Blood Doping • Research Paper

Analysis of human reticulocyte genes reveals altered erythropoiesis: potential use to detect recombinant human erythropoietin doping

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A B S T R A C T

Background and Objectives. Enhancement of oxygen delivery to tissues is associated with improved sporting performance. One way of enhancing oxygen delivery is to take recombinant human erythropoietin (rHuEpo), which is an unethical and potentially dangerous practice. However, detection of the use of rHuEpo remains difficult in situations such as: i) several days after the end of treatment ii) when a treatment with low doses is conducted iii) if the rHuEpo effect is increased by other substances. In an attempt to detect rHuEpo abuse, we selected erythroid gene markers from a SAGE library and analyzed the effects of rHuEpo administration on expression of the *HBB*, *FTL* and *OAZ* genes.

Design and Methods. Ten athletes were assigned to the rHuEpo or placebo group. The rHuEpo group received subcutaneous injections of rHuEpo (50 UI/kg three times a week, 4 weeks; 20 UI/kg three times a week, 2 weeks). *HBB*, *FTL* and *OAZ* gene profiles were monitored by real time-polymerase chain reaction (PCR) quantification during and for 3 weeks after drug administration.

Results. The global analysis of these targeted genes detected in whole blood samples showed a characteristic profile of subjects misusing rHuEpo with a increase above the threshold levels. The individual analysis of *OAZ* mRNA seemed indicative of rHuEpo treatment.

Interpretation and Conclusions. The performance-enhancing effect of rHuEpo treatment is greater than the duration of hematologic changes associated with rHuEpo misuse. Although direct electrophoretic methods to detect rHuEpo have been developed, recombinant isoforms of rHuEpo are not detectable some days after the last subcutaneous injection. To overcome these limitations indirect *OFF models* have been developed. Our data suggest that, in the near future, it will be possible to consolidate results achievable with the *OFF models* by analyzing selected erythroid gene markers as a supplement to indirect methods.

Key words: reticulocyte markers, real time-PCR analysis, SAGE, rHuEpo, doping.

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Like endogenous erythropoietin, the main action of recombinant human erythropoietin (rHuEpo) is to stimulate the proliferation and the differentiation of progenitor cells in the bone marrow toward functional erythroblasts.[1,2] The subsequent maturation requires 5 to 9 days under normal physiologic conditions. Although this glycoprotein hormone is officially prohibited by the International Olympic Committee and other major sporting bodies,[3] rHuEpo is used by athletes to enhance athletic performances.[4-6] The duration of the erythropoietin-induced effects is greater than the duration of any hematologic changes associated with rHuEpo misuse. Although direct electrophoretic methods to differentiate endogenous erythropoietin and rHuEpo have been developed,[7,8] recombinant isoforms of rHuEpo were not detectable using these methods in samples taken some days after the last subcutaneous injection of rHuEpo. This limits the usefulness of these methods for doping control in connection with competitions.

To overcome these limitations and make the abuse of rHuEpo detectable, indirect methods have been proposed and developed.[5,9-20] Several hematologic indices (number of red blood cells, blood hemoglobin concentration, hematocrit, reticulocyte count, number of hypochromic macrocytes) and biochemical parameters (serum Epo and soluble transferrin receptor concentrations) change significantly after rHuEpo intake. The main indirect method currently available for the detection of rHuEpo abuse simultaneously utilizes multiple hematologic and biochemical markers.[15,17] However the choice of a method based on only 2 or 3 parameters would be inadequate to confront an athlete confidently with an accu-

sation of doping. The purpose of this work is to research new parameters based on expression of specific genes due to the rHuEpo administration such as hemoglobin-β (*HBB*) gene expression[16] in order to extend indirect methods. In this first study, we selected erythroid target genes (*HBB*), ferritin-light chain (*FTL*) and ornithine decarboxylase antizyme (*OAZ*) from a SAGE (serial analysis of gene expression) library performed on human purified reticulocytes. This SAGE study represents the first gene profile information concerning reticulocytes that could be used in further transcriptome analysis and red cells studies (*Bonafoux et al. submitted publication*). A real time-PCR assay was developed (i) to detect *HBB*, *FHL* and *OAZ* mRNA expression accurately, and (ii) to observe their regulation in mRNA samples from six athletes treated with rHuEpo and from four controls.

