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Erythropoietin as a performance-enhancing drug: Its mechanistic basis, detection, and potential adverse effects

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ABSTRACT

Erythropoietin (EPO) is the main hormone regulating red blood cell (RBC) production. The large-scale production of a recombinant human erythropoietin (rHuEPO) by biotechnological methods has made possible its widespread therapeutic use as well as its misuse in sports. Since the marketing of the first epoetin in 1989, the development has progressed to the third-generation analogs. However, the production of rHuEPO is costly, and the frequent administration of an injectable formula is not optimal for compliance of therapeutic patients. Hence, pharmaceutical industries are currently developing alternative approaches to stimulate erythropoiesis, which might offer new candidates for doping purposes. The hypoxia inducible factors (HIF) pathway is of particular interest. The introduction of new erythropoiesis-stimulating agents (ESAs) for clinical use requires subsequent development of anti-doping methods for detecting the abuse of these substances. The detection of ESAs is based on two different approaches, namely, the direct detection of exogenous substances and the indirect detection, for which the effects of the substances on specific biomarkers are monitored. Omics technologies, such as ironomics or transcriptomics, are useful for the development of new promising biomarkers for the detection of ESAs. Finally, the illicit use of ESAs associates with multiple health risks that can be irreversible, and an essential facet of anti-doping work is to educate athletes of these risks. The aim of this review is to provide an overview of the evolution of ESAs, the research and implementation of the available detection methods, and the side effects associated with the misuse of ESAs.

1. Introduction

During the Mexico City 1968 Olympics, it became evident that the oxygen transport capacity of exercising muscles was the limiting factor for aerobic performances (Jokl et al., 1969). The performances were significantly affected by altitude-impaired oxygen delivery. Consistent with this phenomenon, Ekblom et al. reported that an increase in the hemoglobin (Hb) concentration can ameliorate the maximal oxygen uptake (VO_{2max}) and consequently athletic performance (Ekblom et al., 1972). Shortly thereafter, athletes started to experiment with blood transfusions to increase Hb for improved performance. However, this practice requires a sophisticated methodology and carries the risk of adverse effects. Following its global commercialization between 1987 and 1989, recombinant human erythropoietin (rHuEPO) use surged in the 1990s/2000s due to its easy access and its significant impact on performance. The drug was readily placed on the International Olympic Committee (IOC) list of banned substances in 1990, although no validated detection method was available. A decade later, a direct test to effectively differentiate between endogenous and recombinant EPO based on isoelectric focusing (IEF) was developed by Lasne and de Ceaurriz (Lasne and de Ceaurriz, 2000). Concurrently, the indirect detection of rHuEPO via blood markers of erythropoiesis was also established (Parisotto et al., 2000). In accordance with the indirect detection of blood doping, the Athlete Biological Passport (ABP) was developed and implemented in 2008 (Sottas et al., 2011). The hematological module of the ABP aims to assess the effects of blood manipulators, such as erythropoiesis-stimulating agents (ESAs), on selected biological parameters. The implementation of this tool provoked a trending change of cheating athletes that now use microdoses of rHuEPO to avoid large

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fluctuations in the levels of blood markers in the ABP and to reduce the detection window for classic direct detection (Ashenden et al., 2011). These microdoses are hardly detectable with classical hematological parameters, and new markers need to be developed to increase the sensitivity of the diagnostic tool. Recently, the pharmaceutical industry has developed new ESAs, which provide good candidates for doping. Alarmingly, some athletes have tested positive for some of these agents, although many are still in clinical trials. Consequently, anti-doping laboratories have to prepare for this situation and to develop new methods for the detection of ESAs. Omics technologies, such as transcriptomics and ironomics, offer promising candidates for the refinement of the hematological module. Although the microdosing trend has decreased the occurrence of death related to ESAs, their long-term misuse associates with significant adverse effects that can be irreversible. Therefore, anti-doping authorities play a crucial role in educating athletes about these risks.

2. Erythropoietin (EPO) and ESAs

2.1. Erythropoietin (EPO)

Human erythropoietin (HuEPO) is a glycoprotein hormone that is mainly produced by peritubular fibroblasts in the kidney. It stimulates the survival, proliferation, and differentiation of erythroblasts (Jelkmann, 2011). The molecule exerts its effects by binding to its receptor (EPOR), which triggers its activation, followed by the activation of different kinases and intracellular signaling pathways such as the Janus kinase (JAK)-2 and signal transducer and activator of transcription (STAT)-5 pathways (Debeljak and Sytkowski, 2012). Besides its hematopoietic effects, EPO-EPOR signaling functions in several tissues and systems, including the central nervous system, heart, kidney, gastrointestinal system, reproductive tract, and endothelium (Ogunshola and Bogdanova, 2013; Arcasoy, 2008). Moreover, the pleiotropic effects of erythropoietin (EPO) are not only mediated by an endocrine, but also by autocrine and paracrine mechanisms (Sytkowski, 2007).

