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Blood cells RNA biomarkers as a first long-term detection strategy for EPO abuse in horseracing

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Recombinant human erythropoietins (rHuEPOs) are glycoproteins drugs, produced by the pharmaceutical industry to restore production of red blood cells by stimulating human bone marrow for which this pathology has been diagnosed. It is suspected that these molecules are diverted as doping agents in horseracing to enhance oxygen transport and aerobic power in racehorses. Although indirect double-blotting or direct liquid chromatography-mass spectrometry (LC-MS) methods have been developed to confirm the presence of rHuEPO in a sample, the short detection time (48 h) is still a problem for doping control. In this context, gene profiling investigation through Serial Analysis of Gene Expression (SAGE) has been conducted on seven thoroughbreds treated with Eprex®. This functional genomic method has been performed from total blood cells collected from each animal to assess the mRNA expression consecutive to rHuEPO injections. Sample pooling was chosen as a powerful, cost-effective, and rapid means of identifying the most common and specific changes in terms of gene expression profile and to eliminate individual variation. Consequently, three SAGE libraries were constructed, before, during, and after Eprex® treatment. More than 71 440 mRNA signatures were observed and subjected to statistical analysis; 49 differentially expressed genes were identified and analyzed by qPCR. From the selected gene list, were defined as potential biomarkers in terms of their low inter-individual variation and capacity as strong markers of rHuEPO administration up to 60 days after the beginning of the doping period. In this paper, a new strategy is proposed to the horseracing industry to prevent rHuEPO abuse.

Keywords: erythropoietin; biomarker; blood; horse; doping

Introduction

Erythropoietin (EPO) is a glycopeptidic hormone of the class I cytokines family[1] produced, predominantly in the kidneys, which stimulates proliferation and final maturation of red blood cell precursors in bone marrow when oxygen availability is low.[2–4] Human EPO (HuEPO) is an acidic glycoprotein with a molecular mass of 30.4 kDa. The peptide core of mature EPO is composed of 165 amino acids which form two bisulphide bridges with three N-glycosylation and one O-glycosylation reported as major post-translational modifications.[5–7]

Since the isolation in 1985 of the human EPO gene,[8,9] the huge development in molecular biology techniques has allowed the large-scale production of recombinant human erythropoietin (rHuEPO) in mammalian cell lines (Chinese Hamster Ovary, CHO; Baby Hamster Kidney, BHK; or human cell). rHuEPO was developed to treat patients with chronic renal failure, where their anaemia is due to erythropoietin deficiency. Consequently, several rHuEPO are available worldwide to meet the needs of patient care. The pharmaceutical molecules of EPO-receptor (EPO-R) ligands are presented in Table 1. EPO is classified using a Greek letter (alpha, beta, delta, omega, zeta) which was attributed by the manufacturers themselves prior the realizing of a new EPO molecule on the market. As originally described by Lasne,[10,11] during the isoelectrofocusing step in a gel, the number of charged molecules, such as sialic acid residues, influences the isoelectric point and consequently the isoform's position in the gel. rHuEPOs are manufactured in several countries with different production processes (cell lines) and with different formulations responsible for this isoelectric pattern heterogeneity.

Therapeutic proteins have a short half-life and it is often thought to inject very high doses to delay further injections but this may cause important side effects, like production of anti-EPO antibodies.[12–14] By extending therapeutic protein half-life, lower doses can maintain effective therapeutic action during a longer period; this is the reason behind releasing darbepoetin alfa on the market. Indeed, it stimulates erythropoiesis by the same mechanism as the endogenous hormone but it has five N-linked carbohydrate chains.[15] Due to its increased carbohydrate content, darbepoetin alfa has a longer half-life than rHuEPO and consequently a greater *in vivo* activity.[15]

In 2008, Continuous Erythropoietin Receptor Activator (CERA) was marketed by Roche. This molecule was created by integrating a single 30 kDa polymer chain (methoxy-polyethylene glycol) into the erythropoietin molecule (EPO beta). This artificial increase of the molecular weight up to twice of epoetin at about 60 kDa considerably enhances its elimination half-life in man to about

