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**MODIFICATION OF GENE EXPRESSION: HELP TO DETECT
DOPING WITH ERYTHROPOIESIS-STIMULATING AGENTS**

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3 MODIFICATION OF GENE EXPRESSION: HELP TO DETECT DOPING WITH
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6 ERYTHROPOIESIS-STIMULATING AGENTS
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Enhanced oxygen delivery to tissues has been shown to improve endurance performance [1]. This has led to widespread abuse of erythropoiesis-stimulating agents (ESA) in elite sport. It is conceivable that athletes have even learned how to continue illicit ESA doping [2]. This possibility has fuelled the search for novel methods to detect and deter ESA abuse using “indirect” markers. It is now widely accepted that drugs influence gene expression [3-4]. In this study, we envisage its use as a means to detect ESA doping. We obtained a global view of the transcriptome of total blood cells both before and after treatment with darbepoetin alpha using the SAGE method. *In silico* analysis identified 95 genes whose differential expression was subsequently tested by quantitative real-time PCR in two athletes. The athletes were treated first with high doses of recombinant human erythropoietin (rHuEpo) to increase their hemoglobin concentration and then with microdoses to maintain the high concentration of hemoglobin. Significance analysis of microarrays at a delta ratio greater than 1.5 indicated that 33 genes could be significant markers of ESA administration. Interestingly, when high and microdoses were taken into account together, five genes could still be considered as significant markers.

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6 It is well known that the interaction of a drug with a biological system can
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8 result in a gene expression profile or signature that is characteristic of the event. The
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10 more recently developed methods that estimate quantitative and qualitative
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12 differences in mRNA are microarray analysis [5] and serial analysis of gene
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14 expression (SAGE) [6]. We used the SAGE technique to obtain a global view of the
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16 blood transcriptome to observe gene profiles before, during and after ESA treatment
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18 and to determine indirect markers of ESA abuse. Indeed, although ESAs differ in
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20 pharmacokinetic, pharmacodynamic and receptor-binding properties, they all share
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22 the same mechanism of action: binding and activating the Epo receptor [7].
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27 Three SAGE libraries were constructed by pooling blood samples from 14
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29 athletes (7 males and 7 females) before (J-1), during (J10) and after (P17)
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31 darbepoietin administration. The mean hemoglobin concentration values were $14.8 \pm$
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33 0.3 g/dl (J-1), 15.7 ± 0.4 g/dl (J10), 16.2 ± 0.6 g/dl (P17) for males and 13.1 ± 0.4
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35 g/dl (J-1), 13.9 ± 0.6 g/dl (J10), 15.1 ± 0.3 g/dl (P17) for females.
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39 SAGE allows quantitative analysis of genome-wide expression profiles of
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41 tissues or cell lines [8-11]. We sequenced 60,000 tags from the three libraries.
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43 Among the 19,713 unique transcripts that were identified, 55% corresponded to
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45 known genes, 12% to ESTs, and 33% to unidentified genes. Next, genes were
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47 classified according to their profiles during the three periods using the K-means
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49 clustering (KMC) algorithm. Thus, according to these criteria, 360 downregulated and
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51 240 upregulated SAGE-tags were selected (Figure 1).
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55 The three SAGE libraries were integrated onto a Biotag bioinformatics
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57 platform, which incorporates SAGE transcriptome data from the scientific community
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59 on a monthly basis. One of the advantages of SAGE data is that pathological and
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3 normal cells/tissues can be compared [12]. Thus, the bioinformatics analyses of the
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5 Epo SAGE libraries were compared first with the 25 available blood cell SAGE
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7 libraries and then with the 960 SAGE libraries of pathological and normal
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9 cells/tissues [13].
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12 The final *in silico* step of selection was to discard those genes whose
13
14 expression could be misinterpreted in the final analysis (Figure 1). For example, we
15
16 identified a similar induction of the *RAD23B* (NM_002874) gene. Indeed, *RAD23B*
17
18 was increased both in blood cells with darbepoetin alpha treatment and in
19
20 monocyte/macrophage cells stimulated with lipopolysaccharide (LPS) (Gene
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22 Expression Omnibus id: GSM136119). Therefore, a set of 95 well-identified SAGE
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24 tags was selected and analyzed with a real-time PCR platform (supplementary data).