Endogenous HuEPO production is mainly regulated by hypoxia and this regulation occurs at the transcriptional level (Jelkmann, 2011). When the oxygen supply of the human body decreases, transcriptional factors defined as hypoxia-inducible factors (HIFs) are activated, inducing the expression of several genes that include the *Epo* gene. Under normoxic conditions, GATA-2 and NF-κB suppress the *Epo* promoter, and HIF prolyl hydroxylases (HIF-PH) degrade the HIFα subunit (La Ferla et al., 2002). Under hypoxic conditions, on the other hand, the oxygen-dependent activity of HIF-PH shuts down, which stabilizes HIFα and triggers its dimerization with HIFβ and *Epo* transcription.

The resulting mature EPO protein consists of 165 or 166 amino acids and contains three N-linked and one O-linked oligosaccharide side chains. The molecular weight of the protein is approximately 34 kDa, with the glycans representing 35–40% of the molecular mass of the glycoprotein. N-glycosylation is particularly important for the biological activity of EPO, because it regulates its affinity for the receptor, clearance, and serum half-life (Franz, 2009).

2.2. First-generation recombinant EPO

The human *Epo* gene was first isolated and cloned in 1985, which enabled the transfection of mammalian cell lines, including Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells, for the production of recombinant human EPO (Lin et al., 1985; Jacobs et al., 1985). Because post-translational modifications depend on the enzymatic make-up of the cells performing the synthesis, the use of various cell lines results in differences in glycosylation and sulfation of the protein (Skibeli et al., 2001; Kawasaki et al., 2001). As a result, the biochemical and biological properties can differ between epoetins produced in different cells. Depending on the composition and nature of the glycosylation pattern, commercially produced epoetins are followed by a Greek letter (α, β, δ, ω, ζ).

In 1989, epoetin alfa was the first recombinant EPO to become commercially available. CHO cell-produced epoetin alfa was subsequently approved by the United States Food and Drug Administration (FDA) for the treatment of anemic patients with chronic kidney disease (CKD). It was then approved for the treatment of anemia associated with chemotherapy in cancer patients or with antiviral treatment in HIV patients and for the preparation of autologous blood donation prior to surgery. The treatment decreases the risks of recurrent blood transfusions and iron overload, and improves the quality of life (Bonomini et al., 2016). The serum half-life of epoetin alfa is approximately 8 and 24 h when the product is administered intravenously or subcutaneously, respectively (Franz, 2009). The bioavailability of epoetin alfa is low, and up to three injections per week are needed to attain therapeutic effects (Macdougall et al., 1991). For doping uses, the short elimination time is advantageous, because the detection window is narrowed, whereas the effect is sustained (Fig. 1).

In 1990, epoetin beta became commercially available. Although epoetin beta was also expressed in CHO cells, its glycosylation and biological activity were somewhat different compared to those of epoetin alfa. These properties did not significantly improve the clinical characteristics of the product. In the 1990s, and particularly in 2004 when the patents for epoetin alpha and beta expired, several companies started to produce copies of rHuEPO with different post-translational modifications. For example, epoetin omega was produced in BHK cells, whereas epoetin delta was expressed in the HT-1080 human cell line (Sikole et al., 2002; Smith et al., 2007). These differences in the manufacturing processes created differences in glycosylation and yielded several EPO isoforms. However, none of these products succeeded in improving the biological activity or the half-life of rHuEPO.

2.3. Second-generation recombinant EPO

To overcome these pharmacological limitations and reduce the frequency of troublesome administrations, the pharmaceutical industry urged researchers to develop a modified recombinant EPO with a longer half-life. This goal was reached when the novel erythropoiesis-stimulating protein (NESP or darbepoetin alfa) became available. Five amino acids in the protein backbone of EPO were mutated, enabling the addition of two more N-linked carbohydrate chains with terminal sialic acid residues. These changes increased its molecular mass (while lowering its receptor affinity), circulating half-life by 3-fold, and *in vivo* potency (Egrie and Browne, 2001; Egrie et al., 2003). Due to the longer half-life, the dosing intervals were decreased to once weekly or monthly. However, this was not desirable for illicit doping use, because it increased detection window and the risk of being tested positive (Fig. 1). The launching of darbepoetin alfa resulted in the first collaboration between a pharmaceutical industry (Amgen) and anti-doping laboratories.

2.4. Third-generation recombinant EPO

The third-generation rHuEPO continuous erythropoietin receptor activator (CERA) was launched on the European market in 2007 (Jelkmann, 2012). The drug consists of CHO cell-produced epoetin beta linked to methoxy polyethylene glycol (PEG). Pegylation results in a molecular size of approximately 60 kDa, leading