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Table 1. List of the most known EPO-Receptor (EPO-R) ligands with their pharmaceutical name, manufacturer, country of availability, and their specific production process

EPO-R ligands	Name	Manufacturer	Availability	Production process
Epoetin α	Epogen®	Amgen	USA	Chinese Hamster Ovary (CHO) cells
	Procrit®			
	Eprex®	Ortho Biologics	Outside USA	
	Erypo®			
	Epopen®			
	Epoxitin®			
	Globuren®			
	Abseamed®	Medice Arzneimittel Pütter	EU	
	Binocrit®	Sandoz	EU	
	Epoetin alpha hexal®	Hexal Biotech Forschungs	EU	
	Epiao®	Shenyang Sunchine Pharmaceutical	China	
	Lixie Bao®	Kirin Kunpeng Biological Pharmacy	China	
Epoetin β	Neorecormon®	Roche Pharmaceuticals	EU	
Epoetin δ	Dynepo®	Shire Pharmaceuticals	EU	Human cells
Epoetin ω	Epomax®	Elanex	Eastern Europe	Baby Hamster Kidney (BHK) cells
	Hemax-omega®		India	
Epoetin ζ	Retacrit®	Hospira	EU	Chinese Hamster Ovary (CHO) cells
	Silapo®	Stada Arzneimittel	EU	
Darbepoetin α	Aranesp®	Amgen	USA, EU	CHO + modified human EPO DNA containing two extra N-glycosylation
	Nespo®	Dompe Biotec	EU	
	Nupiao®	Shenyang Sunshine Pharmaceutical	China	
PEGepoetin β	Mircera®	Roche Pharmaceuticals	EU	CHO + EPO PEGylation
Peptide-ESA	Hematide®	Affymax	USA, EU	PEGylated synthetic peptide

130 h.[16] In addition, an erythropoiesis-stimulating agent (ESA) named Hematide® and formulated as a PEGylated erythropoietic peptide, have been specially designed to bind to the EPO-R in order to activate the intracellular signalling system and thus activate the erythropoiesis.[17]

The relative ease with which rHuEPO preparations or mimetics can be obtained means that this hormone is still misused by some endurance athletes as a doping agent to improve aerobic performance. The use of these substances in horseracing for the improvement of horse aerobic capacity was first reported in the middle 1990s.[18] The high degree of homology in the EPO amino acid sequence amongst mammals[19] means the equine EPO (eEPO) sequence is similar to the human sequence, with close to 84% identity.[20] Consequently rHuEPO forms seem to be suitable for would-be dopers to enhance a horse's aerobic capacity. However, the use of EPO is still controversial; when a horse is making an effort, its splenic red cell reserve can release a large amount of red blood cells in the blood vessels.[18,21]

To date, horse EPO doping can be detected by either the double-blot method, applied primarily to human urine samples,[22] or by liquid chromatography linked to mass spectrometry in equine plasma[23–26] but the detection time of 48 h has limitations and remains a problem for doping control.

In this study, the gene profiling investigation by means of blood cells mRNA expression measurement is used as a new method to identify rHuEPO misuse in official racing laboratories. This method has been defined as a rapid and comprehensive approach for elucidation of quantitative gene expression patterns and does not depend on the prior availability of transcript information.[27,28] Starting from total blood cells collected from horses administered with Eprex®, total mRNA was purified prior to the construction of Serial Analysis of Gene Expression (SAGE) libraries. Bioinformatics data processing led to the identification of biomarkers which were analyzed and identified by real-time polymerase chain reaction (PCR). Simultaneously, haematological parameters and rHuEPO levels were followed.

Materials and Methods

Animals and Treatment

The study was led in agreement with animal welfare rules at the administration and sampling centre of the Fédération Nationale des Courses Françaises (FNCF). Seven thoroughbred horses (two stallions: S1, S2; two mares: M1, M2; and three geldings: G1, G2, G3) weighing 440–595 kg and aged 5–18-years-old received 40 IU/kg/day of erythropoietin alpha (Eprex®, Janssen-Cilag, Issy-Les-Moulineaux, France), subcutaneously in the neck, on six consecutive days.

