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Changes in hemoglobin profile reflect autologous blood transfusion misuse in sports

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Abstract

The changes in hemoglobin (Hb) profile following autologous blood transfusion (ABT) for the first time were studied for anti-doping purposes. Twenty-four healthy, trained male subjects (aged 18–40) were enrolled and randomized into either the transfusion (T) or control (C) groups. Blood samples were taken from the T subjects at baseline, after withdrawal and reinfusion of 450 ml of refrigerated or cryopreserved blood, and from C subjects at the same time points. Hematological variables (Complete blood count, Reticulocytes, Immature Reticulocytes Fraction, Red-cell Distribution Width, OFF-hr score) were measured. The Hb types were analyzed by high-performance liquid chromatography and the Hemoglobin Profile Index (HbPI) arbitrarily calculated. Between-group differences were observed for red blood cells and reticulocytes. Unlike C, the T group, after withdrawal and reinfusion, showed a significant trend analysis for both hematological variables (Hemoglobin concentration, reticulocytes, OFF-hr score) and Hb types (glycated hemoglobin-HbA1c, HbPI). The control charts highlighted samples with abnormal values (> 3-SD above/below the population mean) after reinfusion for hematological variables in one subject versus five subjects for HbA1c and HbPI. A significant ROC-curve analysis (area = 0.649, $p = 0.015$) identified a HbA1c cut-off value ≤ 2.7% associated to 100% specificity of blood reinfusion (sensitivity 25%). Hemoglobin profile changed in trained subjects after ABT, with abnormal values of HbA1c and HbPI in 42% of subjects after reinfusion. Future studies will confirm the usefulness of these biomarkers in the anti-doping field.

Keywords Hemoglobins · Biomarkers · Doping in Sports · Blood transfusion autologous

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Introduction

The unavailability of a method to detect the misuse of autologous blood transfusion (ABT), a prohibited performance-enhancing method in sport, led researchers to focus on the effects of ABT to identify markers enabling its indirect detection [1–3]. Abnormal changes in hematological parameters such as hemoglobin (Hb) or OFF-hr score, which are identified following longitudinal evaluation by an adaptive model, are the basis for the athlete biological passport (ABP) hematological module [4, 5] that was adopted by the World Anti-Doping Agency.

Considering the potential confounding factors affecting hematological parameters (altitude, physiological fluctuations, and masking actions) [3, 6, 7] and the limited usefulness of the OFF-hr score [8], novel biomarkers to enable increased sensitivity and specificity of the ABP have been recently proposed [9–12]. Actually, following blood

manipulation, withdrawal, and reinfusion, the changes in oxygen availability may generate integrated molecular responses including changes of the components of the Hb profile. Fetal hemoglobin (HbF), which differs in structure from adult Hb, is normally present at very low levels (0.0–0.9%) in adults [13] but its synthesis can be induced by hypoxia or pharmacological stimulation causing hypoxia [14, 15] with a possible link through the hypoxia-inducible factor system. Slight HbF changes have been observed in physiological [16, 17], pathological, or stressful conditions including blood transfusion [18–20]. Variations were also observed for glycated hemoglobin (HbA1c) following donation of autologous blood for elective surgery or red blood cell transfusion [21, 22]. However, the Hb profile has never been tested for anti-doping purposes. This study, as a part of the project "Novel Molecular biomARkers for detection of autologous blood transfusion in sport: fetal HemOglobin and microRNAs (MARATHON)", aims to determine whether changes in HbF levels and in the Hb profile occur following blood withdrawal and reinfusion, and whether they represent potential biomarkers that are useful in the anti-doping field for ABT detection.

Methods

The subjects were contacted for a possible enrollment in the study via information sheets distributed to university students participating in sports groups. The inclusion criteria included Caucasian male subjects with an age between 18 and 40, healthy conditions, adequate training level (> 4 h/ week), not participating in competitions of any level during the period of the study. The exclusion criteria included current smoking, anemia or altered blood counts at baseline, diabetes, hypotension and continuous use of medications.

Subjects satisfying the inclusion criteria and providing written informed consent were randomized into two groups via a simple randomization process with an allocation ratio of 1:1 between the transfusion (T) and control (C) groups. The study was approved by the Ethics Committee of Ferrara (number: 06/2014).

For both groups, seriated blood samples were obtained (Fig. 1), corresponding to three study periods: (1) baseline: day − 40 (D-40); (2) withdrawal: day −25 (D-25), day − 15 (D-15), and day 0 (D0); (3) reinfusion: day +3 (D + 3); day +6 (D + 6); day +15 (D + 15);

Blood samples also collected at D-35 and at D + 35 were not considered for the study being potentially influenced by dilution (hyper hydration before withdrawal) or variations of training status or smoking habits, respectively.