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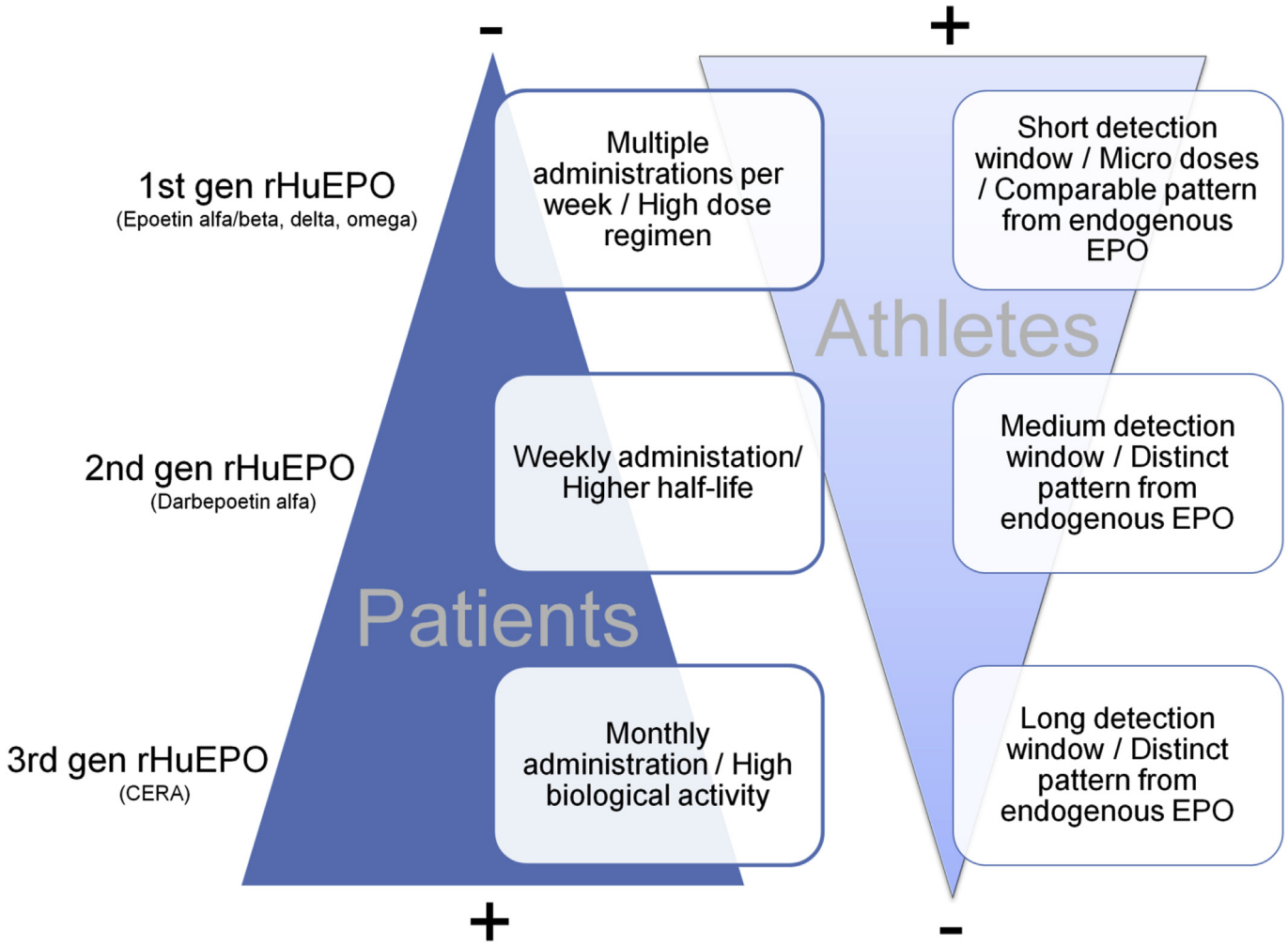

Fig. 1. Advantages and disadvantages of the three different generations of recombinant human EPO for athletes and patients. The benefits are inversely correlated between patients suffering from chronic anemia and cheating athletes for the different generations of rHuEPOs. The first generation is advantageous for athletes, because of its short detection window and its similarity to endogenous EPO. However, high doses are needed to treat anemia, and it requires multiple uncomfortable injections per week. The benefits are reversed with longer half-life rHuEPOs.

to a lower affinity for the EPO receptor, hindering its glomerular filtration by the kidneys, and increasing its serum half-life (Macdougall, 2005). The exceptional stability of CERA in the blood results in a mean serum half-life of 70–122 h, which decreases its administration to once monthly or less frequently (Macdougall et al., 2006). Because of its wider detection window, CERA is less likely to be used for doping purposes (Fig. 1). The manufacturer (Roche) developed a CERA-specific ELISA for pharmacokinetic studies and provided it to the World Anti-Doping Agency (WADA) before the marketing of the drug.

2.5. Erythropoietin-mimetic peptides (EMPs)

Another strategy to stimulate erythropoiesis is the use of EPO biomimetic peptides (Wrighton et al., 1996). Peginesatide (trade name: Hematide, Affymax/Takeda) is a synthetic pegylated dimeric peptide-based ESA that was approved by the FDA in 2012 for the treatment of anemia caused by CKD (Fan et al., 2006; Macdougall et al., 2013). The molecule has no sequence homology with EPO, and is therefore unlikely to induce a cross-reactive immune response against endogenous or recombinant EPO (Macdougall et al., 2009). Although the structure of peginesatide differs from that of EPO, it can stimulate EPOR dimerization and activate similar intracellular signaling pathways to induce erythropoiesis (Fig. 2) (Green et al., 2012).

A study on the safety and pharmacodynamics of peginesatide reported a prolonged half-life and delayed clearance (Stead et al., 2006). The same study demonstrated that the injection of peginesatide was associated with an increase in Hb in healthy patients, whose level was sustained for longer than a month. The monthly administration of the drug was able to maintain the Hb level in anemic patients with CKD undergoing hemodialysis and those not receiving dialysis (Macdougall et al., 2013; Fishbane et al., 2013). However, Affymax and Takeda voluntarily recalled peginesatide from the market 1 year after its commercialization due to serious, life-threatening reactions (Takeda, 2013).