Table 1. Well annotated genes and specific reticulocyte markers.

HUGO symbol	*Leukocyte library*[1]	*Reticulocyte library*[1]	*GenBank acc. no.*	*SAGE genie*[2]	*Fold induction*[3]
HBB	100	100	NM_000518	2227	5.8
FTL	8	2	NM_000146	4911	1.8
UBA52	1	2	NM_003333	1415	1
RPS19	0	9	NM_001022	36010	1
OAZ1	2	2	NM_004152	4373	2.1

[1]For an easier reading of the results, tag frequencies were calculated per 100 HBB tags. [2]Frequencies of NlaIII tags observed among all the CATG-constructed libraries available on http://cgap.nci.nih.gov/SAGE). [3] Fold induction of examined genes in the TF1 cell line treated by rHuEpo versus GMCSF.

Design and Methods

Subjects

Ten recreational athletes ranging from 18 to 29 years old participated in this study. The mean age of the subjects was 25.0±2.1 years and their mean weight was 70.2±2.8 kg. All athletes had been involved in regular training for several years, and during the study period they were allowed to continue their normal physical activity (more than 10 hours/week). None of them took part in an official competition during this period. One week prior to the study, each athlete underwent an initial evaluation, including personal and clinical history taking, physical examination, and hematologic and biological parameters. Exclusion criteria were age (<18 years); hypertension (blood pressure: systolic >160 mmHg, diastolic >95 mmHg); cardiovascular disease; thrombosis; anemia; iron, vitamin B12 or folic acid deficiency; and thrombocytosis. Volunteers had normal blood cell counts, blood smears, hemoglobin electrophoresis, red cell membrane resistance, and no biological evidence of hemolysis. Subjects were instructed not to consume alcohol or other medications before and throughout the study period.

This study was conducted according to the declaration of Helsinki as amended in the 41st World Medical Assembly (Hong Kong 1989) and was reviewed and approved by the Regional Ethics Committee. The subjects were included in the study after having given informed consent.

Study design

After the initial testing, subjects were assigned to one of the two groups: rHuEpo group (n = 6, age 25.8±0.8 years body weight, 70.2±2.3 kg, height 178.0±1.6 cm), placebo group (n = 4, age 24.8±2.1 years, body weight 70.2±2.6 kg, height 177.3 ± 1.8 cm). The rHuEpo group received subcutaneous injections of rHuEpo (Eprex 4000®, Janssen-Cilag, France) 3 times a week for 6 weeks; the first 4 weeks at a dose of 50 U/kg body mass and the next 2 weeks at a dose of 20 U/kg body mass as already described (Connes *et al. accepted publication*).

Selected genes from SAGE analysis of reticulocyte tags

The most abundantly expressed genes in reticulocytes were determined from the SAGE reticulocyte library (Bonafoux *et al.* submitted publication and *http://www.igh.cnrs.fr/equip/transcriptome*). Table 1 presents the selected genes and their occurrences, and tag frequencies from the SAGEgenie database (*http://www.sagegenie*). We also compared the reticulocyte data to a published NlaIII SAGE library, constructed from bulk blood cells (the Leukocyte library GSM709; *http://www.ncbi.nih.gov/SAGE*). As expected, the reticulocyte selected genes were also present in this library and significantly expressed in whole blood cells.

Blood collection and analysis

Each blood sample was collected into a 3 mL K_3EDTA tube (Vacuette®, Grenier Labortechnik, Frikenhaussen Germany) from an antecubital vein before starting the trial protocol (baseline-day or day -2), four times during the acceleration phase (days 4, 11, 18 and 25), three times during the maintenance phase (day 30, 32 and 37) and three times during the wash-out phase (days 44, 53 and 61). All blood was collected in the morning at the same times to minimize diurnal variations.[21,22] Posture was standardized with each subject seated for 5 min before assuming a supine position for venipuncture. Total RNA extraction was performed from 2 mL of whole blood at each sampling time.

mRNA extraction from whole blood

Four steps are required to isolate intact RNA: disruption of cells, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNAse) and removal of contaminating DNA and proteins. For this purpose, we used the SV Total Isolation System (Promega kit) which combines the protective properties of guanidine thiocyanate and β-mercaptoethanol to inactivate ribonucleases and associates a DNAse treatment to remove contaminating DNA.

