological characteristics of this population. It is important for sports physicians and clinical pathologists to know the reticulocyte variability in the general population and in athletes, the pre-analytical warnings, the different methodologies for counting reticulocytes, and, finally, the possible influence of training, competitions, type of sport and altitude. Besides the influence of training, competitions and altitude on reticulocytes, other aspects could be considered, although not specifically treated in this article, such as the influence of several haematological disorders/genetic abnormalities (which may exist in some apparently healthy athletes) or the influence of hydration.

Many articles were written during recent years enlightening the argument from different points of view: technical and methodological problems, follow-up during types of training, the effect of training in different altitude levels and the reaction of reticulocytes induced by Epo. Each study focused on one partial aspect and it is now time to summarize this information.

1. Pre-Analytical Phase

1.1 Blood Drawing

Blood collection should be accurate and rigorously standardized to avoid spurious modifications of reticulocyte values and other haematological parameters, integrated in formulae used in the field of antidoping.^[3,4]

Lippi et al.^[7] investigated the influence of tourniquet time on these parameters. Two separate tubes were filled from 27 professional male cyclists. The first tube was drawn immediately after venipuncture, the second was the last specimen during the blood drawing, following eight tubes, used for various laboratory tests. The tourniquet was placed immediately before the venipuncture until all the tubes were filled. The average time of tourniquet application was 2.30 ± 0.12 minutes. All other phases of the blood drawing were standardized with identical time of resting (10 minutes), 20-gauge needle use and same lot of vacuum tubes. The analyses were performed on a Bayer Advia120®¹. The tourniquet induced a significant difference in Hb and packed cell volume (haematocrit; Ht) measurements, but not in reticulocyte count, as also demonstrated in 13 physically active subjects by Kuipers et al.^[8] Mean observed variations were +2.4% for Ht, +1.4% for Hb and -1.9% for reticulocytes. The coefficient of variation (CV) of the measures on the two independent samples were 1.0% for Ht, 1.7% for Hb and 4.6% for reticulocytes. It should be remarked that in 4 of 27 subjects, the variability in the Ht measurement exceeded the analytical goal.^[7] The blood drawing techniques must be accurate in sports medicine, because the variations induced by the often neglected pre-analytical part of the whole procedure of analyses could influence the final result, although this is not the case for reticulocytes, probably because of the low number of particles not influenced

1 The use of trade names is for product identification purposes only and does not imply endorsement.

by intravascular/extravascular fluid variations, possibly caused by the relatively long period of tourniquet holding.

1.2 Circadian Rhythm and Diurnal Variation

The number of circulating reticulocytes shows a circadian rhythm with an acrophase (phase of release) at 0100 hours (i.e. 1 am) with a 95% confidence interval between 1948 (i.e. about 8:00 pm) and 0428 hours (i.e. 4:30 am). The circadian rhythm constitutes 37% of the total variability encountered over a 24-hour span. The range of circadian variation in single subjects, expressed as the highest of each subject's measurement as a percentage of the lowest was 130%. The existence of a marker rhythm of bone marrow release is interesting for timing analyses or therapies. For example, the acrophase of RBC and Hb is at 1200 hours, about 12 hours after the reticulocyte one. The synchronization of biological rhythms is assured by sleep-wake and rest-activity paces. A radical change in these rhythms requires a long-time resynchronization, up to 42 days: frequent changes in athletes due to intercontinental flights should be taken into account.^[9] The rhythm of reticulocytes reflects the diurnal variations of serum Epo levels, which has the zenith (its highest level) of release at 0100 hours.^[10]

In a study performed on 96 healthy subjects, equally divided for gender, and assayed in three different hospitals (two equipped with Coulter STKS® and one with Technicon H*3®, later Bayer H*3®) Richardson-Jones et al.^[11] calculated the coefficient of variation of diurnal changes. RBC, Hb and Ht showed very similar data (3.5%, 3% and 3.7%, respectively, on average in the population of $4.8 \times 10^{12}/L$, 14.2 g/dL and 0.42). In contrast, reticulocytes showed a higher value of 20% on average in a population of $64.8 \times 10^9/L$. In order to explain the high diurnal change of reticulocytes compared with low values of RBC, the authors

focused their attention on the limited lifespan of immature particles.

However, the percentages of reticulocytes in 23 male professional cyclists participating in a 5-day road race and drawn every morning prior to the race and at the end of exercise did not show specific fluctuations in the results.^[12] Similarly, in 13 physically active males and three healthy physically active females, reticulocyte percentages were stable from 8:00 am to 4:00 pm.^[8]

The diurnal changes seem to be important for reticulocyte counting, although the two studies performed on athletes quoted in the literature do not report significant results.

1.3 Biological Variability

The biological variability of a laboratory parameter includes the variability of cyclical biological rhythms and the fluctuation beyond a homeostatic value. The amplitude of the fluctuation, which is independent from the pre-analytical phase, corresponds to the intra-individual variability, reported as a coefficient of variation (CV_i). The variability of the homeostatic value among a group of different individuals corresponds to the inter-individual 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 haematology ($\leq 9\%$); thus, V_t is highly dependent on V_b . The classical analytical goals for laboratory parameters consider $V_a = 1/2 V_b$. Haematology parameters have low V_a , but also low V_b and, in addition, analytical goals are certainly reached for some parameters such as Hb, RBC and derived parameters. Reticulocytes, however, have a V_b that is greater than RBC and Hb, and similar to leucocytes: the analytical goals should be evaluated carefully method by method.

The precision of the reticulocyte count is often taken for granted and it is not reported in published papers.^[13,14] For example, the precision of Abbott

technology produced a CV of 3.32% at a reticulocyte mean value of 1.68% and a CV of 2.61% at a mean value of 7.88%,^[15] reaching the analytical goals. In a parallel evaluation of five fully automated analysers, using National Committee for Clinical Laboratory Standards (NCCLS) protocol,^[16] the imprecision, based on duplicate determinations of 225 samples and calculated by one-way analysis of variance, gave a CV of 15–24.1% for the low level, 6.5–11.7% for the normal level and 3.5–6.9% for the high level.^[17] The CV_i of Hb, RBC and reticulocytes are presented in table I.^[11,18–20]

It is interesting to note the significant difference between the study of Richardson-Jones et al.^[11] using a Coulter and the study of a group of Italian laboratories that used four different and more modern analysers.^[20]

The V_b in haematology is particularly important because the stability of the blood parameters in a homogeneous group of subjects is high. Thus, a modification induced by an external factor is easily spotted. Stability was controlled and confirmed in a homogeneous population of university students in the period 1979–87.^[23]

Fraser^[24] combined the analytical precision (CV_a) and the average intra-individual biological variation (CV_i) from which a critical difference (CD) can be calculated for a predetermined probability. CD corresponds to the value indicating

that a difference between two consecutive results in the same subject is statistically significant and it is therefore unlikely to be the result of casual oscillation of values. In general, the difference in results, moving either up or down, which is >95% likely to 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 2.77 is equal to $\sqrt{2}$ times the z score for the difference. CD, in other words, identifies if an external factor (e.g. training, therapy) really modified the result of the parameter and if the modification is not dependent on instrumental or biological variables.

CD should be calculated for reticulocytes when the parameter is used for legal purposes. Tentatively, we can assume that CD should be for CV_a = 11.7% (the highest value for short-term imprecision at normal concentration as suggested by the NCCLS protocol in Buttarello et al.^[17]) and CV_i = 5.8%^[20] equal to 36.1%. If the lowest value for CV_a is used (6.5%),^[17] the CD is 24.1%.

CV_i is based on only one multicentre study:^[20] highlighting such an approach needs further evidence.