Sample Collection and RNA Preparation

Venous blood was collected as follows: D_{-3} to D_{-1} before the beginning of administration, D_{+2}, D_{+3}, D_{+4}, D_{+6}, during administration and D_{+8}, D_{+10}, D_{+11}, D_{+13}, D_{+15}, D_{+20}, D_{+30}, D_{+40}, D_{+50}, D_{+60}, D_{+70}, D_{+80}, D_{+100} after the first Eprex® administration by jugular venipuncture using PAXgene Blood RNA tubes (Becton-Dickinson, Le pont de Claix, France). Total RNA was extracted using the PAXgene blood RNA kit (Qiagen, Les Ulis, France) according to the modified protocol provided. The quality of total RNA extracted

from PAXgene® method was checked by capillary electrophoresis analysis using an Agilent BioAnalyser 2100 (Agilent, Palo Alto, CA, USA) and the quantity was measured using a spectrophotometer NanoDrop ND-1000 (Thermo Scientific, Les Ulis, France). Three pools were prepared with total RNA extracted from blood cells obtained from each of the seven horses (1) before EPO treatment with D_{-3} to D_{-1} blank samples; (2) during at D_{+6}; and (3) after at D_{+11} therefore giving three different SAGE libraries for the findings of mRNA biomarkers. Then, RT-qPCR were performed with the selected biomarkers from the pool of samples collected from each of the seven horses on the same day (mean gene kinetic regulation).

Plasma was collected before, during, and three days after the end of administration with 9 mL lithium heparinate tubes (Greiner Bio-One SAS, Les Ulis, France) to assess the rHuEPO level. All samples were stored at −20 °C until their analysis.

Venous blood was also collected from five horses in EDTA-treated tubes (Bio Technofix, Bagneux, France) at D_{-1}, D_{+4}, D_{+6}, and D_{+7} to perform haematological analyses.

Serial Analysis of Gene Expression (SAGE)

Libraries were constructed using the I-SAGE Kit for Genome-Wide Expression Analysis (Invitrogen, Cergy Pontoise, France) according to the protocol developed by Velculescu.[27] Briefly, a pool of mRNA samples was converted into cDNA using biotinylated oligo(dt) primer linked to magnetic beads. The cDNA were cleaved using the *NlaIII* anchoring enzyme. Digested DNAs were split in two and each ligated with one of two adapters containing a restriction site of *BsmFI* tagging enzyme. The two pools of the tags obtained were ligated to one another and served as templates for PCR amplification. The PCR product (containing two tags (ditag) linked tail to tail) was then cleaved with the *NlaIII* anchoring enzyme, thus releasing 21 bp-long ditags that were then concatenated by ligation, cloned and pyrosequenced (Solewe, Illumina, CA, USA).

Bioinformatics Data Processing

Sequence files were analyzed by means of software developed by Skuld-Tech (Montpellier, France). This software (BIOTAG) extracts experimental data and enumerates tags from concatemers.[29] Tags corresponding to linker sequences were discarded, and those originating from duplicate ditags were counted only once. For tag identification, the library tag list was matched against public databases such as UniGene. This information was extracted from the leader sequences of UniGene Cluster or other sequences in the cluster in the both directions. An algorithm then allowed comparison between the three SAGE libraries and measured a threshold of significance for the observed variation ($P\ value < 0.1$).[29] The results were displayed in a table, providing thus the sequence of each tag, its number of occurrences with the matching cluster number, and other data extracted from the source file, including GenBank accession numbers.

cDNA Synthesis and PCR Analysis

The cDNA was synthesized using 500 ng of total RNA and performed with the SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen, Cergy Pontoise, France).

RT-qPCR reactions were performed with LightCycler® DNA Master SYBR Green I (Roche Applied Science, Meylan, France). The reaction mix was prepared as recommended by the manufacturer in a final volume of 20 µL as follows: 1 µL of cDNA was added to 19 µL of reaction mixture which included 4 pmol of each primer, 8.2 µL of ultra-pure water, 1.3 µL of $Mn(OAc)_2$ and 7.5 µL of LightCycler® DNA Master SYBR Green I.