Total RNA of the reticulocytes was extracted from whole blood using the SV total RNA Isolation System. The RNA was extracted within 4 h following blood collection. Briefly, whole blood was centrifuged for 10 min at 3500 rpm. Four hundred microliters of *Red Blood Cell Lysis Solution* and 700 μL of *SV RNA Dilution Buffer* were added to 800 μL of concentrated red blood cells. This solution was centrifuged at 12,000 × g for 10 min. The cleared lysate was transferred to a sterile tube and 500 μL of ethanol were added, then 700 μL of the mixture were transferred in a column and centrifuged at 13,000 x g for 1 min. The column was washed with 600 μL of *SV RNA wash solution* and again centrifuged at 13,000 × g for 1 min, before adding 50 μL of *DNAse incubation mix*. The reaction was incubated for 15 min at 25°C and stopped with 200 μL of *DNAse stop solution* with ethanol. The column was centrifuged at 13,000 × g for 1 min. The membrane was washed with 600 μL of *SV RNA wash solution* and the column centrifuged at 13,000 × g for 1 min. RNA was eluted with 100 μL of *Nuclease Free Water*, centrifuged at 13,000 × g for 1 min and stored at –80°C. For calibration of cDNA synthesis, mRNA for chlorophyll A/B-binding protein (CAB) (Stratagene, The Netherlands) was added during reticulocyte RNA extraction.

cDNA synthesis and determination of gene specific primers

For synthesis of first strand cDNA, annealed mRNA was incubated at 42°C with 40 μL of an RT-Mix (20 μL of total RNA, 8 μL of RT-buffer, 4 μL of oligodT (0.5 μg/μL), 4 μL of DTT (0.1M), 2 μL of dXTP, 1 μL of H_2O RNAse free and 1 μL of Superscript II reverse transcriptase (200 U/μL; Invitrogen). To avoid artifacts due to manipulation, the real time reactions were performed simultaneously from a RT-Mix. To normalize cDNA synthesis, real time-PCR was performed with a *CAB* primer (forward 5' GCA TTT GTT GAG CAC CAG AG 3' and reverse 5' TAT CGC CAA TGT TGT TGT GC 3' which gives a 259-bp-long). A low standard deviation was obtained comparing all samples (<3%).

RPS19, *UBA52*, *HBB*, *FTL* and *OAZ* specific primers were designed with the assistance of Primer 3 software (*http://www-genome.wi.mit.edu:cgi-bin:primer:primer3_www.cgi*). BLASTN searches were performed against a non-redundant set of Genbank database sequences to confirm the gene specificity. Primers were designed to span two adjacent exons to ensure specificity for cDNA rather than genomic DNA. The following primers were designed: *RPS19* forward 5' GGC TGA AAA TGG TGG AAA AG 3' and reverse 5' AAC CCA GCA TGG TTT GTT C 3' which gave a 131-bp-long product, *UBA52* forward 5' CTC ACT GGC AAA ACC ATC AC 3' and reverse 5' GCG GAG AGA AGG CTC AAT AA 3' which gave a 228-bp-long product, *HBB* forward 5' GCA ACC TCA AAC AGA CAC CA 3' and reverse 5' AGC TCA CTC AGT GTG GCA AA 3' which gave a 294-bp-long PCR product; *FTL* forward 5' TGT ACC TGC AGG CCT CCT AC 3' and reverse 5' AGA ACC CAG GGC ATG AAG AT 3' which gave a 290-bp-long PCR product; *OAZ* forward 5' GGA TTC TCA ACG TCC AGT CC 3' and reverse 5' CGG TTC TTT GTG GAA GCA AA 3' which gave a 200-bp-long product. Specific primers were tested by PCR on reticulocyte cDNA. The PCR products were cloned, sequenced and after verification, plasmids were purified and their concentration was determined on agarose/ethidium bromide gel. Dilutions of plasmid were used in real time PCR experiments on reticulocytes in order to obtain the calibration curve.