2. Reference Values

The ratio between intra-individual (CV_i) and inter-individual variability (CV_g) is the individuality index (II = CV_i/CV_g). The II provides information about the biological individuality of a laboratory parameter and, primarily, about the usefulness of reference ranges calculated on a population of apparently healthy individuals. When II is >1.4 the reference interval is useful, when ≤1.0 it is not useful; if the index is ≤0.6 the reference interval should not be used. The II for haematological parameters are illustrated in table I: for reticulocytes, only one result is reported, but is similar to the Hb result.^[20–22]

The II value indicates that the comparison of reticulocyte values should not be performed with

Table I. Intra-individual biological variability and indices for haemoglobin (Hb), red blood cells (RBC) and reticulocytes (Ret)

Study	Parameter (%)		
	Hb	RBC	Ret
Intra-individual biological variability			
Statland et al. ^[18]	2.6		
Costongs et al. ^[19]	4.3	4.4	
Richardson-Jones et al. ^[11]	3.0	3.5	20.0
Buttarello ^[20]	1.9	1.8	5.8
Individuality index			
Fraser et al. ^[21]	0.43	0.31	
Groner and Simson ^[22]	0.30	0.35	
Buttarello ^[20]	0.19	0.20	0.18

reference ranges of the general population, but with previous values of the studied subject.

Reference values in athletes are illustrated in table II. The values are calculated in athletes during competitions and are the result of a cumulative collection of data in two studies,^[25,26] at one time at rest during the competitive season in three other studies,^[27-29] and at rest, before the start of training and competitions in the studies of Sharpe et al.^[30] and Banfi et al.^[31-33]

Reference values of laboratory parameters are strictly linked to analytical quality, evaluated through external quality control schemes, and to the transferability of common reference values among

different laboratories: it has particular value in sports medicine, when the evaluated subjects are by definition normal and healthy, but their laboratory parameters could be greatly influenced by physical exercise.^[34]

Reference values for reticulocytes in the general population are reported as 0.5–2.5% by Perkins,^[35] evidently calculated by the manual classical method. The reference values calculated by means of modern analysers and published by independent researchers (not reported by producers), and reference ranges for reticulocyte parameters are reported in table III and table IV.^[1,13,14,17,36-39]

Table II. Reference values for reticulocytes (Ret) and related parameters in athletes

Study	Sport (level)	Subjects	Parameter: mean (range)	Methodology
Malcovati et al. ^[25]	Soccer players (professional)	Males (n = 923)	Ret (%): 1.00 (0.53–1.47)	Bayer Advia120®
Fiorella et al. ^[26]	Cyclists (non-professional)	Males (n = 1914)	Ret (%): 1.00 (0.46–1.54)	Various (automatic and manual)
		Females (n = 117)	Ret (%): 1.15 (0.48–1.12)	
Lippi et al. ^[27]	Cyclists (non-professional)	Males (n = 80)	Ret (%): 1.00 (0.60–1.40) Ret (10 ⁹ /L): 47 (27–67) ChR (pg): 32.6 (30.9–34.2)	Bayer Advia120®
	Cyclists (professional)	Males (n = 61)	Ret (%): 1.10 (0.60–1.60) Ret (10 ⁹ /L): 55 (29–81) ChR (pg): 31.4 (30.1–32.7)	Bayer Advia120®
Lippi et al. ^[28]	Cyclists (professional)	Males (n = 25)	Ret (%): 1.6 (1.3–1.9) Ret (10 ⁹ /L): 78.7 (53.3–94.1) ChR (pg): 32.9 (31.9–33.9)	Bayer Advia120®
Mayr et al. ^[29]	Ice skaters (non-professional/national level)	Males (n = 60)	Ret (10 ⁹ /L): 63.4 (45.3–81.6) MCVr (fL): 108.1 (104.9–111.3)	Bayer Advia120®
		Females (n = 56)	Ret (10 ⁹ /L): 59 (44.1–73.9) MCVr (fL): 109.2 (106.2–112.2)	
Sharpe et al. ^[30]	37 various sport disciplines (professional/national level)	Males (n = 739)	RetHt (%): 0.81 (0.69–0.83)	Bayer Advia120®
		Females (n = 413)	RetHt (%): 0.79 (0.67–0.91)	
Banfi et al. ^[31]	Rugby, soccer players	Males (n = 106)	Ret (%): 0.81 (0.30–1.54) ^a MRV (fL): 104.0 (93.1–114.8) ^a IRF (%): 0.29 (0.18–0.39) ^a	Coulter LH750®
Banfi et al. ^[32]	Skiers (professional)	Males (n = 20)	Ret (%): 1.16 (0.66–2.13) ^b IRF (%): 0.31 (0.26–0.49) ^b	Abbott Cell Dyn 4000®
	Soccer players (professional)	Males (n = 24)	Ret (%): 1.13 IRF (%): 0.35	Abbott Cell Dyn 4000®
Banfi et al. ^[33]	Rugby players (professional)	Males (n = 44)	Ret (%): 1.08 (0.94–1.22)	Abbott Cell Dyn 3500®

a 2.5th–97.5th percentile.

b Interval for all the studied athletes (skiers and soccer players).

ChR = reticulocyte haemoglobin content; **IRF** = immature reticulocyte fraction; **MCVr** = mean reticulocytes corpuscular volume; **MRV** = mean reticulocyte volume; **RetHt** = reticulocytes haematocrit.

Table III. Reference values for reticulocytes (Ret) in the general population

Study	Subjects	Parameter	Methodology						
			manual	Abx Pentra120 Retic®	Coulter Gen-S®	Sysmex SE 9500®	Abbott Cell Dyn 4000®	Bayer Advia120®	Sysmex XE-2100®
Buttarelli et al. ^[17]	126 (man);	Ret (10 ⁹ /L)	19–111	30–130	20–85	23–95	25–108	27–125	
	213–221 (auto)	Ret (%)	0.4–2.3	0.6–2.6	0.5–1.8	0.5–1.9	0.4–2.2	0.6–2.5	
Noronha and Grotto ^[14]	50	Ret (10 ⁹ /L)							20.5–122.8
Van den Bossche et al. ^[36]	175 f	Ret (10 ⁹ /L)		22–95	24–73	16–66	21–98	19–64	
	142 m	Ret (10 ⁹ /L)		31–130	30–90	16–70	30–110	29–69	
	317	Ret (%)		0.61–2.16	0.61–1.79	0.44–1.55	0.61–2.24	0.50–1.40	
Bovy et al. ^[37]		Ret (10 ⁹ /L)						38.3–65.1	
		Ret (%)						0.7–1.3	

auto = automatic; **f** = females; **m** = males; **man** = manual.

The inter- and intra-individual variation of reticulocytes was defined in 114 rowers (83 males, 31 females) of the French National squad in the period July 2001 to March 2004; from two to eight blood samples were taken for each athlete.^[40]

The parameter $\sqrt{\text{Ret}}$, integrated in the OFF (out of administration of recombinant human erythropoietin [rHuEpo])-model (OFF-hr = Hb-60 $\sqrt{\text{Ret}}$) for identifying possible rHuEpo doping,^[3,4] showed an inter-individual variation of 0.0127 in males and of 0.0139 in females. The same variation for the parameter Hb was 4.51 and 1.83 g/dL, respectively; the lower variation in females has no apparent explanation. The intra-individual variance for reticulocytes was 0.75-fold that of inter-individual variance, regardless of the sport or gender.

The ethnical origin of athletes influenced the ranges of reticulocytes haematocrit (RetHt): the range in the whole group of 413 females, coming from various disciplines, was 0.30–1.08%, whereas the range in African athletes went from 0.34% to 1.20%. The higher upper limit appears in the group of male Oceanian athletes, who had 0.35–1.21%, as opposed to the range of the whole group of 0.31–1.12%.^[30]

The distribution of reticulocyte values and mean reticulocyte volume (MRV) in athletes was found to

be non-parametric,^[31] whereas in another study a Gaussian distribution was described for reticulocytes and mean reticulocytes corpuscular volume (MCVr).^[29] It is worth noting that a Gaussian distribution is found in a group of 60 athletes competing in the same sport, and non-Gaussian in a group of 106 athletes competing in different sports.

In summary, reference values are highly method-dependent; automatic methods are recommended instead of manual counting. However, reticulocyte concentrations <0.4% or >2.6% could be interpreted, in the general population and in athletes also, as abnormal values.