The samples were amplified and quantified in the LightCycler 480 real-time PCR detection system (Roche Applied Science, Meylan, France) under the following conditions. The cycling conditions of 54 cycles consisted of denaturation at 95 °C for 10 s, annealing at 58 °C for 10 s and extension at 72 °C for 15 s. A single sequence was amplified for each probe pair and each amplified sequence had the expected size. In order to discriminate specific from non-specific products and primer dimers, a melting curve was obtained by gradual increase in temperature from 65 to 95 °C at a rate of 2.5 °C/s. To confirm reproducibility, all PCR were performed in triplicate.

Samples were normalized with two control genes Cofilin-1 (CFL-1) and Ferritin H (FTH). The results were analyzed with the LightCycler software version 1.5.0 and quantification of RNA levels were assessed by calculating $2^{-\Delta\Delta CT}$.[30]

Plasma rHuEPO Quantification and Haematological Parameters

The horse plasma rHuEPO level was determined by a human EPO ELISA kit (Quantikine® IVD®, R&D Systems, Lille, France). The procedures were carried out according to the supplier's manual. The enzyme-linked immunosorbent assay (ELISA) plate was placed on a plate-shaker, Shaker/Incubator ZLE164 (Amersham-GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and the ELISA plate was washed using a plate washer, Novapth Washer (BioRad, Marnes la Coquette, France). Finally, absorbance was measured at 450 nm using a microplate reader (Opsys MR Reader, Thermo Labsystems, Issy-Les-Moulineaux, France).

Haematological analyses were performed with a Cell-Dyn® automated analyser (Abbott Diagnostics, Santa Clara CA, USA) which applies the principles of Multi-Angle Polarized Scatter Separation (MAPSS™) with high-resolution flow cytometry for generation of the blood count data. Due to some differences between human and horse haematological parameters (shape, size of cells), analyses were confirmed by manual counting.

Results and Discussion

rHuEPO Kinetics

Recombinant human EPO concentrations were measured by ELISA to assess the rHuEPO level in each of the seven administered horses both during treatment and afterwards. The ELISA kit is designed for human plasma but has been well evaluated for analysis of horse plasma containing rHuEPO and is furthermore widely used in horseracing anti-doping laboratories for its ability to cross-react significantly with Aranesp®.[31]

The profiles of rHuEPO plasma concentrations are shown in Figure 1. The rHuEPO levels, before the first drug administration (Day 0), were found to be very low and considered non-specific because of the poor cross-reactivity of equine EPO and the anti-human EPO monoclonal antibody coated in the wells. After rHuEPO administration, the maximum concentration values observed for the seven animals were between 70 and 90 IU/L from D_{+1} to D_{+3}. rHuEPO concentrations then decreased slightly until the last administration at D_{+5}. The day following the last Eprex® injection, rHuEPO plasma concentrations ranged from 12 to 42

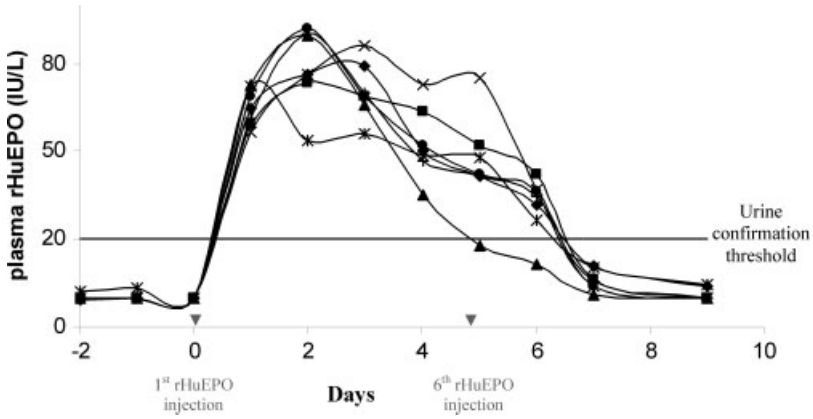

Figure 1. Individual plasma concentration of rHuEPO measured by ELISA, before, during, and after subcutaneous administration (from Day 0) of recombinant human EPO (40 IU/kg/day, Eprex®) for 6 days in 7 thoroughbred horses (M1: ♦; S1: ■; G1: ▲; G2: ×; S2: ✳; M2: ●; G3: |). The rHuEPO plasma threshold at 20 IU/L, from which a sample can be confirmed by double-blotting in urine.