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29 We explored putative functions in the selected genes using the Kyoto
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31 Encyclopedia of Genes and Genomes (KEGG) database [14] and checked the
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33 physiological relevance of using various blood cell types as biomarkers. Indeed, we
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35 compared these genes and their profiles in transcriptomic myeloid and lymphoid
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37 normal lineages (Figure 2). Interestingly, although the kinetics used could not indicate
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39 Epo as a direct or indirect cell-type specification factor, gene expression analysis
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41 revealed the profiles of the blood cell types.
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45 A functional genomic study using KEGG showed events predominantly related
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47 to proliferation and differentiation. Many of the changes concerned genes involved in
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49 the translation process and cell growth regulation; for example, the genes of the
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51 translation machinery (ribosome biogenesis) were found to be increased with rHuEpo
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53 treatment. We also observed the induction of gene markers associated with Epo and
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55 thrombopoietin pathways, known to be involved in the proliferation and differentiation
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57 of erythroid and megakaryocytic lineages, as well as more primitive progenitors.
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3 Moreover, we found an increase in the genes involved in blood cell-specific functions
4 like Epo or CD45 receptors. Finally, we retrieved the markers associated with an
5 upregulation of the genes involved in the hemoglobin complex. It is noteworthy that
6 our set of markers contained only one gene downregulated and involved in the MHC
7 class I protein complex and immune response. The most important groups were
8 cytokine-cytokine receptor interaction with the receptors CX3CR1 [chemokine (C-X3-
9 C motif) receptor 1], EPOR (erythropoietin receptor), IL8RB (interleukin 8 receptor,
10 beta) and IL10RA (interleukin 10 receptor, alpha) and the ligands CCL3 chemokine
11 [(C-C motif) ligand 3], IFI30 (interferon, gamma-inducible protein 30) and TNFSF10
12 (tumor necrosis factor, member 10).
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27 The 95 gene profiles from total blood cells were analyzed by RT-qPCR
28 (TaqMan assays on an Applied Biosystems platform). The expression of selected
29 genes was modified during high-dose administration, which confirmed the SAGE
30 results. During the kinetics of selected gene expression, we observed a two-stage
31 variation. For most of the genes, the modification in expression appeared five days
32 after the first ESA administration, was maximal at the end of the high-dose treatment,
33 and thereafter decreased more or less quickly. Few genes were over-expressed
34 during microdose treatment.
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46 To facilitate interpretation, our gene expression was measured using a time
47 interval of three periods corresponding to three rHuEpo administration levels: "Period
48 0" was baseline, "Period 1" was high dose, and "Period 2" was microdose.
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53 The final investigation used significance analysis of microarrays (SAM), the
54 most popular method both to detect significantly expressed genes and to provide an
55 estimate of the false discovery rate (FDR) for multiple testing. We determined the
56 statistical significance of the observed differential expression in two cases, taking into
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3 account a period with only high doses (Period 0 versus Period 1) and a period with
4 high and microdoses (Period 0 versus Periods 1 and 2). Considering the period with
5 only high doses (Period 0 versus Period 1) and a delta ratio with previous periods
6 higher than 1.5 (SAM parameter), we found that 33 genes could be considered as
7 significant markers (means FDR = 0%) (Figure 3 and Supplementary data). With the
8 same parameter (delta ratio higher than 1.5), when high and microdoses were
9 considered only five genes could be declared as significant (Supplemental material).
10 Interestingly, SAM analysis did not yield any downregulated genes in blood cells.
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22 Of course, we found the effect of ESA of the induction of the erythropoiesis.
23 Among the targeted genes induced by Epo, we retrieved the hemoglobin gene family
24 (HBA2, HBB and HBD); the receptor EPOR, which is directly involved in the
25 proliferation and differentiation of erythroid progenitors [4]; glycophorin C (GYPC),
26 which plays an important role in regulating the stability of red blood cells [15]; and
27 ERAF, which acts as a chaperone to prevent the harmful aggregation of alpha-
28 hemoglobin during normal erythroid cell development [16].