Detection and quantification of HBB, FTL and OAZ expression in reticulocytes

HBB, *FTL* and OAZ expression was detected and quantified on reticulocyte cDNA by means of real time PCR with a Light Cycler (Roche).

RPS19 and *UBA52* were chosen for the global calibration of blood samples. The quantification data of these calibration genes were compared and showed equivalent results. *RPS19* was further selected considering its expression level.[23] Reactions of 20 μL were set up consisting of 2 μL 10× PCR Mix (Roche), 1.6 μL of 25mM $MgCl_2$ stock solution and 10 pmol of each primer and 1 μL template DNA (plasmid or cDNA from specific tissue). The thermal profile used for real time PCR consisted of a step at 95° C for 10 min and 40 cycles of denaturing at 95°C for 15 sec, annealing at 62°C for 4 sec and elongation at 72°C for 8 sec. After the last cycle, the temperature in the Light Cycler chamber was increased to 95°C and then decreased down to 62°C for 30 sec. Finally, the temperature was increased gradually up to 95°C in order to obtain the melting curves of the amplified fragments. Absence of specific PCR products and of primer dimers were checked by the melting curves.

Quantification and analysis of the results were performed with the computer system of the Light Cycler (Light Cycler Relative Quantitation Software 1.0,

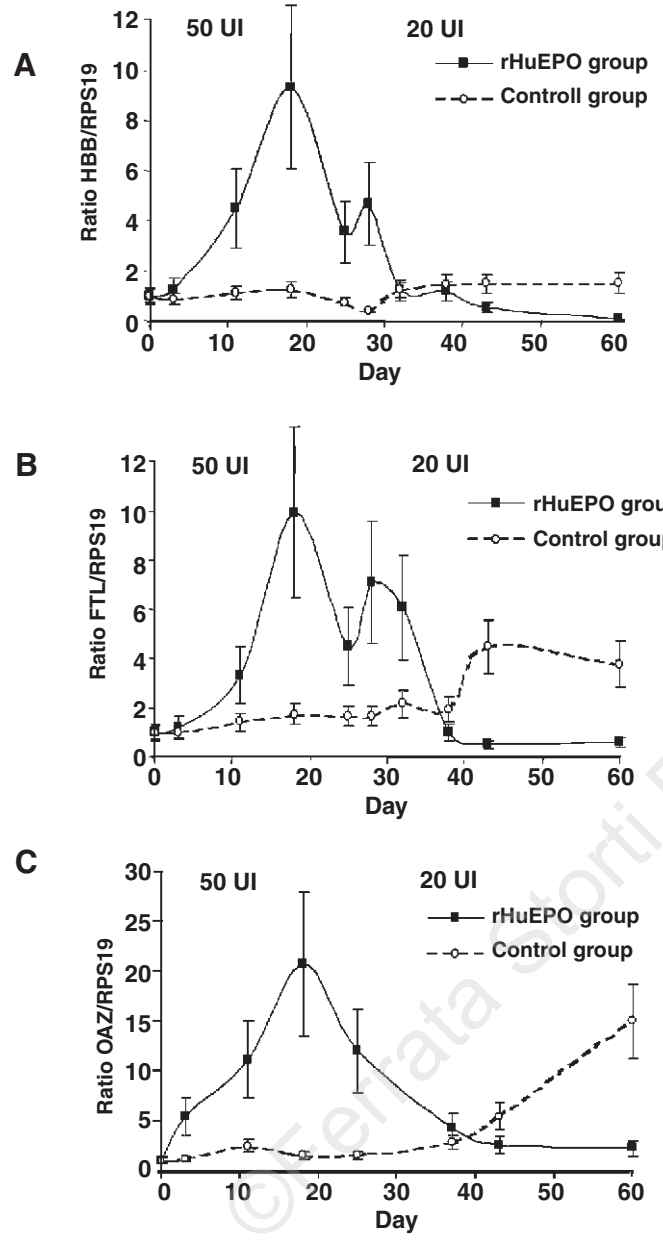

Figure 1. Kinetics of *HBB*, *FTL* and *OAZ* mRNA expression in a group of athletes without (control) or during and after rHuEpo administration (rHuEpo group). The rHuEpo group received rHuEpo injections 3 times a week for 4 weeks at 50 IU/kg, and 3 times a week for 2 weeks at 20 IU/kg; there was a wash-out phase of 3 weeks. All subjects received elemental iron, vitamins B9 and B12 (see Design and Methods). The initial ratio (before rHuEpo injections) of selected gene and calibration gene RPS19 mRNA contents corresponds to the reference value (=1). Each point represents the mean ± standard error of the mean.