Before the drug was licensed, peginesatide was included on the Prohibited List of the WADA in 2009 (WADA, 2009). There were indeed rumors that cheating athletes were using unlicensed substances, including peginesatide (Benkimoun, 2009). Nevertheless, despite its withdrawal from the market, there are still suspicions about the compound being used for doping purposes (Leuenberger et al., 2012).

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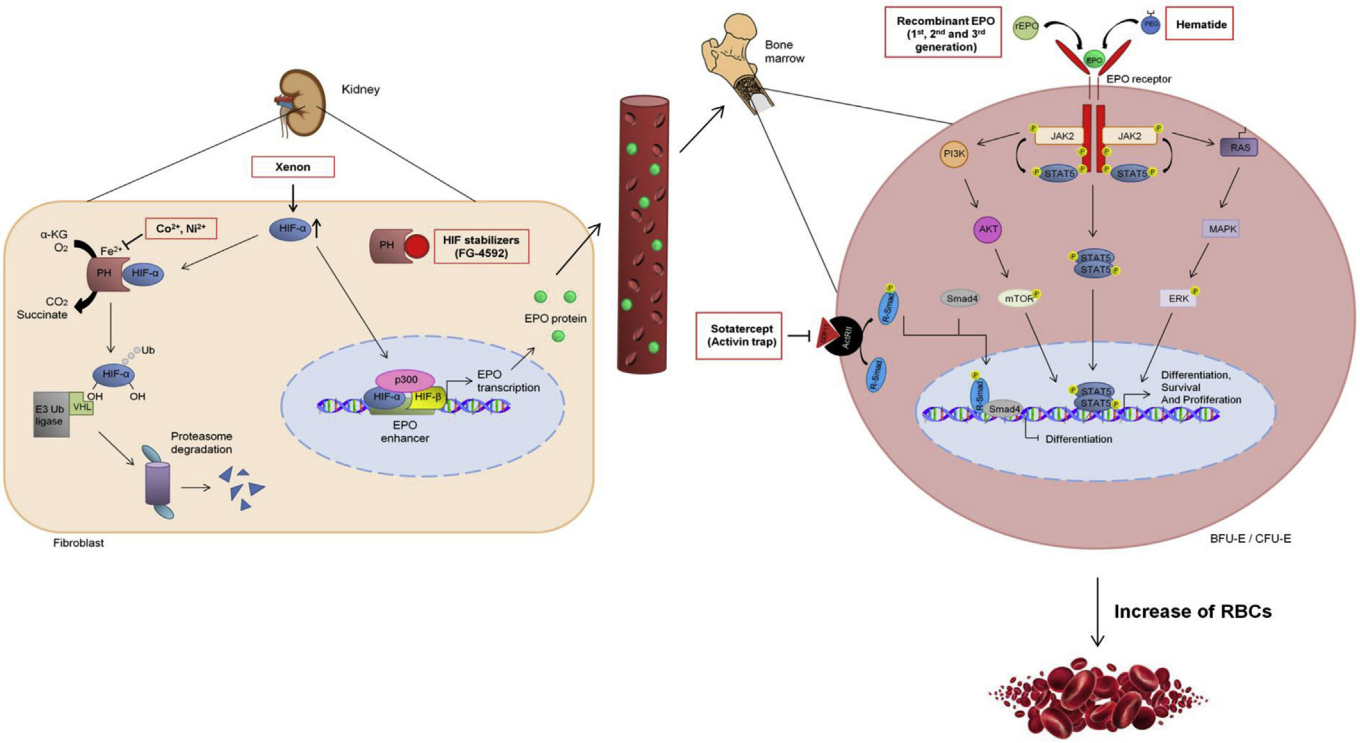

Fig. 2. Schematic model of the control of red blood cell production and the sites of action of erythropoiesis-stimulating agents. EPO is produced in the kidney under the control of HIFs which can be modulated by different substances. As a circulating endogenous hormone, EPO enters the bone marrow and activates the EPO receptor in BFU-E and CFU-E cells. Signal transduction through the JAK2/STAT5 pathway induces the differentiation, survival, and proliferation of red blood cell precursors. Recombinant human EPO and EPO biomimetic peptides also activate EPOR. Sotatercept prevents the binding of GDF-11 to its receptor and blocks the inhibition of differentiation of erythroblasts (Shenoy et al., 2014).

2.6. HIF stabilizers

HIFs are transcription factors that coordinate the physiological response to hypoxia, resulting in increased EPO production in the liver and kidney, enhanced iron uptake and utilization, and changes in the bone marrow (BM) environment that facilitates erythroid progenitor maturation and proliferation (Haase, 2013). As discussed in section 2.1, HIF levels are regulated by the hydroxylation state of the HIFα subunit under the control of prolyl hydroxylases (HIF-PH). The catalytic activity of HIF-PH depends on the presence of 2-oxoglutarate and oxygen among others (Fe(II)) (Bruegge et al., 2007).