Venous blood samples from an antecubital vein were drawn into four 5-mL and one 2.7-mL collection tubes (Sarstedt S-Monovette, Sarstedt AG, Nuembrecht, Germany) containing ethylenediaminetetraacetate (EDTA) as an anticoagulant. From each volunteer in the T group, one bag (450 ml) of blood was collected at D-35. The blood was either refrigerated ($n = 6$) or cryopreserved ($n = 6$) until reinfusion, which was performed 35 days after donation (D0). Packed red blood cells (RBCs) and plasma samples were isolated and RBCs stored at 4 °C in SAGM (storage solution composed of saline, adenine, glucose, mannitol) or at − 80 °C (cryopreserved group) until reinfusion. Refrigerated blood was carefully warmed to 37 °C before reinfusion, whereas cryopreserved blood was defrosted and treated according to the international standard of transfusion medicine procedures, that were followed for all the interventional procedures.

At the day corresponding to blood withdrawal and autologous reinfusion, subjects in the T group were asked to avoid stressful conditions and heavy training sessions, and to maintain an adequate nutritional status. Before blood donation, participants were recommended to drink about 400 ml of water and to have a light breakfast, avoiding milk and its products. All the procedures were performed between 7:30 and 11:00 AM, by unblinded qualified personnel at the Blood Transfusion Service of Ferrara, Azienda Ospedaliero-Universitaria Ferrara.

The EDTA samples underwent a full blood count analysis using a fully automated hematology analyzer (Sysmex XE-2100, Sysmex, Norderstedt, Germany) at Laboratorio analisi chimico-cliniche e microbiologia of the Azienda Ospedaliera-Universitaria Ferrara. The parameters measured included RBCs, Hb concentration [Hb], hematocrit

Fig. 1 Study design. *T* transfusion, *C* controls
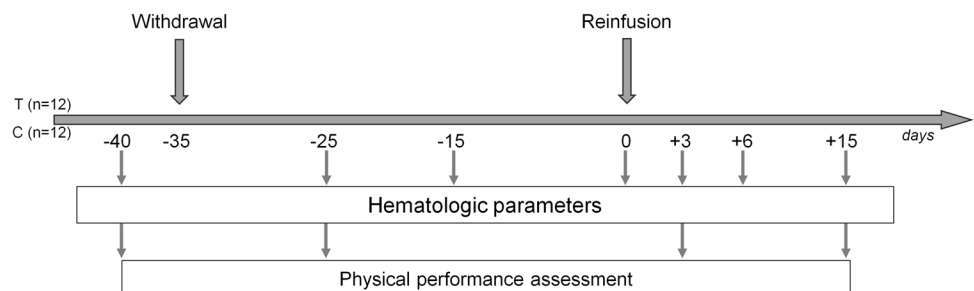
(HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cells (WBC), platelets (PLT), reticulocytes (Ret), immature reticulocytes fraction (IRF), red cells distribution width (RDW), and OFF-hr score (calculated as [Hb] divided by a constant value of 60 multiplied by the square root of reticulocytes).

The complete Hb analysis performed by high-performance liquid chromatography (HPLC) allowed the identification of the following Hb types: HbF; HbA; HbA2; glycated hemoglobin (HbA1c); and Hb A1d aldimine fraction (HbA1d). To enable an overall analysis of the profile, the Hemoglobin Profile Index (HbPI) was arbitrarily determined by calculating the ratio between the product of HbA and HbA2 values and HbA1c.

For each sample, EDTA-treated whole blood was harvested (100 µl) and washed once with sterile $1 \times$ Dulbecco's phosphate-buffered saline (DPBS). The cells were then lysed by adding H_2O (dilution 1:10 v/v), vortex mixed, incubated on ice for 20 min, and centrifuged at $12,000 \times g$ for 5 min. The hemolysate supernatant was separated from the membrane debris, diluted in H_2O (1:5) and injected (20 µl) onto the column. Hb proteins present in the lysates were separated by cation-exchange HPLC (PolyCAT-A column), using a Beckman Coulter Instrument System Gold 126 Solvent Module-166 Detector (Brea, CA, USA). Samples were eluted in a solvent gradient using aqueous sodium chloride–BisTris–KCN buffers and detection was performed at 415 nm. The standard controls were the purified HbA (Sigma, St. Louis, MO, USA) and HbF (Alpha Wassermann, Milano, Italy) [23, 24]. Values of HbF below the lower limit of quantitation were substituted with an arbitrary score of 0.01.

Statistical analysis

Data are presented as the mean $\pm$ standard deviation for continuous variables or number and percentage for categorical variables. Data distribution was tested using the Kolmogorov–Smirnov test. A baseline between-groups comparison was performed using the Student's *t* test or a Mann–Whitney *U* test, as appropriate. Within-group testing was performed using the paired Wilcoxon test, while the between-group comparison of all parameters was performed using an analysis of variance (ANOVA) or a Kruskal–Wallis test, as appropriate.

Trend analyses of hematologic and Hb profile parameters were performed via a repeated measures ANOVA. The analysis of hematological variables and Hb types that showed significant changes in the between- and within-group analyses, was implemented with control charts to identify samples of T subjects with values exceeding 2- and 3-standard deviations (SD) from the entire non-treated population (mean of all values of C group and of values at D-40 for the T group). Samples with values exceeding the cut-off set for each parameter were labelled as atypical (> 2-SD) or abnormal (> 3-SD).