3. Stability

The reticulocytes tend to mature and transform to RBC in whole blood, but the stability is high (72 hours), if the blood, anticoagulated with EDTA, is stored at 4°C.^[15,41] However, over this period of time, the indexes mean reticulocyte corpuscular haemoglobin concentration (CHCMr) and mean reticulocyte haemoglobin content (CHr), decrease, respectively, by 1.2 g/L and 1.8 pg, while reticulocytes distribution width (RDWr) increases by 2%.^[42] It is advisable that reticulocytes should be analysed within 24 hours from blood collection, in EDTA samples.^[43,44] The stability of 24 hours was

recently confirmed using Bayer Advia120® on the blood of 25 male professional road cyclists. Samples were collected in the morning, the first measurement was performed within 2 hours of venipuncture and samples were stored at 4°C and retested after 24 hours. Reticulocyte count, percentage reticulocyte count (Ret%) and CHr differed significantly after 24 hours.

The reticulocyte average value decreased by 4.8% with a range of -20.5–10.8% (95% confidence

limits of agreement) The difference was statistically significant, but clinically negligible; the desirable bias for the reticulocytes was $\pm 7.8\%$: they can be efficiently tested for up to 24 hours on blood taken from sportsmen, even for legal purposes.^[28]

The reticulocyte values ($\times 10^9/L$), measured on Sysmex XT2000®, of three subjects (two males, one female) tested at different temperatures (room temperature of 22°C and cold temperature of 2°C) during a period of 72 hours from the drawing, de-

Table IV. Reference values for reticulocyte (Ret) parameters in the general population

Study	Subjects	Methodology					
		Bayer H*3®	Coulter Gen-S®	Sysmex SE 9500®	Abbott Cell Dyn 4000®	Bayer Advia120®	Sysmex XE- Abx Pentra 2100® 120®
IRF (%)							
Van den Bossche et al. ^[36]	317				0.14–0.35		
Buttarelo et al. ^[38]	225		0.20–0.37	0.05–0.21	0.15–0.35	0.04–0.25	0.06–0.23
IRF (arbitrary units)							
Noronha and Grotto ^[14]	50						1.4–17.8
Ret low ($10^9/L$)							
Van den Bossche et al. ^[36]	317			84.6–97.1		88.3–98.0	
Ret medium ($10^9/L$)							
Van den Bossche et al. ^[36]	317			2.6–13.8		1.5–10.7	
Ret high ($10^9/L$)							
Van den Bossche et al. ^[36]	317			0–2.4		0–2.0	
CHr (pg)							
David et al. ^[13]	54 m					29.8–33.9	
	38 f					28.1–33.3	
D'Onofrio et al. ^[1]	64	25.9–30.6					
Buttarelo et al. ^[39]	80					28.2–33.4	
MCVr (fL)							
D'Onofrio et al. ^[1]	64	103.2–126.3					
David et al. ^[13]	54 m					81.0–91.5	
	38 f					98.5–110.2	
Buttarelo et al. ^[39]	80					104.5–119.6	
CHCMr (g/dL)							
D'Onofrio et al. ^[1]	64	23.5–28.7					
RET-Y (arbitrary units)							
David et al. ^[13]	54 m					1825–1979	
	38 f					1759–1969	
Buttarelo et al. ^[39]	80					1661–1820	
Noronha and Grotto ^[14]	50					1612–1807	

CHCMr = mean reticulocyte corpuscular haemoglobin concentration; **CHr** = reticulocyte haemoglobin content; **f** = females; **IRF** = immature reticulocyte fraction; **m** = males; **MCVr** = mean reticulocytes corpuscular volume; **RET-Y** = mean value of the forward-scattered-light histogram within the reticulocyte population.

creased after 4, 8, 24 and 48 hours at room temperature, whereas at cold temperature the phenomenon was less noticeable. The values after 72 hours were both similar to baseline ones, but it was an artefact of the haematological systems. Reticulocyte, Ht and Hb values were stable at cold temperature for 24 hours, but Ht was unstable at room temperature, due to MCV modifications.^[45]

Kouri et al.^[46] studied the effects of the combination of some pre-analytical factors (transportation, storage, delay in sample pre-treatment) and analytical variability on a series of biochemical and haematological parameters, the latter measured on Bayer Advia120®. The difference between results obtained on samples transported by car for 4 hours and the samples analysed without delay were used for calculating the uncertainty of measurement due to transportation. The variation in specimen collection and the effect of delay in sample pre-treatment were calculated by collecting samples from both arms of ten persons from the author's laboratory personnel. To establish the effect of a delay in pre-treatment, the difference between the data obtained from a sample kept at room temperature for 3 hours and the data from a sample immediately treated was calculated. The storage effect was tested reanalysing 14 samples after storage for 3 days at 4°C. The uncertainty for transportation for reticulocytes was 10% (mean value of 1.6% before transportation, 1.73% after) and the uncertainty due to storage was 12% (mean value of 1.51% after storage). The values of reticulocytes were the highest among the haematological parameters. The uncertainty of specimen collection was negligible, being lower than analytical variability, and uncertainty of delay in pre-treatment was 7.9%. The combination of pre-analytical and analytical uncertainties gave a result of 35%, which could influence thresholds used in sportsmen.

4. Methods

The reference method for reticulocyte counts is the manual count through a microscope at 100× after supravital dying with new methylene blue (basic blue 24, colour index 52030). Identical volumes of EDTA-anticoagulated whole blood and fresh dye solution are mixed and a smear is prepared. With a microscope, all the particles of red series not nucleated having at least two subparticles stained by blue have to be considered reticulocytes. The enumeration of reticulocytes through a microscope is affected by high inter-individual differences, due to personal experience, and also to the need for reticulocyte counting expression per 1000 evaluated RBC.

The automatic count is based on the dying of particles by classic dyes (new methylene blue, brilliant cresyle blue or similar) or by fluorescent molecules. The dyes and fluorophores link RNA residues and the marked particles can be recognized and counted. Among fluorophores, the acridine orange was the first used for reticulocytes.

The automation allows the contemporary enumeration of stained particles and the registration of some dimensional characteristics of particles.

The reticulocyte counting has been integrated in automated systems for haematology, initially in semiautomation with a manual preparation of specimens and, later, in complete automation. The principal advantage of the automation is the high degree of precision, because of the high number of events collected; moreover, the throughput is clearly better than with manual procedures.

However, there are some problems and pitfalls in reticulocyte measurements despite the enormous improvement of analyses, from inaccurate and imprecise manual counting to automation and availability of counts in haematological analysers also supplying additional parameters for describing volume, density and Hb content. The automated haematology systems that supply reticulocyte counting are listed in table V.

Table V. Automatic analysers for reticulocyte counting and their characteristics

Manufacturer	Instrument	Methodology	Marker	Collected events
Coulter Beckman	Max-M	Impedentiometry, conductivity and light scattering	New methylene blue	32 000
	HMX	Impedentiometry, conductivity and light scattering	New methylene blue	32 000
	STKS	Impedentiometry, conductivity and light scattering	New methylene blue	32 000
	Gen-S	Impedentiometry, conductivity and light scattering	New methylene blue	32 000
	LH750	Impedentiometry, conductivity and light scattering	New methylene blue	32 000
	LH500	Impedentiometry, conductivity and light scattering	New methylene blue	32 000
Sysmex	R1000	Fluorescence	Auramine O	30 000
	R2000	Fluorescence	Auramine O	30 000
	R3000	Fluorescence	Auramine O	30 000
	R3500	Fluorescence	Auramine O	30 000
	R-500	Fluorescence	Auramine O	30 000
	SE9500	Fluorescence	Auramine O	32 000
	XT2000	Fluorescence	Polymethine	30 000
	XE2100	Fluorescence	Polymethine	30 000
Bayer	H*3	Absorbance	Oxazine 750	20 000
	Advia120	Absorbance	Oxazine 750	50 000
	Advia2120	Absorbance	Oxazine 750	50 000
Abbott	Cell Dyn 3200	Absorbance	New methylene blue	10 000
	Cell Dyn 3700	Absorbance	New methylene blue	10 000
	Cell Dyn 4000	Fluorescence	CD4K530	20 000
	Sapphire	Fluorescence	CD4K530	20 000
Horiba Abx	Pentra DX120®	Fluorescence	Thiazole orange	32 000

The lack of calibration material to be used for all the systems prevents an alignment between systems that have different technologies, different dyes for marking reticulocytes and so different software for differentiating reticulocytes from RBC and from RBCs having nuclear material. The calibration should be made with fresh blood, but there are some limitations on its use, such as poor stability and technology-dependent calibration procedures.^[17]

Two standards for reticulocyte counting have been approved. The standard of International Committee for Standardisation in Haematology (ICSH)^[47] proposes the manual microscopic reference method for reticulocyte counts. The method is based on the use of new methylene blue (basic blue 24 colour index 52030) with a supravital dying of blood. Two pairs of clinical pathologists count reticulocytes on two different films of the same sample. The pathologists count at least 1000 RBC and retic-

ulocytes in different fields, ignoring the cells touching the lower and right side of the square ('edge rule'); the mean value obtained by both set of pathologists can be used for comparison with automated systems.^[17]

The standard H44-A,^[16] revised in 2004 as H44-A2, was proposed from the NCCLS for evaluating automated analysers. The comparison is made with microscopic examination performed on blood stained with new methylene blue.