To block HIF-PH activity and stabilize the HIFα subunit, chemical competitors of 2-oxoglutarate have been developed (Mole et al., 2003). These compounds are defined as HIF stabilizers, and they mimic the hypoxia-driven expression of endogenous EPO in the kidney (Fig. 2) (Bonomini et al., 2016; Rabinowitz, 2013). The unique feature of HIF stabilizers is that they are administered orally to stimulate erythropoiesis with EPO at physiological concentrations compared to the high concentrations observed with standard ESA therapy (Bonomini et al., 2016; Unger et al., 2010). Therefore, they are interesting compounds for pharmaceutical companies but also for doping purposes. Although HIF stabilizers are in the clinical trial phase of development, they are available on the black market. For these reasons, HIF stabilizers were classified as prohibited substances by the WADA in 2011 (WADA, 2016a, b).

Several HIF stabilizers are currently being developed by different pharmaceutical companies (FG-4592 and FG-2216 by FibroGen, AKB-6548 by Akebia Therapeutics, GSK 1278863 by GlaxoSmithKline, BAY 85-3934 by Bayer Pharmaceuticals). FibroGen has made the most progress in this area (Buisson et al., 2016). Both oral drugs are intended to treat anemia in patients with CKD. FG-2216 was the first promising molecule in this category, but it was withdrawn after a case of fatal hepatitis (Bonomini et al., 2016). Currently, roxadustat (FG-4592) is in a phase 3 clinical trial for the treatment of anemia in patients with CKD (FibroGen, 2016). In a phase 2 randomized placebo-controlled study, roxadustat at doses of 0.7–2.0 mg/kg administered orally two or more times weekly increased the Hb level in non-dialysis-dependent subjects without intravenous iron supplementation (Besarab et al., 2015). The drug was well tolerated without adverse effects normally associated with EPO analogs. Moreover, oral administration of the compound reduced the serum hepcidin level, increasing the availability of iron for erythropoiesis.

Products labeled “FG-4592” are already available on the black market and represent a threat to be used for doping purposes. This was recently substantiated by the first reported case of doping with FG-4592 (Buisson et al., 2016).

In addition to oxygen and 2-oxoglutarate, Fe (II) is an essential co-factor for HIF-PH activity. Fe (II) is also reversibly linked to the active site of the metalloenzyme. Metal ions, such as Co^{2+} or Ni^{2+}, can reduce iron availability by competitive substitution, stabilize HIF transcription factors, and increase EPO gene expression (Fig. 2) (Beuck et al., 2012). Another mechanism of action involves the direct binding of cobalt to HIFα. Nevertheless, chronic cobalt exposure can lead to severe toxic effects, and its use as a hypoxia-mimetic agent is thus limited to experimental applications.

Recently, xenon gas was identified as an activator of the HIF pathway and therefore a stimulator of red blood cell (RBC) production. It came to the public's attention during the past Olympics (2014), following claims that some athletes inhaled this gas for performance-enhancing purposes. Xenon exerts its effects by

stimulating HIF-1α translation, rather than preventing its degradation (Fig. 2) (Jelkmann, 2014). Although there are no human data on the effects of xenon on the serum level of EPO, xenon was classified as a HIF activator on the Prohibited List by the WADA in 2014.

2.7. Activin traps

The current ESAs are not effective for all anemic patients and have limitations, including undesirable and adverse effects. There is thus the need for agents that treat anemia for which conventional ESAs are not effective such as thalassemia. Members of the transforming growth factor beta (TGF-β) family, such as activins, growth differentiation factors (GDFs), or bone morphogenic proteins (BMPs), are potential modulators of adult erythropoiesis via the intracellular SMAD signaling pathway (Fig. 2) (Maguer-Satta et al., 2003).

Sotatercept (ACE-011, Acceleron and Celgene Corp) is a dimeric fusion protein consisting of the extracellular domain of the human activin receptor IIA (ActRIIA) linked to the Fc portion of the human immunoglobulin G1 (IgG1) antibody. It interferes with downstream signaling cascades, in particular the SMAD pathway, by sequestering activin (Fig. 2) (Raje and Vallet, 2010). Sotatercept was mainly developed to increase bone mineral density. In a phase 1 clinical trial involving healthy postmenopausal women, a single dose of ACE-011 resulted in a dose-dependent increase in the biochemical markers of bone formation and a decrease in the biochemical markers of bone resorption. Surprisingly, the treatment provoked an increase in Hb and hematocrit (Hct) levels and RBC number (Ruckle et al., 2009). These increases persisted for approximately 3 months. Using a murine ortholog of ACE-011 (RAP-011), preclinical studies explored the potential cellular and biochemical mechanisms by which sotatercept regulates erythropoiesis (Carrancio et al., 2014). Mice treated with RAP-011 exhibited a rapid increase (within 24 h) in Hct and Hb levels, and RBC number, accompanied by a rapid stimulation of late-stage erythroid precursors in the BM. RAP-011 at a dose of 30 mg/kg induced a significant increase in erythroid burst-forming units and EPO.