IU/L and values corresponding to the physiological concentration of endogenous EPO were reached at D_{+9}.

The results obtained from the analysis of rHuEPO levels in the plasma of the seven horses administered with Eprex® showed a wide variation in the range of concentrations. In the case of G1, its rHuEPO concentration decreased rapidly to reach the rHuEPO threshold of 20 IU/L two days earlier than the others animals. The 20 IU/L threshold is based on the minimum amount of rHuEPO detectable in a plasma sample during the screening step for which a confirmation method in urine can be performed by double-blotting.[22] This study confirms that measurement of rHuEPO levels by ELISA method can only detect the use of rHuEPO during treatment or one day after the last administration and is, therefore, not consistent with the long-term detection of this forbidden drug.

Haematological Parameters

The measurement of haematological parameters was performed to investigate the haematocrit (Ht), haemoglobin (Hb) and red blood cell (RBC) values of five horses treated with Eprex® (40 IU/kg/day). Results obtained are presented in Figure 2. The haematocrit values (%) obtained from the five horses were 41.4 ± 3.3 with a range of 36.5 to 44.7 before administration and 41.8 ± 4.0 with a range of 37.4 to 47.4 after the last administration. The heamoglobin values (g/100 mL) obtained from the five horses were 13.7 ± 1.3 with a range of 11.9 to 15.3 before administration and 14.1 ± 1.5 with a range of 12.4 to 16.5 after the last administration. The red blood cells values (M/mm^3) obtained from the five horses were 8.7 ± 0.7 with a range of 7.5 to 9.4 before administration and 8.7 ± 0.7 with a range of 7.8 to 9.7 after the last administration. These results show that no significant variations were observed for any of these parameters at a dose of 40 IU/kg/day and moreover data obtained were consistent with the reported haematological reference values.[32,33] These results are in good accordance with previous data obtained from horses administered at 30 IU rHuEPO/kg bwt.[34,35] The hypothesis that the selected animals (5–9-years-old) were relatively old horses (≥19-years-old), with a bone marrow of low activity can be ruled out for this study and therefore cannot explain these haematological results. Repeated administration did not stimulate erythropoiesis sufficiently to induce any significant increases in RBC, Hb, or Ht. Consequently, low doses of rHuEPO cannot be detected by the means of the diagnostic measurement of the haematological parameters. Nonetheless, it must be noted that higher rHuEPO doses have been administered to horses (120 IU/kg bwt) or low dose (50 IU/kg bwt) with a longer treatment period (3 times/week for 3 weeks) and have shown an increase in the haematological parameters.[34–36]

Findings of Biomarkers by SAGE

The gene profiling investigation was performed starting from three SAGE libraries constructed by means of the seven horse-blood samples collected before, during, and after Eprex® treatment in specific PAXgene tubes. These tubes (BD Vacutainer®) contain an intracellular RNA conservation medium (nucleic acid stabilization), minimizing the processing urgency.

From the libraries, 200 000 mRNA signatures were obtained and after statistical analysis (P-value <0.1 and up or down-regulation $= 5$ times), 963 mRNA up-regulated and 1335 mRNA down-regulated have been selected. These biomarkers were from genes up-regulated or down-regulated during the administration but also several days after the end of rHuEPO administration. These biomarkers were specific to the different blood cellular types for which the reticulocyte cells mRNA accounted for 45% of the total mRNA amount in the blood, white cells for 45% and platelets for 10%.

SAGE-tags annotation was performed from the sequences available from the UniGene databank (http://www.ncbi.nlm.nih.gov/UniGene/). Horse genome knowledge is poor and hence sequence identifications were often difficult. Finally, less than 4500 genes were well-identified from the available data provided by the UniGene databank. This lack of knowledge of the horse genome led to a reduction in the speed of the experimental tag annotations and sometimes led to mistakes. To assess the gene annotation quality, an appraisal step of the identified biomarkers was performed by real-time PCR.