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38 However, the biological activity of Epo is not restricted to erythropoiesis
39 regulation, and some of the regulated genes are involved in the development of
40 specific blood cells, like LST1 and its putative role in lymphocyte proliferation [17].
41 Also, the genes are required to control a variety of signal transduction pathways for
42 specific blood cell maintenance, like S100A9, which has a role in the regulation of
43 leukocyte migration [18], or GBP1, a cytokine which contributes to the regulation of
44 monocyte adhesion [19]. Last, we observed some of the genes implicated in antigen
45 processing and presentation, like B2M [20] or TSTA3, which catalyzes the two-step
46 epimerase and reductase reactions in GDP-D-mannose metabolism. This produces
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3 GDP-L-fucose, which is in turn involved in the expression of many glycoconjugates,
4 including blood group ABH antigens and developmental adhesion antigens [21].
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8 These findings are consistent with the role of Epo in governing several cellular
9 pathways and they suggest future directions for clinical trials, as described by Maiese
10 et al. [22].
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14 To confirm potential genes as biomarkers of ESA treatment in our study, we
15 set up a real-time PCR platform and explored the differential expression of 95
16 selected genes. The data were supervised with SAM software. With a delta
17 parameter greater than 1.5, SAM revealed 33 genes with a signature of high-dose
18 ESA administration. This number was reduced to five when high and low doses were
19 taken together. Our results suggest that measurement of gene expression changes
20 could be a very interesting parameter to help in detecting ESA misuse, particularly by
21 reinforcing the interpretation of the hematological passport [23]. One important
22 characteristic of this novel approach is its robustness despite the use of masking
23 agents and/or microdose regimens. The five selected genes were still over-
24 expressed at least one week after the end of microdose treatment (Figure 2),
25 whereas the direct test based on isoelectric separation of Epo isoforms was unable to
26 detect the administered ESA (rHuEpo) 24 hr after the last administration [2].
27 Moreover, the increase of EPO's biosimilars and EPO's copies could make definitive
28 identification problematic [24].
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50 The inter-individual variation in the selected genes needs to be determined on
51 a large population of male and female athletes from different disciplines and parallel
52 research must also establish the stability of these markers in different circumstances,
53 such as training in normoxia and natural or artificial hypoxia. Further research is
54 required to reinforce our findings and to explore the hypothesis that not only current
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3 ESAs but also new Epo mimetics in advanced stages of clinical trials and Epo gene
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5 therapy may yield detectable “fingerprints” of prior use.
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8 **Patients and Method**

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10 The studies were approved by the local medical ethics committees of
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12 Copenhagen, Denmark (KF01-070/03), and Montpellier, France (040702), in
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14 accordance with the revised Helsinki Declaration. All subjects were non smokers and
15
16 did not take part in any kind of organized sports.
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20 The three SAGE libraries were constructed by pooling blood samples from 14
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22 healthy, recreationally active subjects. Subject characteristics (mean \pm SD) for males
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24 were: age, 26 ± 3.2 yr; body mass, 79.2 ± 8.6 kg; height, 181.3 ± 3.5 cm. The
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26 corresponding values for females were: age, 24.5 ± 4.5 yr; body mass, 57.6 ± 12.8
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28 kg; height, 167.1 ± 7.2 cm. All subjects had subcutaneous injections of $0.72 \mu\text{g}\cdot\text{kg}^{-1}$
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30 $\cdot\text{wk}^{-1}$ of darbepoetin alfa (Aranesp, Amgen Europe B.V., Netherlands), administered
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32 weekly for 4 weeks). One week before the study until 2 wk after, female and male
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34 subjects were orally supplemented each day with 200 and 100 mg of iron,
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36 respectively.