Receiver operating characteristic (ROC) curves were calculated to assess sensitivity and specificity of HbA1c to verify whether the variations of this parameter observed in the reinfusion phase may be of potential interest in the ABT detection. ROC curves were performed comparing samples free from any treatment (all samples of C athletes and samples at D-40 of T subjects) to samples following reinfusion (D + 3, D + 6 and D + 15 of T group).

A value of $p \le 0.05$ was considered significant. Data were analyzed using Medcalc 18.0 (MedCalc Software bvba, Ostend, Belgium).

Results

Subjects and interventions

Thirty subjects were contacted for a possible enrollment in this study, and 24 of them met the inclusion criteria and were randomized into T and C groups. At baseline, the two groups did not differ in terms of anthropometric variables, training hours and directly measured maximal oxygen consumption (Table 1). The enrolled subjects underwent blood sample collection as scheduled by the timeline proposed.

Hematological parameters and hemoglobin types at baseline

At baseline, both hematological variables and the five components of the Hb profile did not differ between groups (Tables 2, 3). None of the components of Hb profile was significantly correlated with other blood parameters at baseline.

Hematological parameters and Hb types after withdrawal and reinfusion: group analysis

As expected, RBC, [Hb], HCT, and OFF-hr score significantly decreased after blood withdrawal and increased after reinfusion in the T group, with significant differences between groups, particularly for the OFF-hr score at D-25 ($p < 0.001$) (Table 2). A quadratic trend overtime was observed for [Hb] ($t = 4.28$; $p = 0.001$), Ret% ($t = -3.50$; $p < 0.001$) and OFF-hr ($t = 4.00$; $p = 0.001$) in subjects of the T group. No differences were observed between the two T subgroups (cryopreserved versus refrigerated blood).

HPLC analysis was successfully performed and values of HbF were below the lower limit of quantitation for 88 out

Table 1 Characteristics of the subjects included in the study

	Transfusion ($n=12$)	Control ($n=12$)	p
Anthropometric			
Age (years)	25 ± 3	23 ± 2	n.s.
Height (m)	1.79 ± 0.07	1.77 ± 0.05	n.s.
Weight (kg)	79 ± 11	70 ± 6	n.s.
Body mass index (kg/m^2)	24.6 ± 3.4	22.3 ± 2.2	n.s.
Hematologic			
RBC, $\times 10^6$/μL	5.11 ± 0.44	5.03 ± 0.21	n.s.
Hb, g/dl	14.9 ± 0.9	15.1 ± 0.6	n.s.
HCT, %	44.0 ± 2.2	44.1 ± 1.4	n.s.
MCV, fL	86 ± 4	88 ± 3	n.s.
MCH, pg	29.3 ± 1.4	30.1 ± 1.0	n.s.
MCHC, g/dL	33.9 ± 0.9	34.3 ± 0.8	n.s.
WBC, $\times 10^3$/μL	6.12 ± 1.12	6.18 ± 1.22	n.s.
PLT, $\times 10^3$/μL	228 ± 72	215 ± 32	n.s.
Reticulocytes, %	0.86 ± 0.22	0.87 ± 0.17	n.s.
IRF, %	5.5 ± 3.2	3.5 ± 1.8	n.s.
RDW, %	13.0 ± 0.5	12.8 ± 0.5	n.s.
OFF-hr score	94.1 ± 10.9	95.6 ± 9.1	n.s.
Glycaemia, mg/dl	84 ± 8	87 ± 6	n.s.
Sport parameters			
Training hours/week	8 ± 3	7 ± 3	n.s.
Sport vintage (years)	15 ± 5	13 ± 7	n.s.
Type[a] (AE, AN, ALT)	3–2–7	3–3–6	n.s.
VO$_2$max (ml/kg/min)	56.7 ± 9.2	59.1 ± 6.1	n.s.

RBC red blood cells, *Hb* hemoglobin, *HCT* hematocrit, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular haemoglobin concentration, *WBC* white blood cells, *PLT* platelets, *IRF* immature reticulocytes fraction, *RDW* red cells distribution width, *AE* aerobic, *AN* anaerobic, *ALT* aerobic-anaerobic alternate, *VO$_2$max* maximal oxygen consumption

[a]Sports classification according to Dal Monte (1969)

of 168 samples collected. Considering the values at all time points (Table 3), between-group differences were not observed at any time. Sporadic within-group differences were observed for HbF, HbA, HbA2, and HbA1d particularly in the T group. Trend analyses performed for all Hb profile parameters, highlighted a significant trend for HbA1c in T group (linear $t = -4.00$; $p = 0.021$), with significant within-group differences observed at D+6 and D+15 respect to D-40 (Table 3).