A study of intermethod comparison on five fully automated analysers, by using the NCCLS and ICSH standards on 256 samples, showed a satisfactory correlation of the systems (Bayer Advia120®, Abbott Cell Dyn 4000®, Coulter Gen-S®, Sysmex SE 9500® and Abx Pentra®) with the reference method: the intercept was between 0.20–0.49 (reticulocyte counts 10⁹/L; specifically 0.38 for Advia120®, 0.39 for Cell Dyn 4000®, 0.36 for Gen-S®,

0.20 for SE9500[®], 0.49 for Pentra[®]. The automated systems tended to overestimate at low levels and underestimate at high levels.^[17]

The problems stemmed from the lack of calibration material and different technologies integrated in haematological systems for counting reticulocytes are clearly crucial for antidoping research, especially if reticulocytes were to be registered in the so-called haematological passport^[25] (a personal document for athletes where Hb and OFF-model parameters [including Hb and reticulocytes] are recorded) to calculate the variance of these parameters of a single athlete to identify the possible use of banned substances when values fluctuation is higher than expected.^[48]

Ashenden et al.^[49] proposed a protocol for standardizing results supplied by different systems. They used Bayer Advia120[®] as a reference, because the aim of the study was the possible substitution of Advia120[®] in models proposed for rHuEpo doping, originally built up by using the Bayer instrument.^[3] The Bland and Altman analysis showed that Advia120[®] reported higher reticulocyte values than other instruments, either using fluorescence or absorbance techniques, on 210 samples. The exception was the group of FacsCount, a flow cytometer from Becton Dickinson, Abbott Cell Dyn 4000[®] and Sysmex XE2100[®], which showed intermethod agreement. The homogeneity of data is based on the sampler mode. Using Advia120[®], the authors obtained data from 54 endurance athletes by duplicate analyses and calculation of standard deviation (SD) between duplicates. In the compared system, the SD of duplicates should be calculated; if the value is ≤ 0.05 , the SD of samples can be calculated. If the SD of the samples is within the range 0.134–0.196, the bias between Advia120[®] and the compared system is calculated as the difference between the value of 1.12 and the half of the sum of the duplicate (square root transformed). The resulting number

should be added to all the subsequent values for OFF-hr model score (Hb-60 $\sqrt{\text{Ret}}$).

The choice of a single instrument for controls reduces variability, but athletes are also examined periodically during competitions in laboratories equipped with various systems, other than those chosen by antidoping official reference laboratories. The differences could have potentially harmful consequences. On the other hand, the use of a single system, but in different laboratories, does not solve the problem.

4.1 Comparison Among Methodologies in Athletes

The Union Cyclistique Internationale (UCI) performed reticulocyte counts by means of a transportable analyser, the Sysmex R500[®]. The results were satisfactory, but the system throughput was not sufficient in some cases. When many samples had to be handled, the measurements were performed in a laboratory. The tests were performed within 12 hours of blood collection to avoid the maturation of reticulocytes. The reticulocyte data of 177 professional cyclists competing in the 2001 Tour de France, analysed using three different systems, has been published.^[50]

The comparison showed the best agreement between Abbott Cell Dyn 3500[®] and Bayer Advia120[®] showing an intercept of 0.2 and a slope of 0.8. The intercept was higher comparing Sysmex XE2100[®] and Abbott (0.41) or Bayer (0.49). The slope was good in comparing Sysmex with Abbott (0.90) and satisfactory in comparing Sysmex with Bayer (0.76). The poorest coefficient of correlation was 0.53 (Sysmex vs Bayer), the highest 0.77 (Sysmex vs Abbott).

The values of intercepts clearly demonstrate the need for an improvement in systems comparability. If we consider the biological variability of 5.8%, the differences among systems could exceed in an athlete having a normal value of 1% of reticulocytes

from three to eight times this variability. It should be pointed out that UCI has chosen Sysmex XT2000i® for official controls during and within competitions including OFF-model, derived from Bayer Advia120®: UCI has adapted the cut-off limits to 133 for male subjects to prevent instrument bias.

Bayer Advia120® and Sysmex XE2100® were compared using blood samples obtained from rowers in the French National team (83 males and 31 females; from two to eight blood drawings for each athlete in the period July 2001–March 2004). The samples of 66 males and 26 females were measured on Advia120®, and 1 month later the samples of 58 males and 18 females were measured on Sysmex XE2100®. Reticulocytes were consistently higher when measured on Advia120® ($p \leq 0.001$).^[40] In a previous study, the same author found good agreement between Advia120® and Sysmex XE2100®.^[49] The authors appropriately stressed that the type of instrument that supplies reticulocyte counting should be recorded. The bias adjustment must always be performed if different instruments are used for obtaining data from a single athlete.^[49] It could easily be performed for sizeable groups of athletes, for example during championships,^[48] but it is quite difficult to reach individual athletes during the season or during the training sessions. The passport concept^[25] is based on intra-individual variability of parameters calculated on at least five consecutive blood drawings: the comparability of data is mandatory for obtaining valid follow-up.

The evaluation of the reticulocyte count after a mountain marathon (48 km of rough trail involving ascent and descent of 7000 m) by means of three different systems have been performed by Banfi et al.^[51] The results were partially satisfactory because two of three systems were concordant. Bayer counts distribution differed from the other two. In 33 athletes, the median values of reticulocytes ($10^9/L$) were 52.9 on Abbott Cell Dyn 4000®, 42.9 on Bayer Advia120 and 48.7 on Sysmex SE9500®.

More recently, a comparison between Abbott Cell Dyn 3700® and Coulter LH750® has been performed on 80 professional male athletes (30 rugby players from the Italian National team, 30 footballers from a Second Division Italian Championship and 20 skiers from the Italian National Alpine Ski team) before training and competitions. There were no significant differences for Ret% (mean \pm SD: 1.05 ± 0.22 for Abbott and 0.93 ± 0.22 for Coulter) and for immature reticulocyte fraction (IRF%; mean \pm SD: 0.30 ± 0.05 for Abbott, and 0.28 ± 0.05 for Coulter). It should be pointed out that the comparison was performed between one system utilizing fluorescence and another one utilizing classical staining for reticulocytes. The high number of subjects and different sporting disciplines could reinforce the validity of the agreement.^[52]

The data observed in athletes, collected not only in the laboratory but also on the field, do not differ from the data reported for the general population. This result could support the use of various instruments for antidoping purposes. Some comparisons between methods prove satisfactory, but they are not sufficient nowadays to propose the interchangeability of systems.

5. Comparison of Reticulocyte Values Between Athletes and Sedentary People

The comparison between the values found in athletes and sedentary controls are illustrated in table VI.^[29,31,53]

Similar values are characterizing the two populations, but the studies are very few for a definitive judgment. It is noticeable that there are some differences in reticulocyte values between the two studies that used the identical method.