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41 Our second study recruited two well-trained male subjects (28 yr, 74 kg, 176.5
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43 cm, regional level triathlete; 31 years old, 62 kg, 170 cm, national level long distance
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45 runner). Initially, red blood cell production was rapidly accelerated in both subjects by
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47 high doses of rHuEpo (~ 260 IU/kg injections on days 0, 2, 4, 7, 9 and 11) in
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49 conjunction with a single intravenous iron treatment (100 mg), with the goal of
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51 elevating hemoglobin (Hb) concentration to approximately 170 g/L. Over the next
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53 three weeks, injections were given every two to three days (injections on days 15, 17,
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55 19, 22, 24, 26, 29, 31 and 33) and doses were adjusted by a pharmacologist guided
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57 only by basic hematologic information (blood and reticulocyte counts; no urine
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3 profiles were provided as feedback). Microdoses were less than 10% of the initial
4 dose (exact dosage undisclosed to prevent replication by athletes). Longitudinal
5 samples from the first subject were collected five times during the high-dose period
6 (days 2, 4, 6, 8, 10), seven times during the microdose period (days 14, 18, 21, 23,
7 25, 28, 31) and once six days after the last injection (day 37). The second subject
8 was sampled four times during high-dose period (days 2, 4, 6, 9), six times during the
9 microdose period (days 17, 19, 23, 28, 32, 37) and once eight days after the last
10 injection (day 45).
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22 Posture was standardized with each subject seated for five min before
23 assuming a supine position for venipuncture (blood sampled from an antecubital
24 vein).
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29 Total RNA was extracted from 2.5 ml of blood using the PAXgene blood RNA
30 kit (Qiagen, Courtaboeuf, France), following the protocol provided by the
31 manufacturer. The quality of total extracted RNA using the PAXgene[®] method was
32 checked by capillary electrophoresis analysis via an Agilent Bioanalyzer 2100
33 (Agilent, Palo Alto, CA, USA). RNA quantity was measured using a NanoDrop ND-
34 1000 spectrophotometer (Thermo Scientific, Les Ulis, France).
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43 Libraries were constructed using the I-SAGE kit for genome-wide expression
44 analysis (Invitrogen, Cergy Pontoise, France) according to the protocol developed by
45 Velculescu [6]. Briefly, a pool of mRNA samples was converted into cDNA using
46 biotinylated oligo(dt) primer linked to magnetic beads. The cDNA were cleaved using
47 the NlaIII anchoring enzyme. Digested DNAs were split in two and each ligated with
48 one of two adapters containing a restriction site of *BsmFI* tagging enzyme. The two
49 pools of the obtained tags were ligated to one another and served as templates for
50 PCR amplification. The PCR product [containing two tags (ditag) linked tail to tail]
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3 was then cleaved with the NlaIII anchoring enzyme, thus releasing 21 bp-long ditags
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5 that were then concatenated by ligation, cloned and sequenced.
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8 Bacterial colonies were screened by blue/white selection to identify those that
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10 harbored vectors containing ditags. PCR was performed directly on white bacteria for
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12 35 cycles of 30 s at 95°C, 30 s at 58°C and 45 s at 72°C with an initial heat activation
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14 of the enzyme for 5 min at 95°C and a final extension of 5 min at 72°C. Primers used
15
16 for PCR amplification were the universal M13 primers. Sequencing reactions were
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18 performed on PCR products by PCR amplification according to Applied Biosystems
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20 (Big Dye Terminator) or Amersham Pharmacia Biotech (ET terminator) protocols by
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22 Genome Express (Grenoble, France).
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27 Generation of expression matrices, data annotation, filtering, and processing
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29 were performed using Biotag in-house software. Data annotation was based on the
30
31 UniGene build #172. Sequence files were analyzed using software developed by
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33 Skuldtech (Montpellier, France). This software (C+tag program) extracts
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35 experimental data and enumerates tags from concatemers. Tags corresponding to
36
37 linker sequences were discarded, and those originating from duplicate ditags were
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39 counted only once. For tag identification (Preditag program), the library tag list was
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41 matched against public databases like UniGene. Preditag annotated tag-to-gene with
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43 a confidence of annotation. This information was extracted from the leader sequence
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45 of UniGene Cluster or another sequence in the cluster in both directions. Then, an
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47 algorithm (Probatag program) allowed the comparison between libraries and
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49 measured the threshold of significance of the observed variation ($P < 0.01$). The
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51 SAGE libraries were deposited on the NCBI database (GEO numbers are
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53 respectively GSM389906, GSM389907 and GSM389908 for the Before, During and
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55 After SAGE libraries).