Roche). For data analysis, the fit-point method was used to set the threshold manually between the background and significant fluorescence. A standard curve was generated from plotting plasmid standard concentrations against the threshold cycle.

All samples were determined in duplicate. The *HBB*, *FTL* and *OAZ* messenger quantifications, calculated from the respective standard curves, were expressed in number of copies per µg mRNA. Values are presented as mean ± the SE of the mean. The kinetics of *HBB/RPS19*, *FTL/RPS19* and *OAZ/RPS19* were explored.

Results

Detection of HBB, FTL and OAZ mRNA content, upon rHuEpo administration, by quantitative RT-PCR

A quantitative RT-PCR assay was developed to detect *HBB*, FHL and *OAZ* mRNA content accurately in the RNA samples extracted at each time point. In order to interpret data easily, results were compared with the first sample: the value before injection of rHuEpo.

The increase in *HBB* mRNA content at the different sampling times is illustrated in Figure 1A: the results,

expressed as mean values for each group, clearly indicate that *HBB* mRNA expression increased during rHuEpo administration, reaching its highest value on day 17 (9.5-fold the initial ratio). *HBB* expression decreased drastically on day 25, returning to the initial ratio at the last time points (day 42).

The increase in *FTL* mRNA content at the different sampling times is illustrated in Figure 1B: the results indicate a similar profile to that of *HBB* mRNA expression: an increase during rHuEpo administration, reaching a peak on day 18 (9.5-fold the initial ratio). FTL expression decreased on day 25, returning to the initial ratio at the last time points.

The increase in *OAZ* mRNA content at the different sampling times is illustrated in Figure 1C: the results show that *OAZ* mRNA expression increased during rHuEpo administration and reached a peak on day 18 (21-fold the initial ratio). OAZ expression decreased drastically on day 35, returning to the initial ratio at the last time points (day 42).

Surprisingly for the last two genes, results indicated that mRNA expression continued to increase after the end of iron injections in the control group.

Individual analysis with the reticulocyte markers

We observed a significant global variation of mRNA expression in the treated group compared to among the untreated athletes but a different expression profile among individuals. Therefore, to complete the study, we presented *HBB*, *FHL* and *OAZ* mRNA expression individually for the subjects.

Treated subjects and controls were monitored. Different profiles of HBB and *FTL* mRNA expression were found among the treated subjects: the results clearly indicate that *HBB* mRNA expression increased during rHuEpo administration in some subjects (Figure 2A), but did not increase greatly in others (Figure 2B) The opposite was observed for *FTL* mRNA (Figures 2A and 2B). It seems that *HBB* and *FTL* mRNA are subject-independent markers. In the placebo group, *FTL* mRNA remained stable during the treatment and increased slightly after the end of the injections (Figure 2C). The same result were observed for *HBB* mRNA variations (*data not shown*).

The same mRNA *OAZ* expression profile was obtained for the treated subjects: the results indicated that *OAZ* mRNA expression increased during the acceleration phase of rHuEpo administration, reaching the highest value on day 18, and then *OAZ* expression progressively decreased during the maintenance and the wash-out phases. It seems that *OAZ* mRNA is a subject-independent marker. In the placebo group, *OAZ* mRNA remained stable during the treatment and increased after the end of the injections.