For the calculated parameter, HbPI a significant linear trend ($t = 2.53$; $p = 0.028$) was also observed, along with a significant within-group comparison for T subjects at D+6 ($p = 0.047$) respect to D-40. No differences were observed when comparing the two T subgroups.

Hematological parameters and Hb types after withdrawal and reinfusion: identification of atypical and abnormal values

The control charts of parameters which showed significant changes in the between- and within-group comparisons, highlighted abnormal absolute values in the reinfusion phase particularly for HbA1c (two subjects) and HbPI parameters (five subjects) respect to [Hb] (one subject), Ret% (zero subjects) and OFF-hr score (one subject) (Fig. 2).

A different pattern for Hb profile changes of possible interest for the ABP management was frequently observed in the T group (Online Resource Figure 4). The analysis of each subject at the different time points for hematological parameters showed several atypical/abnormal values mainly located in the withdrawal phase (particularly at D-25) for 9 out of 12 subjects, rather than after reinfusion (4/12 subjects). On the contrary, HbA1c and HbPI showed a high number of atypical/abnormal values in the samples after reinfusion (7 out of 12 subjects) as well a delayed (20 days after blood donation) and weaker response after withdrawal (3/12 subjects) (Fig. 3).

The ROC analysis of HbA1c, to detect the blood reinfusion procedure performed, showed an area under curve of 0.649 ($p = 0.015$; 95% CI 0.561–0.730) with sensitivity/specificity of 50.0/82.2 for values ≤ 3.32 (positive/negative likelihood ratio of 2.82/0.61). A 100% specificity was reached for HbA1c values ≤ 2.7 (sensitivity 25.0%; negative likelihood ratio of 0.75) with possible detection of 5 out of 12 subjects of the T group (Online Resource Figure 5).

Discussion

This study has confirmed the hypothesis that after blood collection and autologous reinfusion, detectable changes in Hb species occur.

The change of hemoglobin profile (i) becomes evident 10 days after blood withdrawal and particularly after reinfusion, (ii) shows an individual pattern of response which reduces the statistical significance in the between-group analysis when comparing treated and controls, and (iii) exposes several subjects with atypical/abnormal values with respect to the mean values of the untreated population. Such behavior, of possible interest in the anti-doping field, differs from that of hematological parameters, that despite presenting average values significantly different between T and C groups, they do not exhibit individual values useful to discriminate treated subjects in the entire population after reinfusion.

Table 2 Values of hematological parameters in the two groups for the different time points. Within- and between-group comparisons are shown