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3 The cDNA was synthesized using 300 ng of total RNA and performed with the
4 SuperScript™ II First-Strand Synthesis SuperMix (Invitrogen, Cergy Pontoise,
5 France). PCR reactions were performed according to the Applied Biosystems
6 protocol for the 7900HT ABI Prism Real-Time PCR System and a 384-well plate
7 (Micro Fluidic Card). Standardization and quality control studies of RT-qPCR were
8 based on the study of the Europe Against Cancer program [25, 26], which includes a
9 total of 26 European university laboratories from ten countries. This study
10 demonstrated no statistically significant difference in expression levels in peripheral
11 blood from paired samples. RNA quantification was assessed by calculating $2^{-\Delta\Delta CT}$
12 [27]. The Ct values of both the calibrator and the samples of interest were normalized
13 to an endogenous housekeeping gene, GAPDH mRNA (NM_002046).
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29 **Statistical analysis**

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31 The Biotag platform is a dedicated platform constructed to provide integral
32 access to SAGE data. More than 960 SAGE libraries are integrated on this platform
33 [13]. Normalized and filtered expression files were analyzed for multiple experiment
34 analysis. To analyze the expression signature before and during/after Epo treatment
35 in blood cells, K-means clustering (KMC), an unsupervised clustering statistical
36 method, [28], was applied using the open source Java-based software package,
37 Multiexperiment Viewer, version 3.01 [29].
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48 For statistical analysis of qPCR, we used the SAM software package from
49 Stanford University [30]. This method was chosen over conventional statistical tests
50 because of its acceptance in the microarray community, its general simplicity and its
51 ability to provide an estimate of the FDR. The FDR is particularly important when
52 comparing the expression of thousands of genes simultaneously.
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5 with Aranesp.
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11 study, and by the French Antidoping Agency (AFLD) for the gene selection.
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16 17 AUTHORSHIP AND DISCLOSURES

18
19 Contribution: EVM: designed the research, recruited patients, analyzed data and co-
20 wrote the manuscript; MA and MA: designed the research and analyzed data; MTS:
21 performed the human experimentation for this study; DP: performed the biological
22 and bioinformatics analyses, analyzed data and co-wrote the manuscript. The
23 authors reported no potential conflicts of interest.
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31 32 FIGURE LEGENDS

33
34 **Figure 1.** Flow diagram of selected genes: successive steps describe the selection of
35 genes from the three SAGE libraries to real-time PCR analysis and statistical
36 analysis. BEFORE is the library with blood samples taken before darbepoetin alpha
37 treatment (baseline), DURING with blood samples taken during the darbepoetin
38 alpha treatment phase (10 days after the first injection) and AFTER with blood
39 samples taken after the darbepoetin alpha treatment phase (17 days after the last
40 injection). SAGE libraries are available on NCBI databases. HUGO is the Human
41 Genome Organisation for gene nomenclature. K-means and SAM are statistical
42 approaches.
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55 **Figure 2.** Global view of targeted genes in different normal blood cells types.
56 Hierarchical clustering (HCL) of gene expression profiles was constructed from
57 available human SAGE libraries (Biotag) which are Gene Expression Omnibus
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3 (GEO) labeled: from left to right, CD34 cells (GSM43175), leukocytes (GSM709),
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5 monocytes (GSM118371), macrophages (GSM136120, GSM1361201 and
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7 GSM264073), B cells (GSM136199), T cells CD4 (GSM136194 and GSM3245), T
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9 cells CD8 (GSM136198) and NK cells (GSM136197). Red and green indicate,
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11 respectively, weak and high expression.
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15 **Figure 3.** Measures of (A) hemoglobin concentration and reticulocyte percentage and
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17 (B) gene expression profiles of five markers in two athletes injected successively with
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19 high (period in dark gray) and micro- (period in clear gray) doses of recombinant
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21 human Epo. (A) Full curves represent reticulocyte percentage. Dotted curves
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23 represent hemoglobin concentration and dotted full lines represent the recommended
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25 limit for athletic competition. (B) Tendency curves of the five genes selected by SAM
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27 during high and microdoses. RNA was quantified real-time PCR and assessed by
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29 calculating $2^{-\Delta\Delta CT}$. Data are expressed in log and Day 0 is equal to 0. Experiments
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31 were done in duplicate.