Discussion

The only method currently accepted by the World Anti-doping Agency to detect rHuEpo abuse is the isoelectric focusing and *double blotting* method based on direct proof of rHuEpo misuse. This method is, however, unable to select rHuEpo abuse some days (2-7 depending of the dosage and the administration route) after the end of rHuEpo treatment. Dishonest athletes who stop rHuEpo administration some days before a competition have the advantage of the performance-enhancing effect of the rHuEpo treatment without risking of a positive test. To discourage rHuEpo abuse, several biological parameters have been used. Some federations adopted a *cut-off value* of hemoglobin (Hb) and/or hematocrit (Hct) and reticulocytes: the international ski federation (I.S.F.) excluded athletes with Hb values above 175 g/L for men and 155 g/L for women, the international cycling union (I.C.U.) excluded athletes with Hct values above 50% for men and 47% for women with reticulocytes above 2%. These values do not represent definitive proof of rHuEpo use but are biochemical findings that raise the suspicion of doping. Some athletes apparently take several products or drugs concomitantly, such as volume expanders, growth hormones, interleukin 3 and insulin and we do not know the physiologic and/or hematologic consequences of these associations which lead to additional difficulties in detecting rHuEpo. A more interesting approach to overcome this limitation and make the abuse of rHuEpo detectable is the method proposed by Gore *et al.*, based on 2 or 3 parameters (*ON models He or Hes* and *OFF models Hr and Hre*).[15] The aim of this study was to consolidate this approach and more specifically the *OFF models* with a new parameter, gene expression, now measurable by real time-PCR. We chose to study *HBB, FTL* and *OAZ* mRNA for various reasons : i) *HBB* is considered a selective marker of erythroid activity and its expression (up-regulated during erythropoiesis stimulation) seems to change most significantly after rHuEpo intake as already described by Magnani *et al.*[16] Moreover the estimated *HBB* level, considering its occurrence in the reticulocyte library, was 30% of total mRNA and, with *HBA1* and *HBA2*, represent the major reticulocyte transcript, ii) *FTL*: the red cell pool incorporates iron during erythroid expansion. The use of rHuEpo may cause a change in iron metabolism: the mRNA content of *FTL*, a protein implicated in iron metabolism and storage, iii) *OAZ*: as a preliminary *in vitro* approach, we studied the regulation of newly described mRNA during erythroid differentiation. We previously tested these markers on a human erythroleukemic *TF1* cell line treated with human recombinant erythropoietin which provides an *in vitro* model of erythroid differentiation (Table 1). Results showed