Following this exhaustive study, some of the identified genes were removed; for instance those linked to the inflammatory process. It has been supposed that those gene functions were far from the haematopoietic pathways.

Biomarkers Selection

The real-time PCR biomarker selection allowed validation and investigation of the inter-individual kinetics associated with whole gene expression from blood samples collected from the seven

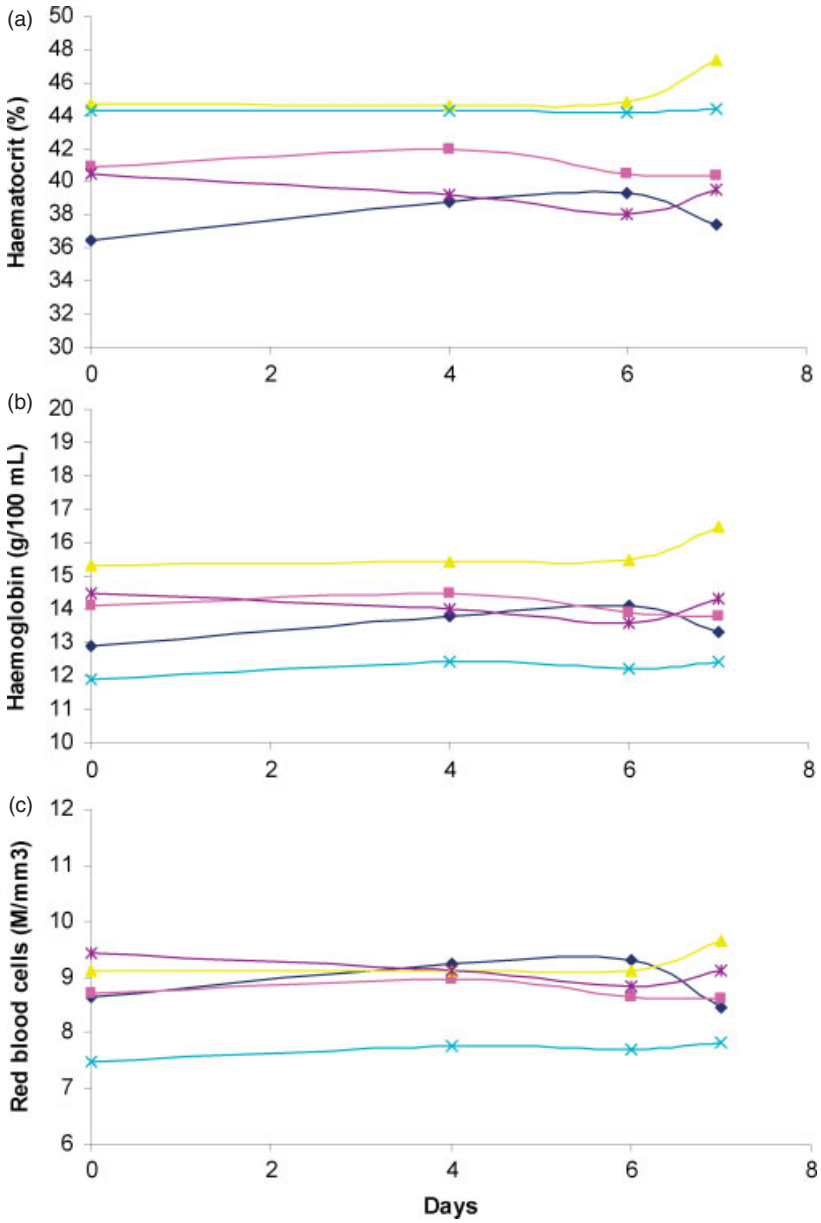

Figure 2. Measurement of three haematological parameters from five horses: haematocrit (a), haemoglobin (b), red blood cells (c) before, during and after the six days of Eprex® treatment at 40 IU/kg/day, (G1: ♦; G2: ■; S2: ▲; M2: ×; G3: ✳).