In a phase 2 trial involving healthy postmenopausal women, sotatercept rapidly increased erythropoiesis dose-dependently, which persisted for up to 4 months, suggesting that ActRIIA ligands are important negative regulators of erythrocyte levels in healthy individuals (Sherman et al., 2013). This compound has a long half-life, which contributes to the sustained erythropoietic response and allows a less frequent dosing schedule compared to classic ESAs. These results indicate that the mechanism underlying the erythropoietic effects of sotatercept, which is an alternative for the treatment of anemia associated with various pathologies, is different from that of conventional ESAs (Fields et al., 2013). Recent phase 2 clinical trial demonstrated its benefits in chemotherapy-induced anemia (Raftopoulos et al., 2016). This approach also provides a new product for athletes to artificially increase their Hb content.

2.8. EPO gene doping

Advances in DNA manipulation have extended the benefits of gene therapy to various common diseases such as anemia. The treatment of anemia with recombinant HuEPO is relatively expensive, involving continuous monitoring and repeated administration. In addition, the doses often overly raise or lower the serum EPO (s-EPO) level. Nevertheless, EPO gene therapy constitutes an attractive approach for continuous secretion where a single administration of the EPO gene would ensure long-term delivery and a steady-state EPO level.

Vectors (Repoxygen) in which murine *Epo* expression was under the control of the Oxford BioMedical hypoxia response element (OBHRE) were developed for the treatment of anemia in mice (Binley et al., 2002). Mindful of the potential of this approach to increase the endogenous EPO levels and RBC production, gene therapy was placed on the Prohibited List by the WADA in 2006.

An autologous *ex vivo* strategy was used in the first *Epo* gene therapy trial on patients with CKD (Jelkmann and Lundby, 2011). Dermal cores (Biopump) were transfected with an adenovector designed to express EPO under the control of the cytomegalovirus (CMV) promoter (Lippin et al., 2005). The dermal cores were harvested and then implanted into CKD patients, resulting in a significant increase in the s-EPO level for up to 14 days.

In conclusion, it was demonstrated that *Epo* gene therapy is effective for the treatment of anemia in CKD patients, suggesting that gene doping is possible. However, the efficacy, safety, and immunogenicity of *Epo* gene transfer have not yet been explored in detail. It seems therefore unlikely that this practice is currently being used by doping athletes.

3. Testing procedures

3.1. Direct detection methods

3.1.1. IEF and sodium dodecyl sulfate (SDS)/sarcosyl (SAR)-polyacrylamide gel electrophoresis (PAGE)

The first method to detect rHuEPO in specimens from doping athletes was developed in 2000 by Lasne et al., more than 10 years after the beginning of rHuEPO abuse in sports (Lasne and de Ceaurriz, 2000; Lasne et al., 2002). IEF, a separation technique, exploits the differences in carbohydrate composition between rHuEPO and endogenous HuEPO. These modifications result in a change of the final net charge of the molecule at a certain pH that can be observed by IEF, in which proteins are separated according to their isoelectric point (pI) (Pascual et al., 2004). Indeed, there are fewer acidic isoforms of rHuEPO than urinary HuEPO (Reichel, 2011). Consequently, the overall charge of rHuEPO is less negative than that of HuEPO, and the isoforms of rHuEPO migrate differently from those of HuEPO in the electric field (Pottgiesser and Schumacher, 2013).

Due to the large volume of urine and the low EPO concentration in this specimen, the urine is first concentrated by ultracentrifugation and the resulting retentate is then separated on an IEF gel with a pH gradient ranging from 2 to 6 (Franz, 2009). This separation produces a distribution of various isoforms, which generate the profile of a particular EPO analog. After double-blotting using the monoclonal AE7A5 antibody, the isoforms are visualized by chemiluminescence. The result of this analysis is an image of the IEF pattern of the EPO molecules present in the urine (Lasne and de Ceaurriz, 2000). Due to its greater overall negative charge, HuEPO migrates in a more acidic region of the gel, whereas the main rHuEPO (α, β, ω) isoforms migrate in a more basic area (Fig. 3). With its additional N-linked carbohydrate chains, darbepoetin alfa has a greater negative charge than HuEPO and migrates in a more acidic region of the IEF gel (Catlin et al., 2002) (Fig. 3). However, the method is not useful for the detection of epoetin delta, which is produced in human cell line, because its IEF pattern partially overlaps with that of endogenous HuEPO (Franz, 2009). Proteinuria, either disease-related or exercise-induced, can also affect the IEF distribution leading to an atypical IEF profile (Lamon et al., 2009; Beullens et al., 2006).

Another approach based on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and the mobilities of target analytes was developed to complement the IEF assay (Kohler et al., 2008). SDS-PAGE was developed in the 1970s for the

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separation of proteins according to their molecular weights. Using mass spectrometry, it was observed that the molecular weights of rHuEPOs were higher than those of HuEPO due to differences in the glycosylation pattern, and these differences could be detected in the urine and/or blood using SDS-PAGE (Leuenberger et al., 2012; Reichel, 2011; Kohler et al., 2008). Two internal standards were used, enabling the calculation of the relative mobility values and the discrimination of the analytes in the specimens (Fig. 4) (Reichel, 2011; Tsitsimpikou et al., 2011). Negative feedback of rHuEPO-administration could be detected as the absence of HuEPO-bands on the gel, and consequently during washout period, an intense rHuEPO band evolved in a glycoform smear above the HuEPO band (Fig. 4) (Leuenberger et al., 2012).