Mayr et al.^[29] have found that macroreticulocyte (particles having volume >120 fL) are higher in athletes (young ice skaters) than in nonathletes. These changes are compatible with increased erythropoiesis, even the number of reticulocytes is lower

Table VI. Comparison between reticulocytes (Ret) and related parameters between athletes (A) and sedentary controls (C)

Study	System	Sport	No. of subjects	Parameter	Results
Parisotto et al. ^[53]	Bayer H*3®	Cycling, swimming, rowing, track and field, boxing, cross-country skiing	A = 155 C = 23 (sex not specified)	Ret (10 ⁹ /L)	A: 46–51 ^a C: 40–52 ^a
				CHr (pg)	A: 30.7–31.2 ^a C: 30.4–31.4 ^a
				RetHb (g/L)	A: 1.14–1.59 ^a C: 1.24–1.61 ^a
Banfi et al. ^[31]	Coulter LH750®	Rugby, soccer, alpine skiing	A = 106 males C = 73	Ret (%)	A: 0.81 (0.30–1.54) ^b C: 0.84 (0.26–1.79) ^b
				MRV (fL)	A: 104.0 (93.1–114.8) ^b C: 103.6 (93.0–117.8) ^b
				IRF (%)	A: 0.29 (0.18–0.39) ^b C: 0.30 (0.19–0.42) ^b
Mayr et al. ^[29]	Bayer Advia120®	Speed skating	A = 60 males C = 14	Ret (10 ⁹ /L)	A: 63.44 ± 18.15 ^c C: 70.64 ± 14.17 ^c
				MCVr (fL)	A: 108.12 ± 3.17 ^c C: 103.81 ± 2.58 ^c
				CH (pg)	A: 30.22 ± 1.25 ^c C: 29.63 ± 1.79 ^c
			A = 56 females C = 17	Ret (10 ⁹ /L)	A: 58.99 ± 14.88 ^c C: 59.64 ± 15.14 ^c
				MCVr (fL)	A: 109.22 ± 3.00 ^c C: 105.31 ± 3.68 ^c
				CH (pg)	A: 29.89 ± 1.02 ^c C: 29.53 ± 1.11 ^c

a Interval.

b Mean (2.5th–97.5th percentile).

c Mean ± SD.

CH = haemoglobin content; **CHr** = reticulocyte haemoglobin content; **IRF** = immature reticulocyte fraction; **MCVr** = mean reticulocytes corpuscular volume; **MRV** = mean reticulocyte volume; **RetHb** = reticulocytes haemoglobin.

in male athletes. The destruction of blood cells may be increased in athletes, resulting in a greater turnover. No difference was found in Hb between athletes and non-athletes, but MCV and MCVr in both male and female athletes were significantly higher than in the control group. The authors stated that the mechanism underlying this phenomenon is not clear: metabolic acidosis typical of exercise may induce an increase in MCV and a corresponding decrease in mean erythrocytes corpuscular haemoglobin concentration (CHCM).

6. Differences Among Sporting Disciplines

Among 155 athletes competing in swimming, running, cycling, track-and-field events, boxing and

cross-country skiing, the cyclists had values significantly lower on Bayer H*3®.^[53] The authors did not explain these results, likely due to high variations of haematological parameters of cyclists during the competitive season.^[54]

In a group of 80 top-level male athletes, there were no significant differences for Ret% and IRF% (30 rugby players from the Italian National team, 30 footballers from a Second Division Italian Championship, and 20 skiers from the Italian National Alpine Ski team) before training and competitions. The technology used was Abbott Cell Dyn 3700®.^[52]

Similar findings were described in a group of 106 top-level athletes from the same sporting disciplines (55 rugby players from the Italian National team, 30

footballers from a Second Division Italian championship, 21 alpine skiers from the Italian National team) by using Coulter LH750® for Ret%, MRV and IRF,^[31] in a series of 55 athletes (24 professional soccer players and 31 professional skiers, including 20 men and 11 women) by using Abbott Cell Dyn 4000®,^[32] and, finally, in a group of 63 professional athletes, including 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 team belonging to the First Division National Italian championships, by using Abbott Cell Dyn 3700®.^[55]

In general, there are no striking differences among athletes competing in different sports, but the published data are still too limited to be able to make a definitive statement on the question.

7. Effects of Acute Exercise on Reticulocytes

There are few articles concerning the behaviour of reticulocytes and related parameters during short- and long-term exercise, and, more extensively, during a competitive season.

No differences after a mountain marathon in 33 athletes completing the race in a time ranging from 6 to 9 hours were found by using Bayer Advia120® for Ret%, MCVr, CHr and CHCMr and by using Cell Dyn 4000 for IRF. The effort was very strenuous, but the time was limited for determining modifications on reticulocytes: the intensity of the exercise is not a discriminant for reticulocyte fluctuations, as expected given the stability of haematological parameters in mountain marathon runners.^[51]

There were no observed changes in the reticulocyte manual count in 20 trained athletes before and after completing a 42.2-km race; reticulocytes remained inside the normal range.^[56]

If the exercise is intense and the period of time is longer, some modifications of reticulocytes are evi-

dent. In a 1600-km ultramarathon, manually counted Ret% for seven males and two females were (mean) 0.8 before the start of race, 1 after 4 days, 1.3 after 11 days and remained 1.3 at the end of race. The increase in reticulocytes was accompanied by a fall in Hb from 14.9 g/dL before the race to 13.4 g/dL at 11 days and 13.9 g/dL at the end of the race.^[57]

Intensive training could enhance iron depletion, particularly in female athletes. To establish whether reticulocyte parameters were sensitive to iron-deficient erythropoiesis in athletes, reticulocyte profiles of five female athletes diagnosed with depleted iron stores were compared before and after iron therapy to seven controls. CHCMr and MCVr, obtained by Bayer H*3®, showed little variation over time in iron-replete females, with 95% of all fluctuations being within 5.8% and 4.3% of original values, respectively. Iron supplementation in athletes with depleted iron stores elicited an increase in CHCMr ($p = 0.01$), and a decrease in the distributions of reticulocyte volume (RDWr, $p = 0.01$) and cell haemoglobin concentration (reticulocyte haemoglobin distribution width [HDWr], $p \leq 0.01$). The ratios of reticulocyte to mature cell MCV ($p \leq 0.01$) and CHCM ($p \leq 0.01$) also changed following iron therapy: the monitoring of reticulocyte parameters can be of use in detecting iron-deficient erythropoiesis in female athletes.^[58]

The evaluation of short-term endurance exercise on haematological variables included in the mathematical indirect detection models for suspecting rHuEpo was investigated in 23 male professional cyclists and 16 sedentary control subjects during a 5-day road cycling stage race at sea level. The blood samples were obtained every morning prior to the race and between 1 and 3 hours after the end of the stage. The samples were analysed on Bayer Advia120®. During the race, Ht and Hb decreased significantly from the start to the end of the experimental period, whilst Ret% were not modified.^[12]

Haemoconcentration occurs during acute exercise and could be considered when reticulocyte counting is performed and interpreted, as well as haemodilution during long-term exercise. No specific studies on this matter have been published.

The effects of exercise on reticulocytes seem to be proportional to the intensity of physical exertion and some days of intense effort are needed to induce reticulocyte modifications.

8. Effect of Long-Term Exercise and Training on Reticulocytes

In the 2004–5 season, four blood samples were taken from athletes in the Italian Rugby National team. The first sample was collected in August at the start of the training period, the second in September after the training sessions and before the start of the championships, the third in January after the first part of championship and before the start of the Six Nation tournament and the fourth in May at the end of the national championship. Ret% measured by Abbott Cell Dyn 3500® was 1.08 at first blood drawing, 1.05 at second, 0.90 at third, and 0.85 at the final drawing. The variations of reticulocytes during the season were significant when the first value was considered as basal.

In this study, reticulocytes were in accordance with the trend of Hb and RBC. Hb showed higher levels during the first half and decreases in the second half of the season. However, Hb concentration fell always inside the physiological range.