horses and assessment of the gene expression level during a long period. From the current results, 49 genes were well-identified. Starting from these 49 selected genes, 8 markers were selected for their low inter-individual variation and strong rHuEPO administration response markers. Mean gene expression profiles of the 8 selected biomarkers, named genes 1 to 8, are presented Figure 3. Genes 1, 2, 3, 7, 8 were genes correctly identified from the gene databank (Table 2). Nevertheless, research is still ongoing to investigate more precisely their identification from the horse genome databank. Functions of genes 4, 5, 6 are unknown at present but referenced in EST (Expressed Sequence Tags).

All of the 8 selected biomarkers were down-regulated during and after the end of Eprex® administration. In most cases, before the beginning of the EPO treatment (D_{-2}, D_{-1}) the gene expression was near 1 arbitrary unit (AU), except for gene 2 where the initial value was two times below (0.5 AU). Then, the gene expression decreased to reach 0 UA at D_{+3} with an earlier decrease at D_{+2} for gene 8. This gene down-regulation was observed until D_{+30} after the first administration for genes 4 and 7, D_{+50} for genes 1, 2, 5, 8 and D_{+60} for genes 3, 6. Starting from 0 AU the gene expression of the selected biomarkers increased slowly until the end of the sample collection at D_{+100}. Solely gene 8 reached the same gene expression at D_{+100} and at D_{-2}, D_{-1}. The identified biomarkers were neither similar to those identified in human reticulocytes in the attempt of the detection of rHuEPO by means of gene expression measurement[37] nor to β-globin mRNA previously described in a multiparametric method to assess rHuEPO doping through a screening method.[38] The investigation to understand the biological significance of the 8 biomarkers is still in progress.

These results mean that the effect of repeated EPO administration may be detected by a transcriptomic strategy during the treatment and also up to 60 days after the beginning of the doping