Transfusion (n = 12)	D-40	D-25	D-15	D0 (r)	D + 3	D + 6	D + 15
RBC, 10^6 μl^{-1}	5.11 ± 0.44	4.78 ± 0.37**	4.90 ± 0.32	4.92 ± 0.39	5.25 ± 0.42***†	5.15 ± 0.45**	5.16 ± 0.39**
[Hb], g dl^{-1}	14.9 ± 0.9	14.0 ± 0.8**†	14.3 ± 0.5††	14.4 ± 0.9	15.2 ± 0.8***	14.8 ± 0.8*	14.9 ± 0.8
HCT, %	44.0 ± 2.2	41.3 ± 2.1**†	42.3 ± 1.6†	42.5 ± 2.4	45.1 ± 2.1***	44.2 ± 2.6*	44.1 ± 2.1*
MCV, fL	86 ± 4	86 ± 3	86 ± 3	86 ± 3	86 ± 3	86 ± 3	86 ± 3
MCH, pg	29.3 ± 1.4	29.3 ± 1.3	29.3 ± 1.4	29.2 ± 1.3†	29.1 ± 1.1†	28.9 ± 1.4	28.8 ± 1.3*
MCHC, g dl^{-1}	33.9 ± 0.9	33.9 ± 0.6	33.9 ± 0.7	33.9 ± 0.7	33.8 ± 0.6	33.6 ± 1.0	33.7 ± 0.8
WBC, 10^3 μl^{-1}	6.12 ± 1.12	6.42 ± 1.61	6.47 ± 1.70	6.26 ± 1.24	6.49 ± 1.51	6.06 ± 1.40	6.11 ± 1.49
PLT, 10^3 μl^{-1}	228 ± 72	244 ± 61***	242 ± 65*	241 ± 72*	234 ± 76	227 ± 64	226 ± 64
Reticulocytes, %	0.86 ± 0.22	1.35 ± 0.33***†	1.11 ± 0.27**	0.96 ± 0.35	0.90 ± 0.28	0.74 ± 0.17**†	0.79 ± 0.29
IRF, %	5.5 ± 3.2	8.4 ± 3.1***††	7.3 ± 2.9**†	6.0 ± 3.6	5.8 ± 2.9	4.2 ± 2.0	5.3 ± 2.6
RDW, %	13.0 ± 0.5	13.2 ± 0.4**†	13.2 ± 0.5†	13.0 ± 0.3	13.0 ± 0.4	13.0 ± 0.5	13.0 ± 0.4
OFF-hr score	94.1 ± 10.9	70.5 ± 8.8***†††	80.4 ± 9.0††	86.2 ± 10.0	96.1 ± 12.0*	96.9 ± 9.3**	95.9 ± 13.0*
Control (n = 12)	**D-40**	**D-25**	**D-15**	**D0**	**D + 3**	**D + 6**	**D + 15**
RBC, 10^6 μl^{-1}	5.03 ± 0.21	4.94 ± 0.26	5.03 ± 0.19	5.00 ± 0.22	4.95 ± 0.26*	4.96 ± 0.24	4.99 ± 0.16
[Hb], g dl^{-1}	15.1 ± 0.6	14.8 ± 0.8*	15.1 ± 0.6	15.1 ± 0.7	14.8 ± 0.8**	14.8 ± 0.7	14.8 ± 0.4*
HCT, %	44.1 ± 1.4	43.1 ± 1.9*	44.0 ± 1.5	43.8 ± 1.6	43.4 ± 2.1*	43.4 ± 2.1	43.6 ± 1.3*
MCV, fL	88 ± 3	87 ± 3	87 ± 3	87 ± 2	88 ± 2	88 ± 2	87 ± 3
MCH, pg	30.1 ± 1.0	29.9 ± 0.9	30.0 ± 0.9	30.2 ± 0.9	30.0 ± 0.9	29.9 ± 1.1	29.7 ± 1.0*
MCHC, g dl^{-1}	34.3 ± 0.8	34.2 ± 0.6	34.3 ± 0.7	34.5 ± 0.7	34.2 ± 0.7	34.2 ± 0.7	34.1 ± 0.6
WBC, 10^3 μl^{-1}	6.18 ± 1.22	5.90 ± 1.20	6.08 ± 1.77	5.71 ± 0.83	5.99 ± 1.49	6.19 ± 1.07	6.25 ± 1.28
PLT, 10^3 μl^{-1}	215 ± 32	212 ± 35	213 ± 25	219 ± 30	215 ± 38	221 ± 36	221 ± 33
Reticulocytes, %	0.87 ± 0.17	0.91 ± 0.15	0.99 ± 0.27	1.03 ± 0.22	0.89 ± 0.19	0.91 ± 0.17	0.98 ± 0.26
IRF, %	3.5 ± 1.8	4.6 ± 1.8	4.9 ± 1.4	4.9 ± 1.5	4.8 ± 1.4	5.3 ± 2.0	5.0 ± 2.5
RDW, %	12.8 ± 0.5	12.7 ± 0.6	12.8 ± 0.5	12.8 ± 0.6	12.9 ± 0.6	12.9 ± 0.6	12.9 ± 0.5
OFF-hr score	95.6 ± 9.1	90.8 ± 9.7	91.6 ± 10.3	90.4 ± 9.6	91.8 ± 8.7*	91.2 ± 10.3	89.4 ± 8.1*

D days of blood collection, *r* blood reinfusion, *RBC* red blood cells, *[Hb]* hemoglobin concentration, *HCT* hematocrit, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *WBC* white blood cells, *PLT* platelets, *IRF* immature reticulocytes fraction, *RDW*:= red cell distribution width

Within-group comparison: paired samples Wilcoxon test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Between-group comparison: one-way analysis of variance. †$p < 0.05$; ††$p < 0.01$; †††$p < 0.00$

The analysis of Hb types in response to ABT, showed that HbF variations in the T group occurred in both phases of ABT treatment, but mainly following blood withdrawal, with values exceeding 0.8% observed only in two subjects of the T group. This observation confirms the responsiveness of this parameter to changes in the status determining hypoxia (e.g., high altitude or hypobaric chamber exposure) [14, 25]. An average trend for HbF to decrease following reinfusion was also observed, which is consistent with changes in this direction noted in the presence of an increase in oxygen availability [26]. Unfortunately, besides the expected modest increase in HbF levels, the statistical analysis was limited by the undetectable values observed at baseline in some subjects. Contrarily, HbA1c was easily detectable and quantifiable by HPLC analysis and showed readily measurable levels and interesting changes in response to ABT. HbA1c,

a clinical biomarker routinely used to monitor long-term glycemic control in individuals with diabetes mellitus but with no current use in the anti-doping field, showed a decreasing trend following both blood withdrawal and especially after reinfusion unlike most of the hematological parameters (e.g., Hb, Ret%, OFF-hr score) which exhibited a response characterized by opposite trends. A slightly not-significant decreasing trend due to HbA1c fluctuations overtime was observed also in C group. However, these changes occurred in a range of values corresponding to a normal mean blood glucose level (around 80–90 mg/dl) unlike values around the lower limits observed in T. These HbA1c values (< 2.7% by HPLC) correspond to a mean blood glucose level around 60–70 mg/dl [27] consistent with a prolonged hypoglycemia incompatible with a healthy status and acceptable training conditions.