Both methods generally follow the same workflow (concentration by ultracentrifugation, separation, single/double blotting, chemiluminescent detection), except for the different principles of separation (charge versus molecular mass). Unlike in IEF-PAGE, urine and blood samples analyzed by SDS-PAGE must be first immunoaffinity purified (an ELISA plate or anti-EPO monolithic disk) due to the high protein content (Lonnberg et al., 2010). Vogel et al. proposed an alternative purification method using EPO receptor-coated magnetic beads (Vogel et al., 2014).

The two complementary methods can easily detect first- and second-generation recombinant EPO. They can even detect gene therapy-derived EPO isoforms that are different from the natural hormone (Lasne et al., 2004). CERA is also different from the endogenous molecule with respect to its IEF pattern and relative mobility due to its pegylated structure. However, the main disadvantage for detecting CERA is that it is too large (~60 kDa), not filtered by the kidneys, and not excreted in the urine, except in cases of exercise-associated proteinuria (Lamon et al., 2009; Bellinghieri et al., 2008). Therefore, the detection method required amendment regarding the matrix of choice; by adding a step of immunopurification, Lasne et al. were able to study the isoelectric profiles of EPO and CERA in human serum samples (Lasne et al., 2007). To separate proteins according to their molecular weight, SDS was replaced with sarcosyl (SAR) because it does not bind to the PEG moiety of CERA. Contrary to SDS, SAR only binds to the amino acid chain of the PEGylated protein, thus leading to enhanced antibody binding and a sharper electrophoretic band (Reichel, 2012; Reichel et al., 2009).

3.1.2. Membrane-assisted isoform immunoassay (EPO WGA MAIIA)

Researchers in Sweden have developed a new test called the membrane-assisted isoform immunoassay (EPO WGA MAIIA) (Franco Fraguas et al., 2008; Lonnberg et al., 2012). The test combines a chromatographic separation of the glycosylated isoforms of EPO with wheat germ agglutinin (WGA) and a sensitive lateral flow immunoassay using anti-EPO carbon black nanostrings. This assay is executed on a dipstick (Lonnberg et al., 2012). Lectins are proteins possessing at least one domain with carbohydrate binding properties. They can interact reversibly and specifically with carbohydrate groups in different glycocompounds (Franco Fraguas et al., 2008). Thus, this assay enables researchers to differentiate endogenous EPO from recombinant EPO based on the affinity of different isoforms with lectin, which is due to the differences in terminal sialic acid residues and poly-N-acetyl lactosamine (LacNAc) residues (Reichel, 2011; Dehnes et al., 2013). rHuEPO isoforms interact more strongly with WGA than HuEPO. Darbepoetin alfa has the strongest affinities for WGA, while the affinity of CERA for WGA is lower than that of HuEPO (Franco Fraguas et al., 2008; Dehnes et al., 2013).

Lectin-bound EPO isoforms are eluted with sugar N-acetyl glucosamine (GlcNAc) at two different concentrations (low/high) for each sample. A low concentration of GlcNAc buffer will elute only EPO isoforms with a low affinity for WGA, whereas a high concentration will elute all EPO isoforms The eluted EPO isoforms react with an immobilized anti-EPO antibody in the capture zone, and the EPO isoforms are detected with another anti-EPO labeled antibody with carbon black nanostrings (Dehnes et al., 2013). The scanner image is used for quantification, and the intensity of the signal is proportional to the concentration of bound EPO.

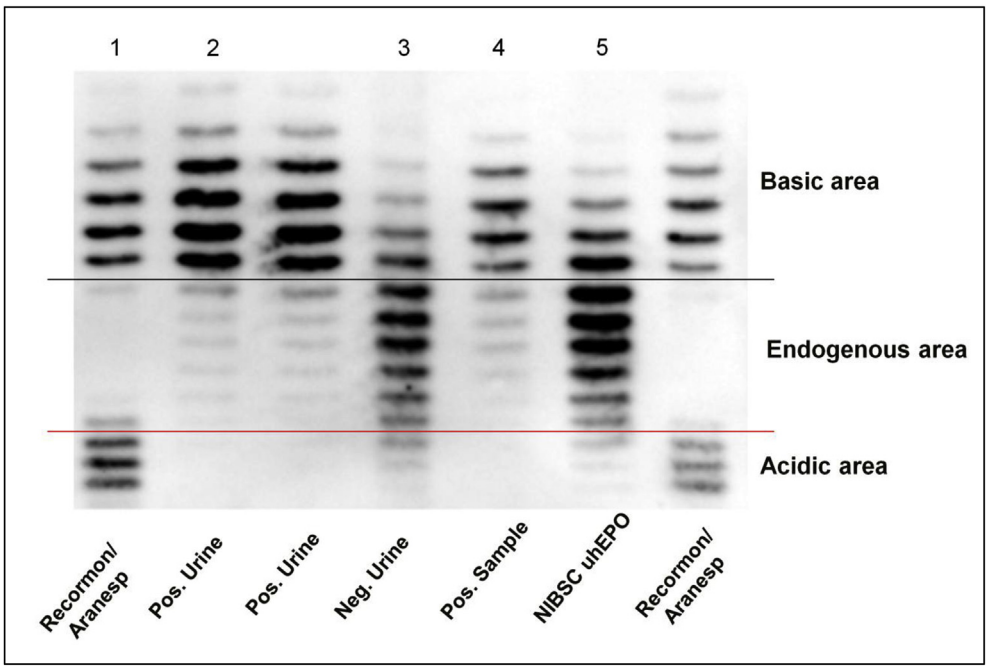

Fig. 3. Example of isoelectric focusing analysis of a sample positive for rhEPO. Lane 1: Reference marker corresponding to a mix of epoetin beta (Recormon) and darbepoetin alfa (Aranesp). Lane 2: human urine spiked with epoetin beta. Lane 3: endogenous urinary EPO. Lane 4: sample positive for epoetin alfa/beta. Lane 5: urinary EPO standard (National Institute for Biological Standard and Control; NIBSC).