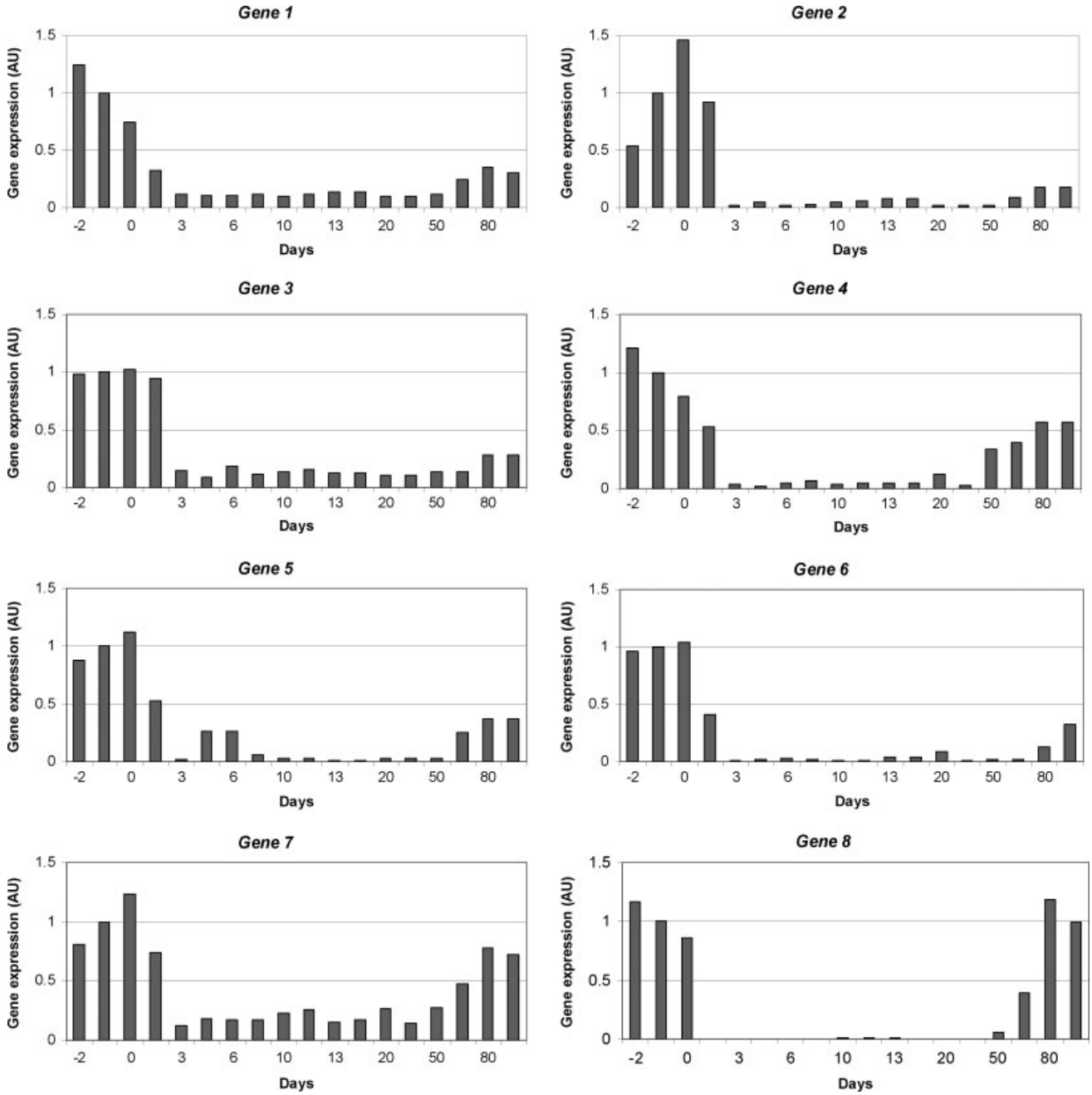

Figure 3. Histogram patterns of the mean gene expression regulation of the 8 biomarkers (gene 1 to gene 8). Data presented are from the pooled samples of 7 horses on the same day. Biomarkers were selected for their low inter-individual variation and strong rHuEPO administration response markers. Gene expression regulation was expressed in arbitrary unit (AU).

period. It is thought that this new method would be useful for horseracing authorities to improve the control of rHuEPO misuse and also to detect rHuEPO drugs recently released on the market such as Mircera®. Indeed, the major advantage of CERA is its long-acting effect requiring only a monthly dose. Consequently, since human French anti-Doping Agency (AFLD) caught cyclists on the 2008 Tour de France who had used Mircera®, it is to be feared that this new substance will be abused in horseracing. Moreover, in humans, erythropoietin gene therapy has been well developed[39] and this process may be shifted to horses to illegally enhance their aerobic capacity. Regarding the new generation of doping strategies, it is necessary to investigate new analytical methods in order to develop an alternative strategy for anti-doping control in routine analysis.

Conclusion

Although numerous methods have been developed to detect specific rHuEPO in plasma or urine, these data suggest that gene expression modification offers a promising method to enhance the detection of erythropoietic stimulant abuse. The major interest of this method seems to be its ability to detect the administration of the recombinant EPO available worldwide; even erythropoiesis

Table 2. Identity of the eight target genes and their accession number from Ensembl (www.ensembl.org) and GenBank (www.ncbi.nlm.nih.gov/genbank) databases

Target Gene	Gene Name	Accession Number	
		Ensembl database	GenBank database
1	IGHDt	–	AY631943.1
2	IGHDs	ENSECAE00000015343	–
3	RPL14	ENSECAT00000013174	
4	EST_03	–	1350045
5	EST_04	–	1350038
6	EST_x7	–	1350050
7	CSRP3	ENSECAT00000017004	–
8	BSG	–	NM_001098795

stimulating agents (ESA) or erythropoietin gene therapy by means of the RT-qPCR measurement of some specific blood cells genes, expressed consecutively to the over activation of the EPO target receptors. In order to assess this innovative technology, the method validation is in progress; other rHuEPO molecules such as Mircera®, Dynepo® and Aranesp® have already been administered to horses to approximate the rHuEPO-specificity of the 8 selected biomarkers and further analysis on post-race samples will be performed to obtain baseline gene expression levels.

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