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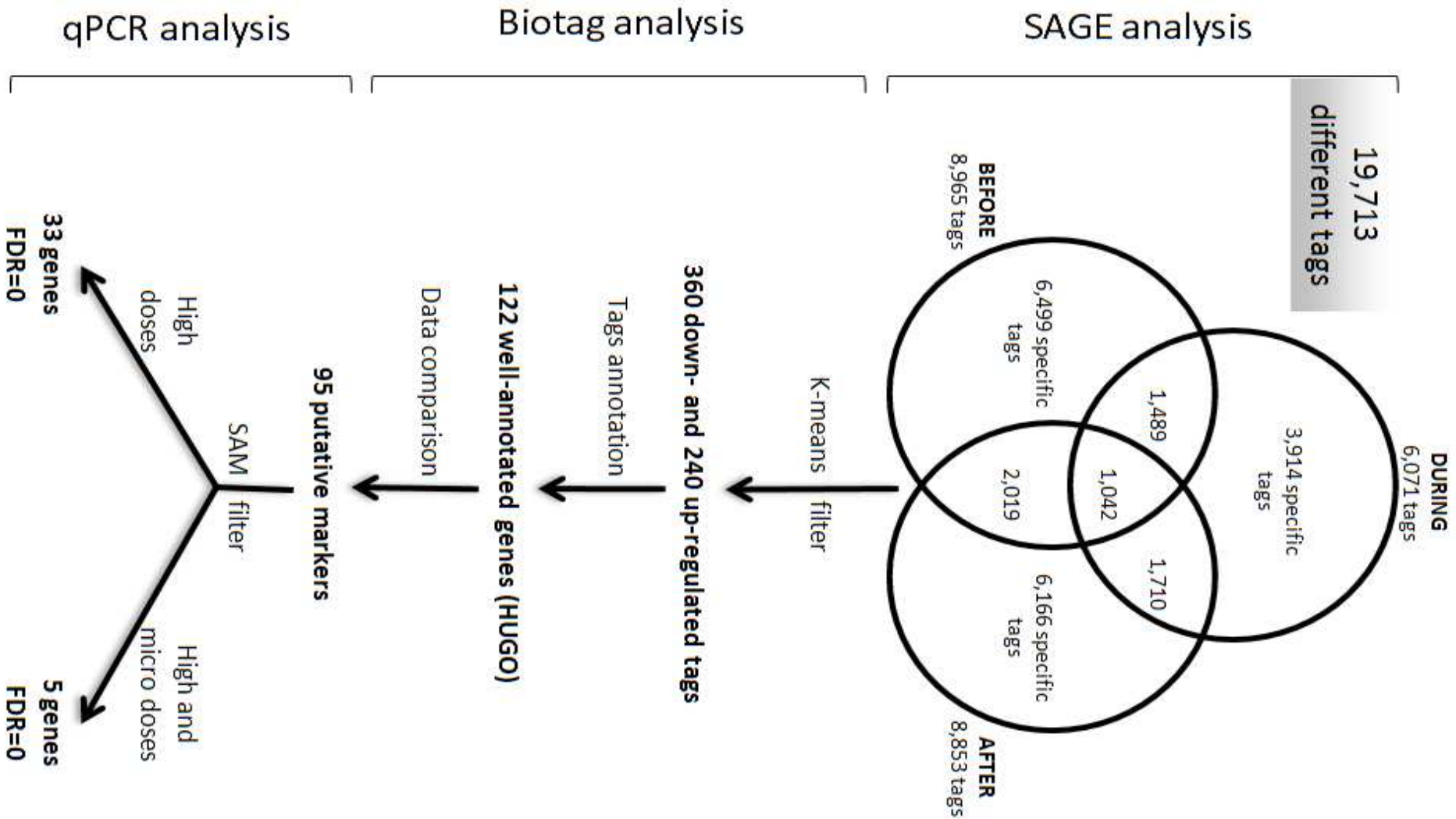
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