Two considerations are worth highlighting: (i) reticulocytes, Hb and RBC show the same fluctuations during a season; and (ii) reticulocytes have some variations during a season, although the concentrations, as well as other RBC parameters, are always physiological. These considerations are of value for antidoping purposes, when threshold based on reticulocytes and/or Hb are used.^[33]

The individual variation of reticulocytes during a season in 96 elite athletes of various sports was

found to be 21.3%, not physiologically significantly relevant, as judged by the authors.^[59]

The variations of Ret% and IRF were not significantly modified during a whole competitive season (2003–4) in alpine skiers from the Italian National team, using Abbott Cell Dyn 4000®. Samples were taken in May, at rest, before the start of training, in July, during the specific training programme, in September, during the specific training programme, in November, before the start of international competitions, and, finally, in May, at rest. The males came from the team of downhill (n = 4), special (n = 5) and giant (n = 8) slalom teams, whereas females came from the special slalom team (n = 9). The mean values of Ret% passed from 1.11 to 0.91, 1.21, 1.09 and 1.01. The IRF values were stable, respectively, 0.30, 0.30, 0.33, 0.27 and 0.29.^[60]

The IRF was described as relatively high in athletes,^[32] testifying the continuous stimulation of bone marrow in sportsmen because of exercise-induced haemolysis.^[6] The values of IRF were higher than those proposed as a reference range in the general population, by using the same technology, in earlier studies performed on this parameter.^[61] In a more recent study, the range of IRF, released by the same technology, is wider;^[36] so, the athletes values are inside the general reference interval obtained from sedentary people. A specific study concerning the behaviour of reticulocyte counts and IRF during a season in athletes from different disciplines was performed in a group of top-level sportsmen: 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 team belonging to the First Division National Italian championship. The data were collected from athletes who were always present at the sessions when blood samples were taken for monitoring their health status, during the whole season. These check-ups generally occurred three to four times: (i) before the start of the training

period (pre-competitive phase); (ii) at the beginning of the competition season; (iii) in the middle of the competitive season; and (iv) at the end of the competitive season. All the athletes were males; the age range was from 19 to 36 years. The recruited athletes performed principally aerobic sports, which have some common aspects: an extended competitive season, a period of heavy training before the competition season, and high intensity and frequency of competitions. Reticulocyte counts in athletes of various sports show, during a competitive season, values always falling into the known reference intervals.

In rugby players and skiers, the decrease of reticulocytes during the season was parallel to the decrease of Hb, whereas in cyclists and soccer players this behaviour was not confirmed. Lack of correlation between the two parameters is shown for all the sports during the season. IRF increased in cyclists and skiers during the competitive season, whereas it was substantially stable in rugby and soccer players.^[55]

Regarding soccer players, similar results were reported on 27 athletes, controlled for three consecutive years. In that study, the mean reticulocyte counts were 1.11% in the initial phase of the season (July–September), 0.97% in the central portion (October–January) and 0.99% in the final phase (February–May).^[25]

The reticulocyte values are consistently stable during the competition season, but some modifications, although they do not exceed the reference intervals, are described. The narrowing of variability in the teams, as judged by standard deviations of data collected during the season, indicates that the modifications are typical of exercise in professional athletes, accurately followed by physicians, nutritionists and trainers.^[55]

Studies during the season of competitions with more blood drawings can explain the real behaviour

of changes of the haematological parameters, and especially of reticulocytes.

The data about the haematological response to training are contentious.

Training could induce an increase in reticulocyte number (manual count), as observed in six individuals undergoing a 45 minutes/day ergometer exercise five times a week; the reticulocyte increase was noticeable from the second day until the programme was completed.^[62]

In a group of 30 healthy male athletes, involved as control subjects in a study of effects of administration of Epo, the physical activity for 13.6 hours per week (range 5–40 hours) did not induce changes, as judged by the authors; however, reticulocytes, manually counted, had two peaks of values at days 2 (increase of 100%) and 14 (increase of 89%) of the training, not followed by an increase in Hb and RBC.^[63]

8.1 Effect of Altitude

Hypoxia enhances the Epo gene transcription and HIF-1 is the factor responsible for the response. The gene expression is upregulated when hypoxia is present for more than a few minutes. A period of time spent by athletes at high altitude is a natural and permitted way to increase the blood oxygenation. The altitude exposure is a controversial argument in sports medicine, but it is accepted that the hypoxia effect appears for most individuals at an altitude between 2100 and 2500 m for a stay of at least 24 hours.^[64]

The exposure to the altitude did not stimulate RBC production on elite female road cyclists who spent 12 nights at 2650 m and on endurance athletes who spent 23 nights at 3000 m.^[65,66]

A marked increase in Hb was evident in a group of 11 male track cyclists for the exposition to an altitude of 2690 m for a month. The increase in Hb (from a mean of 14.5 to 16 g/dL) was parallel to the plasmatic Epo increase, but Ret% were substantially

stable (from 1.3 ± 0.3 to 1.4 ± 0.3 ; technology: Bayer Advia120®).^[67]

In six Kenyan runners training at 2100 m, reticulocytes were even lower during the period spent in altitude compared with the sea-level concentrations. It could be remarked that the runners were born and lived at an altitude of about 2000 m.^[67]

Friedmann et al.^[68] reported that altitude training for 18 days at 1800 m induced an increase of Hb and reticulocytes. The study consisted of two parts both investigating the athletes of the German National boxing team, before, during and after the annual endurance training camp. A group of 17 boxers trained at 1800 m for 18 days, nine of them with iron supplementation, and seven without iron supplementation. In a follow-up study, 13 boxers trained at 800 m for 14 days without iron supplementation (control group). Reticulocytes were manually counted. During training at moderate altitude, reticulocytes were significantly increased on days 7 and 18 in both studied groups and then reached baseline values after the return to sea level. Hb was significantly increased on day 18 in both groups, and only in the group without supplementation after 7 days of altitude. In the group trained at low altitude, reticulocytes were significantly increased on days 7 and 13 of training and remained high after the training period; Hb had no changes in this group.

The authors interestingly supposed that the increase in reticulocytes is more consistently linked to the increased turnover of RBC in trained athletes than to different altitude, as testified also by changes of haemolysis biochemical parameters haptoglobin and lactate dehydrogenase. Also Parisotto et al.^[53] found an increase in RetHb (reticulocytes haemoglobin), measured by Bayer H*3®, in athletes (12 female cyclists, 12 male triathletes, 12 swimmers and 13 elite cyclists) who trained and lived at a natural altitude of 1780 m or 2690 m. In contrast, RetHb were unmodified in the corresponding group of 24 athletes (swimmers, cyclists, runners and

triathletes) submitted to artificial altitude of 2500–3000 m.

Even though the published data are contentious, it can be noted that ‘moderate’ altitude has an evident effect on Hb and Ht, but the reticulocytes do not show a corresponding increase.

8.2 Intermittent Hypoxia

The intermittent hypobaric hypoxia simulates the altitude exposure, and could be used in lowlands for improving oxygenation, stimulating bone marrow through the artificial hypoxia. Abellan et al.^[69] studied changes in haematological parameters after 4 weeks of intermittent hypobaric hypoxia (3 hours/day, 5 days/week simulating altitude of 4000–5500 m) on 16 male triathletes divided into two groups: eight were exposed to hypoxia and eight to normoxia. Blood samples were taken before the start of hypobaric hypoxia, at the end of hypoxia, and 2 weeks from the last exposure; the blood was analysed by Sysmex XE2100®. In the group exposed to hypoxia, a significant increase in Epo was observed, but no changes were recorded for other parameters (Hb, RBC, soluble transferrin receptor, platelets). Reticulocytes and RET-Y (the mean value of the forward-scattered-light histogram within the reticulocyte population) showed significant differences, but occurring also in the control group, possibly linked to training and not to the hypobaric exposure: intermittent hypobaric hypoxia causes Epo production without producing an effective subsequent erythropoiesis.

The ineffectual power of intermittent hypoxia exposure to enhance RBC and Hb production, despite the Epo concentration increase, is confirmed in a study of 23 collegiate-level athletes (swimmers and runners) exposed for 4 weeks to hypoxia for 3 hours/day, 5 days/week, at a simulated altitude of 4000–5500 m. In the group of treated athletes, the absence of any evidence of accelerated erythropoiesis was demonstrated by the lack of change in solu-

ble transferrin receptor concentration, and haematological parameters, including reticulocytes and related indexes, measured on Bayer Advia120®.^[70]

The increase in reticulocytes after intermittent hypoxia (180–300 min/day at simulated altitude of 4000–5500 m for 9–17 days) was described as sufficient to increase Ht, Hb, RBC and also to double the percentage of reticulocytes, manually counted.^[71] It can be concluded that simulated high altitude has an effect on Epo concentrations, but the increase of hormone is not followed by reticulocyte production. In fact, Lundby et al.^[72] verified that artificial exposure at 4100 m for 2 hours/day over a period of 14 days did not change haematological parameters, including reticulocytes measured with Sysmex R-3000®, in eight well trained subjects; Ashenden et al.^[73] found that in 11 athletes who were exposed to a simulated altitude of 2650 m for 8–11 hours Epo levels increased by 57%, significantly more than athletes who remained at sea level, but reticulocytes, CHCMr and RetHt were not modified.