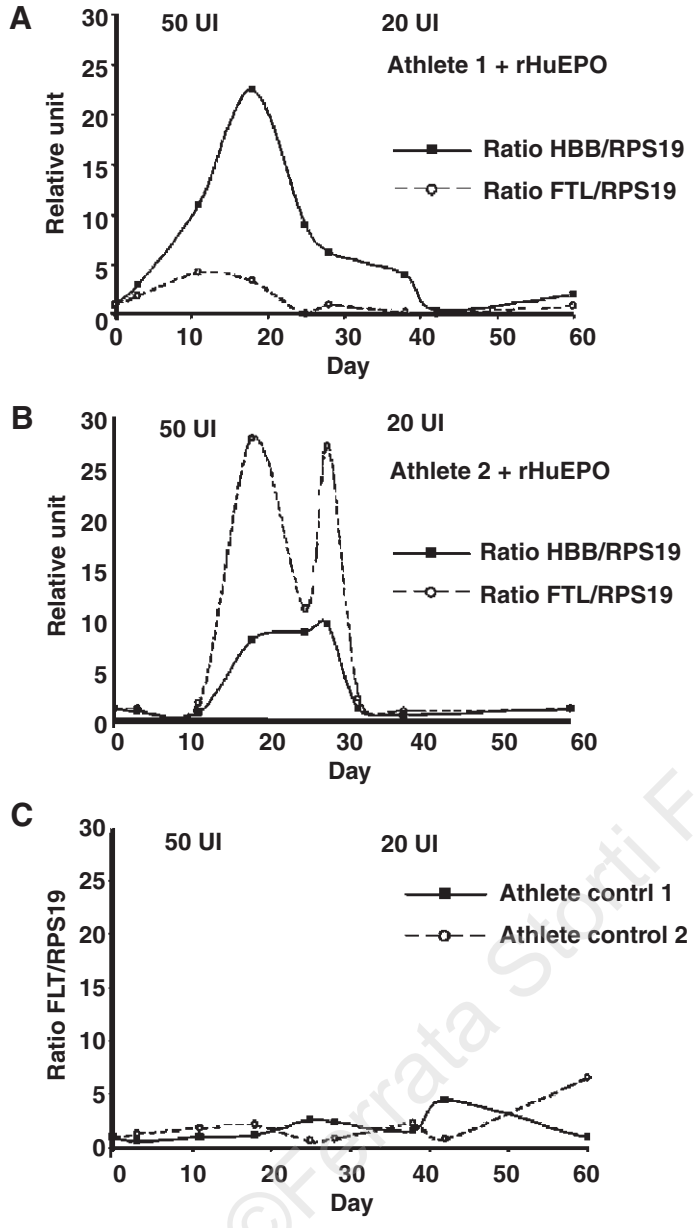

Figure 2. mRNA expression profile of athletes without (control) or upon and after rHuEpo administration (rHuEpo group). Each athlete received rHuEpo injections 3 times a week for 4 weeks at 50 IU/kg, and 3 times a week for 2 weeks at 20 IU/kg; there was a wash-out phase of 3 weeks. Each subject received elemental iron, vitamins B9 and B12 (see Design and Methods). (A) and (B) correspond to 2 athletes during and after rHuEpo treatment and show the mRNA profile of *HBB* and *FTL*. (C) corresponds to 2 control athletes and shows the mRNA profile of *FTL*. The initial ratio (before rHuEpo injections) of selected gene and calibration gene RPS19 mRNA contents corresponds to the reference value (=1).

an increase of these markers during *TF1* treatment with rHuEpo. Concerning *HBB* mRNA, our results confirm, in part, the findings of Magnani *et al.*[16] *HBB* mRNA, detected by competitive real time-PCR in whole blood samples, significantly increased above a threshold level during the first three weeks of treatment with rHuEpo before returning to the normal range at the end of the administrations of low rHuEpo doses. The same was true of the two others markers (*FTL* and *OAZ* mRNA). It became apparent that the variations of these three markers resembled reticulocyte varaiations as described by Russel *et al.*[24] So it appears that these parameters could used to detect rHuEpo abuse only during the treatment. But during this period, the direct determination of rHuEpo in urine is possible, and these parameters could only reinforce the *ON models* as a screening method. Since urinary detection of rHuEpo is impossible some days (2-7 depending of the dose and the route of administration) after the end of rHuEpo administration, it would be interesting to find markers able to reinforce the *OFF models*. Unfortunately, at the end of treatment, the three markers we examined were in the normal range. Curiously we noticed an increase of *FTL* and *OAZ* mRNA in subjects who were not given rHuEpo. This increase after the end of iron injections is difficult to explain. To our knowledge, no data have been published on mRNA expression of *OAZ* and *FTL* genes and *in vivo* iron studies.

At present there are two solutions that raise a suspicion of rHuEpo misuse: the hematologic passport and *OFF models*.[25] It is obvious that if we want to use *OFF models* as a method of confirming rHuEpo misuse, they need to be reinforced. The used of transcriptome data will further help us to define additional genes of interest *(Bonafoux et al., submitted publication*). The recent development of real time-PCR, a rapid, reliable and valid method, could constitute a new perspective in blood anti-doping research and such an approach may be an additional weapon in the war againss doping

This work needs further investigations using additional selected genes from expression profile analysis. If the method is successful, it would allow the detection of other agents stimulating erythropoiesis such as mimetics of erythropoietin.

EVM: conception, design, carrying out the human studies, analysis and data interpretation, drafting and article revision and final approval; MA: conception, design, funding of the project, carrying out the Hemopure study, data interpretation, drafting and article revision and final approval; ML: design, carrying out of the real time-PCR analysis, data acquisition and interpretation, article revision and final approval; BB: design, data acquisition and interpretation, article revision and final approval; MTS: handling, design, data acquisition and interpretation, article revision and final approval; JM: design, data acquisition and interpretation, article revision and final approval; DP: conception of bioinformatic analysis, design, data acquisition and interpretation, article revision and final approval; TC: design, analysis, data acquisition and interpretation, article revision and final approval. The authors reported no potential conflicts of interest.

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