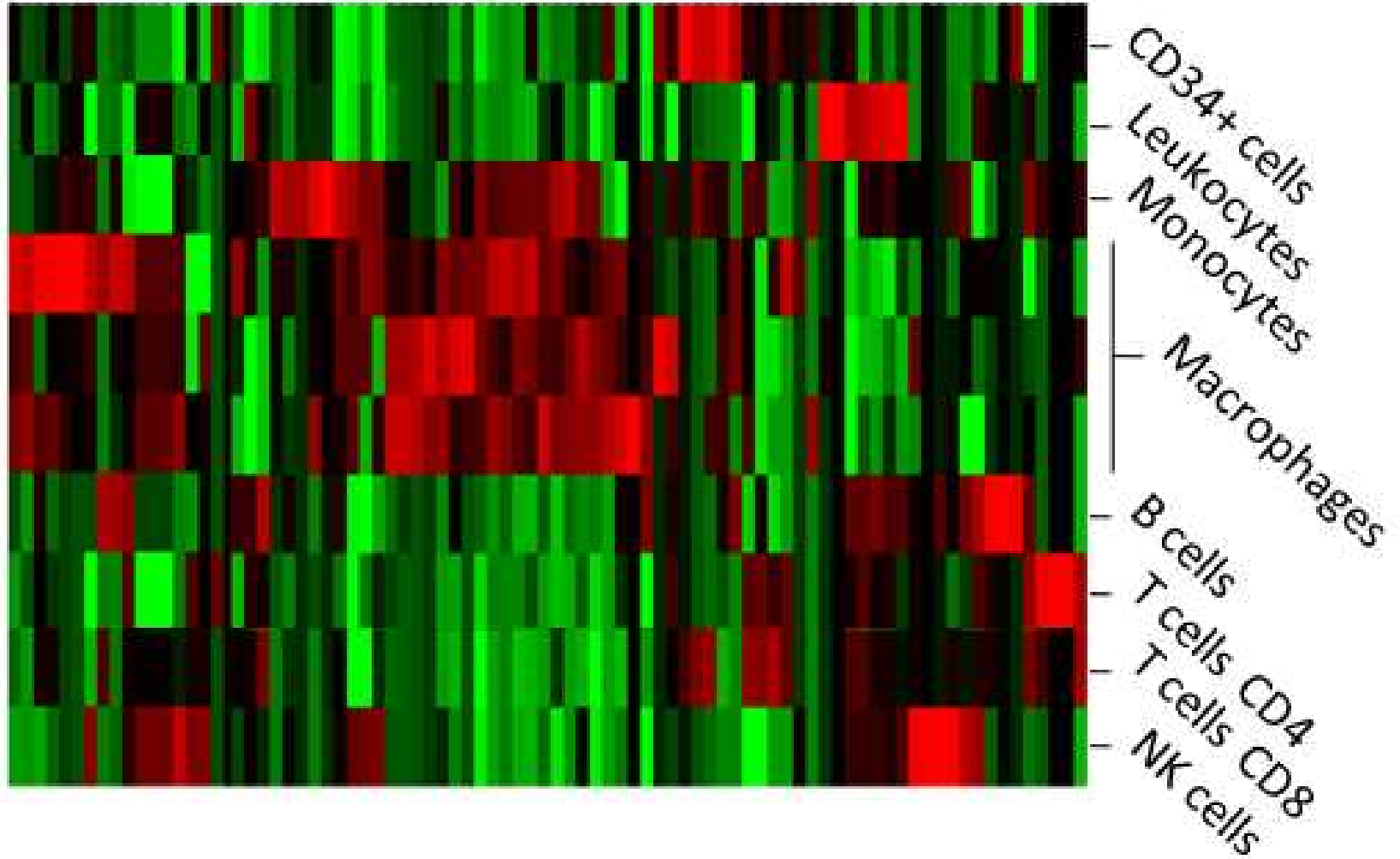
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Athlete 1

Athlete 2