8.3 Living High-Training Low

A method of discontinuous exposure to hypoxia, named 'living high-training low' (LHTL) was first proposed by Levine and Stray Gundersen.^[74]

This training model is now popular among athletes and coaches; LHTL is thought to improve sea-level aerobic performance, because hypoxia increases erythropoiesis and because training performed at low altitude or at sea level preserves training intensities, oxygen flux and muscle activity. Robach et al.^[75] investigated the effect of LHTL on erythropoiesis and aerobic performance in 18 swimmers (2 women, 16 men) of the National French team, and the relationship between haematological modifications with the duration of performance enhancement. Blood tests were performed on Abx Pentra120® analyser. The athletes participated in a training camp held at 1200 m and were divided into two groups of nine subjects. One group was submit-

ted, in hypoxic rooms for 16 hours/day, to a simulated altitude of 2500 m for 5 consecutive days and then at 3000 m for the next 8 days; the other one served as control group. The period of 13 days of LHTL was sufficient to determine an increase in RBC volume in the swimmers. Ret% increased significantly in the LHTL-treated group, whereas a significant decrease was found in the control group, and returned to baseline values after 15 days from exposure. Hb and Ht were not significantly modified. The study clearly showed the bone marrow stimulation, but this did not involve a parallel improvement in aerobic performance. Moreover, the normalization of Ret% and the level of aerobic performance, after 2 weeks, indicated the absence of a persistent effect of LHTL.

In a companion paper, the same authors did not find the Ret% modifications in 11 Nordic skiers of French elite teams (seven biathletes, two Nordic-combined skiers and two cross-country skiers; six women, five men), exposed at altitudes of 2500, 3000 and 3500 m. It is probable that the exposure of 11 hours/day, lower than the recommended threshold of 12 hours/day, was not sufficient to stimulate reticulocytes. The authors stated that LHTL stimulated erythropoiesis, without advantages for maximal oxygen transport, because Hb and Ht were significantly increased after exposure at an altitude of 3000 m and remained elevated at 3500 m.^[76]

In the study of Wehrin et al.^[77] on ten athletes (five men and five women) of the Swiss National orienteering team, Ret%, measured in a flow cytometer (Epics XL; Beckman Coulter), showed a significant increase after altitude exposure (living at 2456 m, training at 1800 m with low intensity, and training at 1000 m at high intensity), when measured 8 days after the LHTL period. The increase started after 24 days of exposure to the elevated altitude, when also Ht was significantly increased, but Ht was not different from the baseline value after the return to sea level. The authors stated that it is not clear if

the increase in Ret% was caused by hypoxia or by training.

The data reported in these studies do not allow a clear conclusion to be drawn about the effects of LHTL on blood parameters and particularly on reticulocytes. Differences in training workload, altitudes, time of exposure to hypoxia and aerobic power of athletes do not induce homogeneous conditions to correctly judge the reticulocyte modifications.

9. Doping

The earliest haematological sign of heavy stimulation of the bone marrow is the reticulocyte count. A significant increase in the reticulocyte count, on Bayer H*3[®], was already reported in the first experiments of rHuEpo administration in athletes.^[78]

Reticulocytes, in nine athletes treated with rHuEpo subcutaneously (SC) at 50 U/kg for 26 days, passed from 50 to $137 \times 10^9/L$ during 10 days of treatment. Reticulocytes, automatically measured without specification of the system used, were significantly increased from day 10 to day 24 during the treatment. Hb and Ht significantly increased after 26 days and remained significantly higher than the basal values for 7 and 14 days, respectively.^[79] The significant increase in reticulocytes was evident after 2 days from the start of Epo treatment with high doses (200 U/Kg); the number of reticulocytes, manually counted, remained elevated for the following 12 days, and decreased to baseline levels after 24 days when they were lower in treated subjects than in the control group. After 24 days, in accordance with the lifespan of RBC, Hb and Ht were elevated, whilst reticulocytes were low.^[63] In a study designed for modelling pharmacokinetics of Epo in athletes, reticulocytes increased on the third day after rHuEpo SC treatment at a usual dose of 50 U/kg.^[80]

The introduction of reticulocytes and related parameters in antidoping protocols originated from

the work of Australian researchers before the 2000 Olympic Games.^[3] The aim of their studies was the definition of thresholds for haematological parameters to identify suspected stimulation of bone marrow, by using routine methodology. The method was not approved by the Olympic Committee as an antidoping method because it is an indirect method and the 'specificity' does not fulfil the strict eligible criteria, but it was accepted as a screening method for identifying suspected haemodoping and for selecting subjects for checking with the direct method (recognition of rHuEpo in urine by means of isoelectrofocusing). The 'Australian protocol' has been re-evaluated twice,^[4,48] simplifying the algorithms used for defining threshold, and reducing the laboratory parameters involved, and, finally, introducing the concept of individual variability (variance of parameters, namely Hb and OFF-score).

The original study plan^[3] envisaged 27 voluntary individuals who performed regular exercise and sport. After 5 weeks of training, over a period of 25 days rHuEpo or placebo were administered and, later, the treatment was discontinued for 4 weeks (wash-out). The studied subjects were divided into three groups; the first had a treatment of rHuEpo 50 U/kg and intramuscular iron, the second rHuEpo 50 U/kg and iron by oral administration and the third placebo. The blood drawings were performed 14 days before the start of rHuEpo treatment (basal value) and at days 1, 3, 10, 15, 17, 22 and 24 during the treatment and at days 5, 7, 12, 14, 19, 21, 26 and 28 during the wash-out. The analyses were performed on a Bayer H*3[®]. Hb and Ht were higher in the two groups treated with rHuEpo from the third week of therapy until 21 days after its cessation, reticulocyte count doubled in rHuEpo-treated subjects, MCVr increased in rHuEpo-treated subjects, the Hb content of reticulocytes increased in rHuEpo-treated subjects and the relative packed reticulocyte volume was increased in rHuEpo-treated subjects.

The principal haematological modification after the rHuEpo administration is the increase in reticulocytes.

On the basis of differences between rHuEpo-treated groups and a placebo-treated group, two scores were proposed, named ON and OFF. Values superior to the threshold of the ON-model indicated that the subject was taking the hormone, whereas values higher than the OFF-model indicated that the subject had taken the hormone, because this model was obtained from the data collected in the period included from days 12 and 21 after the end of administration. In the models, the reticulocyte parameter used was the RetHt; the formula, for example, for the OFF-model was $6.149 \text{ Ht} - 92.87 \text{ RetHt} - 0.1463 \log(\text{Epo})$. The same group of researchers studied reticulocyte parameters to discover Epo abuse by the applications of cut-off of single parameters, chosen as the 95th percentile of the distribution of reticulocyte count, CHr, RetHb, RetHt on 155 athletes not taking rHuEpo against 24 treated. For example, the reticulocyte count cut-off of $221 \times 10^9/\text{L}$ is discriminant. The limit of the cut-offs of single parameters was the possibility that long-term but low doses of hormone had been taken by athletes, not sufficient to cause reticulocyte homeostasis alterations.^[53]

The use of low doses of rHuEpo to adjust Hb and/or Ht values avoiding the crossing of the thresholds imposed by sports federations, but increasing oxygenation and performances and the need for using parameters not influenced by storage and, especially, by RBC volume induced the authors to propose a new version of the scores.^[4] Reticulocytes were still included in the OFF-model scores: OFF-hr = $\text{Hb} - 60\sqrt{\text{Ret}}$, OFF-hre = $\text{Hb} - 50\sqrt{\text{Ret}} - 7 \ln(\text{Epo})$, where OFF-hre = OFF-model containing Hb, Ret% and Epo concentration. The square root was introduced to obtain a normal distribution. The use of the reticulocyte count, and not of RetHt, allowed the implementation of the formulae also for systems commer-

cialized by producers other than Bayer, which was a conceptual and practical limitation to the universal adoption of the model.