Table 3 Values of hemoglobin types in the two groups for the different time points

Transfusion ($n = 12$)	D-40	D-25	D-15	D0 (r)	D+3	D+6	D+15
Measured							
HbF	0.27 ± 0.30	0.23 ± 0.25	0.35 ± 0.38	0.18 ± 0.26	0.13 ± 0.16	0.10 ± 0.14	0.11 ± 0.17
HbA	89.3 ± 0.9	89.8 ± 1.0	90.2 ± 2.1	91.0 ± 1.9*	90.5 ± 1.2**	90.7 ± 1.9*	91.3 ± 1.1***
HbA2	2.69 ± 0.28	2.60 ± 0.30	2.48 ± 0.44	2.31 ± 0.56*	2.53 ± 0.39	2.80 ± 0.94	2.45 ± 0.45
HbA1c	4.14 ± 0.64	4.04 ± 0.49	3.87 ± 0.71	3.67 ± 0.80	3.57 ± 0.98	3.44 ± 1.00*	3.40 ± 0.88**
HbA1d	3.63 ± 0.37	3.40 ± 0.64	3.06 ± 1.10	2.90 ± 1.41*	3.25 ± 1.24	2.97 ± 1.82	2.78 ± 0.95*
Calculated							
HbPI	59 ± 10	58 ± 10	58 ± 9	59 ± 17	69 ± 22	82 ± 36*	71 ± 25
Control ($n = 12$)	D-40	D-25	D-15	D0	D+3	D+6	D+15
Measured							
HbF	0.21 ± 0.32	0.22 ± 0.28	0.15 ± 0.21	0.14 ± 0.21	0.23 ± 0.30	0.10 ± 0.15	0.08 ± 0.12
HbA	89.5 ± 0.6	89.7 ± 0.6	90.1 ± 1.6	91.1 ± 1.7*	90.3 ± 1.1	91.0 ± 1.1*	90.9 ± 1.5*
HbA2	2.84 ± 0.27	2.77 ± 0.29	2.58 ± 0.29*	2.54 ± 0.65	2.67 ± 0.30	2.63 ± 0.59	2.38 ± 0.46*
HbA1c	4.10 ± 0.49	4.11 ± 0.38	4.09 ± 0.88	3.73 ± 0.88	4.08 ± 0.56	3.67 ± 0.60	3.69 ± 0.57
HbA1d	3.40 ± 0.19	3.19 ± 0.73	3.11 ± 1.09	2.54 ± 0.91*	2.76 ± 0.62*	2.60 ± 0.87*	2.99 ± 1.08
Calculated							
HbPI	63 ± 11	61 ± 8	59 ± 14	64 ± 21	60 ± 10	66 ± 15	59 ± 13

Within- and between-group comparisons are shown

D days of blood collection, *r* blood reinfusion, *HbF* fetal hemoglobin, *HbA1c* glycated hemoglobin, *HbA1d* aldimine fraction, *HbPI* Hemoglobin Profile Index (HbA*HbA2)/HbA1c

All hemoglobin types are expressed in percentage of total hemoglobin

Within-group comparison: paired samples Wilcoxon test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Between-group comparison: one-way analysis of variance. †$p < 0.05$; ††$p < 0.01$; †††$p < 0.001$

A decrease of HbA1c was previously reported following both blood collection, donation and reinfusion and attributed to a hyperdynamic state of hemoglobin due to blood volume loss following withdrawal and accelerated turnover of RBC [21] or bone marrow stress [22, 28]. Conditions affecting shortened erythrocyte survival or decreasing mean erythrocyte age were associated with low-HbA1c values [29]. RBC lifespan represents a determinant of percentage HbA1c and a heterogeneity in mean RBCs age in hematologically normal people both with and without diabetes with impact on HbA1c values was reported [30]. In our study, this heterogeneity may be amplified among T subjects, and within each treated subject, during the ABT phases for different factors. The release from the bone marrow of reticulocytes with a certain HbA1c level should be considered [30], being stimulated in the T group after withdrawal (Ret% increase to 1.35%) and downregulated after reinfusion (Ret% decrease to 0.74%). A possible early disappearance of a number of RBCs after transfusion might be also involved. Despite the insufficient understanding of the storage damage on RBC and their performance [31], RBCs during their stay in the blood bank become vulnerable to stressful conditions [32] with a reduced lifespan after reinfusion. A possible early disappearance of 5–15% within the first 24 h after transfusion,

and up to 25% or more for longer storage time [33] has been reported. As hypothesized, the effects of ABT-related changes in oxygen availability and of blood manipulation procedures might switch on integrated molecular responses. Besides altered Hb profiles, an overexpression of a pool of erythroid and HbF-related miRNAs only after ABT in a subgroup of athletes under study was observed [34 and unpublished data]. Therefore, the heterogeneity of RBCs age at baseline, and the different inter-individual erythropoietic responses to ABT may contribute to explain the abnormally low HbA1c values (below 2-SD and 3-SD of the average population) observed only in the T group in 5 and two out of 12 subjects, respectively. In the population under study, we also defined a reference value of HbA1c with a criterion associated to a 100% specificity, potentially useful in the anti-doping field. Other Hb forms assessed by HPLC showed changes during the two ABT phases, and their integration in a parameter as HbPI exposed values 3-SD above the average of the whole untreated population only among transfused subjects up to 15 days after reinfusion.