With the use of logistic regression analysis, the OFF-model allows identification of 67–72% of users several weeks afterwards with a 100% specificity, even when low doses are administered.^[81]

The concept of the use of some thresholds of haematological parameters to combat and limit blood doping and to exclude athletes from competitions if they have abnormal values has been applied in a third-generation approach of indirect models to detect rHuEpo abuse in athletes.^[48] The proposed approach is based on a baseline value of a single athlete; its subsequent values are compared with the baseline.

A universal value for intra-individual variance using the highest value obtained from four cohorts of male athletes has been established. The baseline of the athletes and general variability are integrated in statistical formulae. Hb and OFF-hr model, including $\sqrt{\text{Ret}}$ are proposed for this haematological passport: almost five results^[25] for each athlete are necessary to establish a baseline. The principal factor influencing the reticulocyte values on the OFF-hr model is the type of instrument used for reticulocyte counting: the standardization of systems proposed by Ashenden et al.^[49] is claimed to obtain comparable values. It should be specified that the data used for establishing universal values of intra-individual variability were obtained from various technologies.

Varlet Marie et al.^[82] proposed the use of selected erythroid gene markers for detecting rHuEpo effects. They selected the *HBB* gene, which regulates the synthesis of β chain of Hb, the gene *FTL*, which determines the synthesis of L chain of ferritin, and the gene *OAZ*, which causes the inhibition of enzyme ornithine decarboxylase. The evaluation of genes in reticulocytes of ten recreational athletes divided in two groups, the first ($n = 6$) receiving

rHuEpo three times a week for 6 weeks with dosages of 50 U/Kg for 4 weeks and a maintenance dosage of 20 U/Kg for 2 weeks, the second (n = 4) receiving placebo, demonstrated their activation in the treated group. The use of gene activation is a promising technique, especially for the silent period of 4 days, when isoelectrofocusing cannot identify Epo in urine. Nevertheless, the specificity of reticulocyte gene movement could be further studied because an increase in *FTL* and *OAZ* was evident in the placebo group, who received intravenous iron. Moreover, inter-individual variability is high for *HBB* and *FTL* in the treated group.

There are other expressed genes in reticulocytes that could be useful also in antidoping research. For example, using serial analysis of gene expression among adult human reticulocytes, over 70% of sequenced transcript tags encoded the adult β -globin gene, even in the absence of α -globin transcripts.^[83] The role of newly discovered α -like globin gene, μ -globin, is still not defined, but it is interesting that although the μ - and γ -globin genes are detected at lower levels in adult blood, their signals were still ranked among the highly expressed genes.

Among the highly represented transcripts, there is the mitochondrial solute carrier protein or mitoferrin: the importance of this gene for erythropoiesis is conserved in humans. The relative overrepresentation of *AHRR* binding motifs in the promoter regions of highly represented genes is of particular interest. Since hypoxia is of crucial importance for the regulation of erythropoiesis, further exploration of *AHRR* transcription factors in erythroid cells is warranted. The increased *MAP17* expression in adult erythroid cells is another interesting finding, possibly inducing plasma lipid modifications in adults. Four transcripts (*MAP17*, *FLJ32009*, *ARRB2*, *FLJ27365*) were identified as being upregulated in the adult blood transcriptome. Further studies on these transcripts could be useful also in sports medicine.^[84]

The reliability of tests for raising suspicion of and establishing rHuEpo doping has caused the resurgence of the old blood-boosting technique of transfusions. Reticulocyte parameters have an ancillary role in the detection of this illicit means for increasing oxygen delivery to the muscles; however, recent studies have focused attention on their modification during and after blood donation and reinfusion. In autologous blood transfusion, where the blood donor and transfusion recipient are the same, the increase in Hb, as expected, and the decrease in serum Epo concentration are described.^[85] Reticulocyte count, measured by Bayer Advia120®, and also soluble transferrin receptor also decline progressively from day 7 to day 21 following transfusion; the authors pointed out that alterations of Epo, reticulocytes and soluble transferrin receptor were observed at all times through this investigation, suggesting that determination of these biomarkers could be used as supportive evidence for erythropoietic manipulations with short-term Hb increases of >7.5%.^[86]

The modifications of reticulocytes are testified by studies in blood donors. For example, blood donation of 450 mL from 21 healthy donors controlled on days 2, 4, 7, 9 and 14 after the donation resulted in an increase in hypochromic reticulocytes from day 1 to day 2, which peaked on day 9 with a maximum increase of 178% on Bayer Advia120®. The total reticulocyte count increased from 62 to 96 $\times 10^9/L$ with a maximum change of 55% and the time for significant change was 1 day, as for Hb. In 28 blood donors, a treatment (rHuEpo or placebo) was held. Hypochromic reticulocytes in rHuEpo-treated group increased significantly from day 1 to day 4 and peaked on day 11 with a maximum increase of 232%. The same course was seen in the placebo group, but the increase was significantly lower than in the rHuEpo-treated group.^[87] The reticulocyte count, in association with immunological

and genotyping tests, could prove useful in this field.

The Epo doping does not necessarily lead to increased reticulocyte count since recent data demonstrate that the increase in reticulocytes during prolonged treatment with low doses of Epo is only transient and is not detectable after a few weeks of treatment.^[88]

A new interesting approach for fighting blood doping is the proposition of discriminative algorithms, based on statistical classification of indirect biomarkers of altered erythropoiesis, appropriately validated strictly following the standards of antidoping agencies, and called 'abnormal blood profile score'. The score could be used not only for Epo abuse, but also for autologous transfusions.^[89]

10. Conclusions

The peculiar sensitivity of reticulocytes in the case of bone marrow stimulation by using rHuEpo indicates that this haematological parameter is irreplaceable, together with Hb, for testing athletes during competitions. The studies on the behaviour of reticulocytes in sports medicine have been stimulated from the introduction of the parameter in the Australian protocol. The reliability of the parameter in pathologies led to its introduction in sports medicine, but in subjects with less evident data modifications, even during and after rHuEpo administration, often performed by using low doses of hormone. Moreover, the administration in subjects having a normal RBC mass may not always prove effective or as effective as the user wants, because of homeostatic mechanisms, such as neocytolysis, which are not completely known.^[90]

Reticulocytes were first used in the antidoping context and were later evaluated in athletes, in physical exercise, and in training at sea level and at altitude.

The data reported in this research confirmed the validity of reticulocyte use in the antidoping con-

text, because the reference intervals in athletes do not differ from those of the general population. However, the similarity of reference ranges of athletes and non-athletes is not sufficient for judging reticulocyte abnormalities as doping in athletes.

Moreover, a mix of doping and pathologies that influence the number of reticulocytes could be advocated and considered, although specific descriptions of cases are not found in the literature.^[5]

Additional studies are needed, however, to define the modifications that may be caused by training and competitions: the data are scarce, and, in the case of training in altitude, controversial results emerge. The definition of the variability of reticulocytes is mandatory, due to their relatively high biological variability and to low individuality index, for a correct introduction, one that is not open to criticism, of the parameter in the haematological passport, which rightly includes variance of data.

A special effort should be made for planning intermethod comparisons in athletes.^[91] The automation of reticulocyte counting has made a wide use of the parameter, also in sports medicine, but discrepancies among systems exist, which may limit the impact of laboratory results. Some years on from integration of reticulocytes in automated systems for haematology, the scenario of reticulocytes and related parameter characteristics is practically defined. The use of reticulocyte counts can be recommended, considering satisfactory data observed when comparing systems, whereas the reticulocyte-related parameters have not yet become definitely comparable. It might be advisable that the system used for analyses should always be reported, not only in published studies, but also in the official records of sports agencies and in the haematological passport of the athlete, once it is established. The use of fixed and quite wide thresholds (e.g. 0.2–2.4% in cycling) should be recommended until intermethod comparison is improved. More data should be published to

enable a better knowledge of the behaviour of reticulocytes and related parameters in athletes.

The present author would like to see more studies in 'sports haematology' and fewer studies on 'blood doping'.

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