The presence of an altered Hb profile in subjects receiving reinfusion of both refrigerated and cryopreserved blood, might support the analysis of the hematological parameters of the ABP for the ABT detection (Online

Fig. 2 Control charts of hemoglobin concentration, reticulocytes, OFF-hr score, glycated hemoglobin and HbPI for both groups. The vertical line identifies the reinfusion period. [*Hb*] hemoglobin concentration, *HbA1c* glycated hemoglobin, *HbPI* Hemoglobin Profile Index, *SD* standard deviation

Resource Figure 4). Moreover, the wide window of an altered profile may represent an added value for these parameters in the anti-doping field. Finally, the one-way decreasing trend of HbA1c, which is evident in transfused subjects and particularly recognizable in the cryopreserved subgroup, may be of interest also in the case of frequent repeated procedures of blood collection and reinfusion of even small amounts of blood to bypass the ABP.

Unlike hematological variables, following ABT, Hb types did not show significantly different average values compared to C group, but exhibited a wider range of changes compared to the baseline as well a high individual variability of response. This pattern may derive from the factors previously discussed, as well from factors not related to the treatment (training status, nutritional aspects, measurement issues, etc.). Hence confounding factors able to interfere with measurement or interpretation of HbA1c results are possible [29, 35], but the accuracy of HbA1c measurement is preserved by HPLC even in the presence of genetic Hb variants or high HbF levels. However, the HPLC measurement here employed, makes difficult to compare the HbA1c value of this study with those obtained with methods routinely used in diabetes detection [27]. Other possible confounding factors related to chronic diseases (chronic renal failure, chronic liver disease) are not applicable to a healthy population competing at a high agonistic level. Changes of HbA1c observed in pregnancy and in iron deficiency or following iron replacement therapy should be considered [35].

We are aware that this study has several limitations. The sample size is limited for the analysis and includes only male Caucasian volunteers. This was intended to reduce the data variability, considering that the two different procedures of blood conservation might contribute to a less-homogenous response in the T group.

In conclusion, for the first time, to the best of our knowledge, a study points out not quantitative but qualitative changes of the hemoglobin pattern following ABT. These changes were evident only in treated subjects, independently from blood conservation, in all the phases of ABT and especially in the reinfusion phase when the hematological variables were almost "silent" in terms of variations.

Novel parameters related to the study of Hb profile might be, therefore, suggestive of blood manipulation,
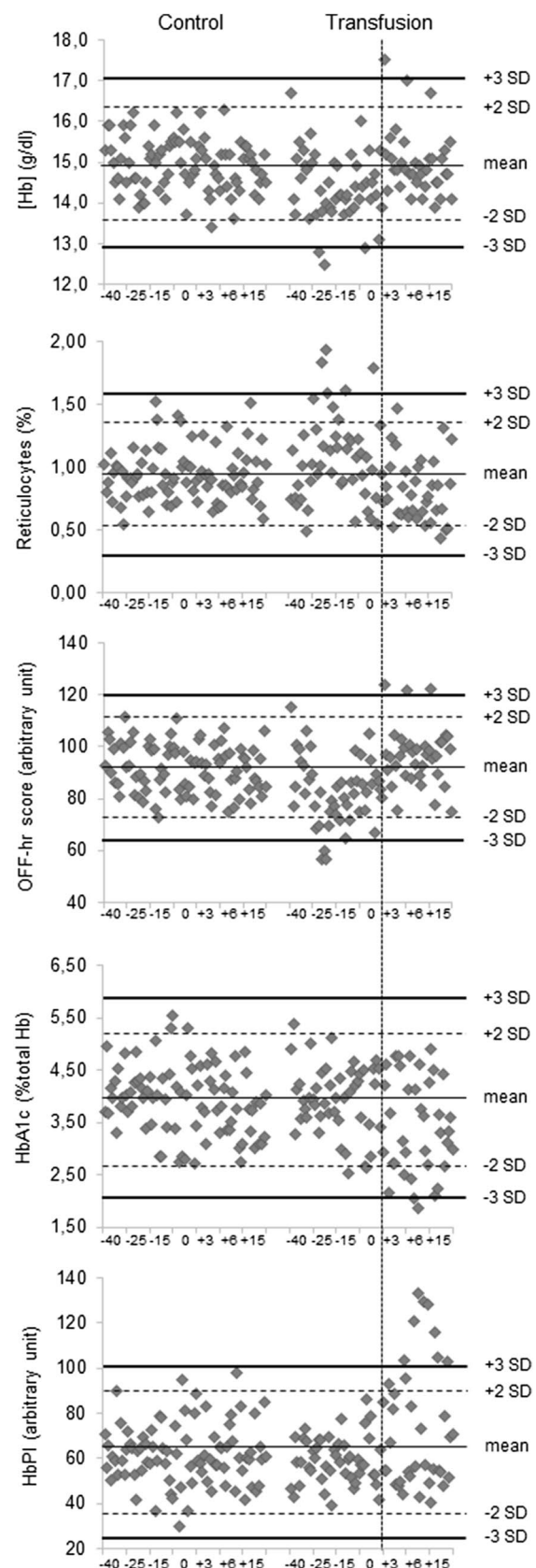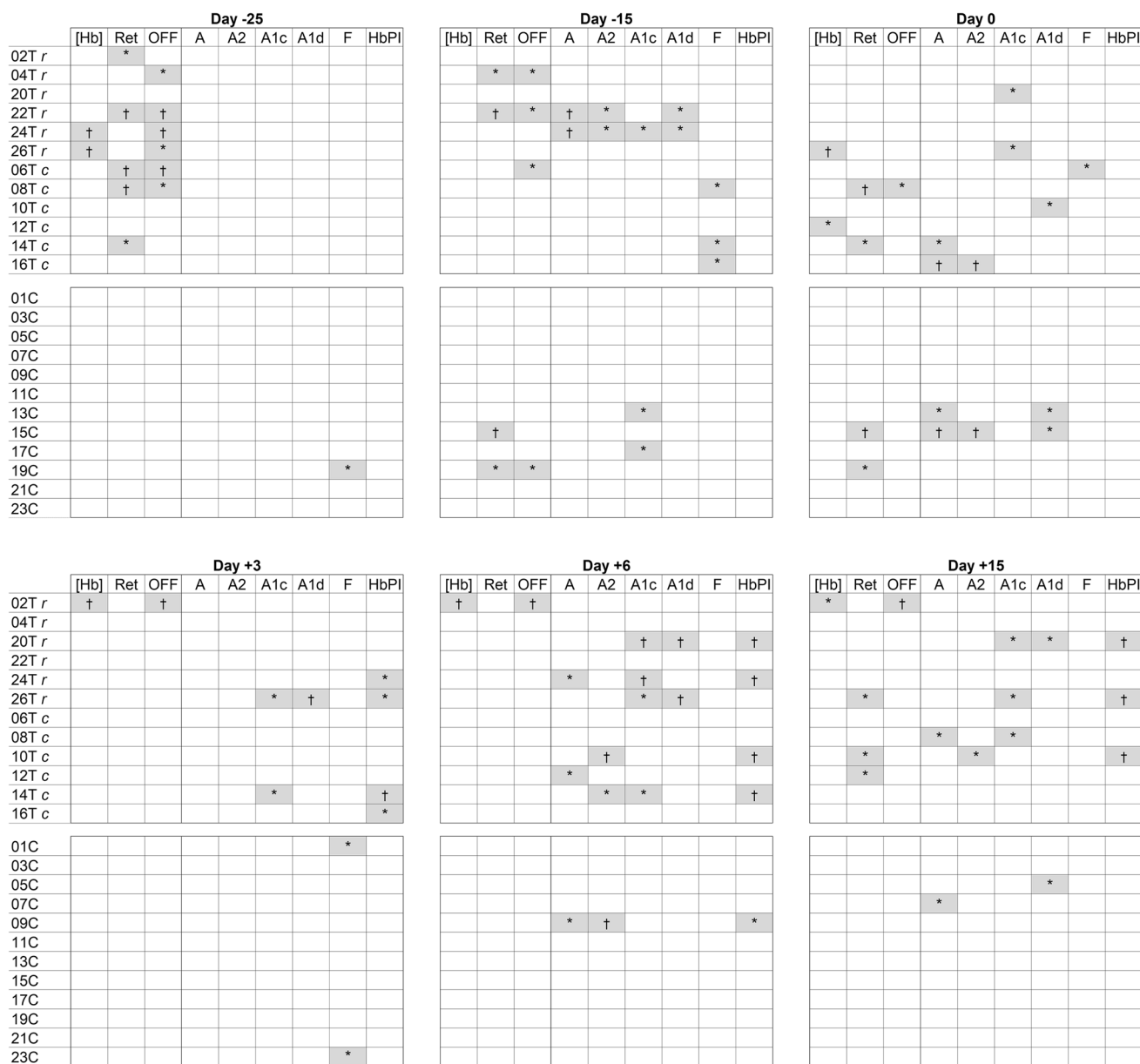
Fig. 3 Samples exceeding 2- (*) and 3-standard deviations (†) from the mean of all untreated samples at the different time points for each subject. [Hb], hemoglobin concentration, Ret reticulocytes, OFF OFF-hr score, A hemoglobin-A, A2 hemoglobin-A2, A1c glycated hemoglobin, A1d hemoglobin aldimine fraction, F fetal hemoglobin, HbPI Hemoglobin Profile Index, SD standard deviation, r refrigerated blood, c cryopreserved blood, T transfusion group, C control group

however, further studies to confirm their relevance as biomarkers of ABT are needed.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statement of human and animal rights All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Ethics Committee of Ferrara (06/2014).

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