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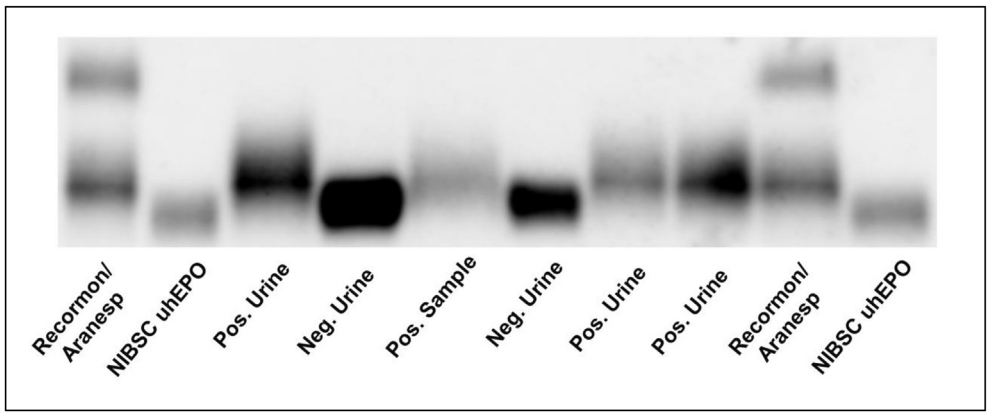

Fig. 4. Example of SDS-PAGE analysis of a sample positive for epoetin alpha/beta. A mix of epoetin beta (Recormon) and darbepoetin alfa (Aranesp) is used as reference standard. Urinary EPO standard (NIBSC) and endogenous EPO (negative urine) are used as negative control. The positive sample is characterized by mixed bands creating a glycoform smear above the endogenous band. Positive urine corresponds to urine spiked with epoetin beta.

Urine and serum/plasma specimens are compatible with the EPO WGA MAIIA assay but require immunoaffinity purification using anti-EPO monolith columns. Compared to the current WADA-accredited IEF and SDS/SAR-PAGE methods, the MAIIA assay can quickly screen a batch of 15 immunopurified samples (controls included) within 1 h (Dehnes et al., 2013). Therefore, the EPO WGA MAIIA has the sensitivity and potential required for a high-throughput screening test to complement IEF and SDS/SAR-PAGE. In their study evaluating the MAIIA dipstick test for the detection of microdose quantities of rHuEPO in plasma, Ashenden et al. proposed a longitudinal evaluation of the percentage migration isoforms (PMI) score and to interpret the results against the subject's historical values rather than a population-based threshold similar to that of the ABP for an optimal sensitivity (Ashenden et al., 2012). Compared to SDS-PAGE, the scores from the MAIIA test are more difficult to interpret than the bands from the electrophoresis gel, and they are more difficult to defend in court. Although the method needs further improvement, the dipstick test seems to be an interesting complement to the existing antidoping tests.

3.1.3. *Liquid chromatography-tandem mass spectrometry (LC-MS/MS)*

As previously discussed, the pharmaceutical industry is focusing on the development of small molecules that directly and indirectly stimulate RBC production, such as HIF stabilizers and erythropoietin-mimetic peptides (EMPs), in addition to their efforts in recombinant EPO production. These peptides are readily filtered by the kidney and are present in the urine, and they can be detected with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Although most of these new molecules are still under investigation, they are available on the black market. For these reasons, the use of these agents by athletes was prohibited by the WADA, and accredited anti-doping laboratories have taken preventive measures and developed methods for doping control (Beuck et al., 2011; Moller et al., 2011).

Based on the collaboration between the pharmaceutical industry and anti-doping laboratories, Hematide/peginesatide detection assay was developed and validated using ELISA and immunopurification followed by SDS-PAGE for the confirmation of results (Leuenberger et al., 2011a; b). Together with this approach, a LC-MS/MS method incorporating proteolytic digestion was introduced for the detection of Hematide in plasma and then further developed for the detection of the compound in dried blood spots (DBSs) (Moller et al., 2011; Moller et al., 2012). After being prohibited for 5 years, the first case of an athlete testing positive for the HIF stabilizer FG-4592 was reported (Buisson et al., 2016). LC-MS/MS was used to detect and confirm the presence of FG-4592 in the urine, while there were no profound alterations in the hematological parameters of the ABP. This case highlights the sensitivity and efficiency of the direct LC-MS/MS measurement of an exogenous substance, but also the limitations of the ABP. Regarding the activin traps and the potential misuse of sotatercept as a new doping agent, a research project was recently funded by the WADA to develop initial testing and confirmatory procedures using LC-MS/MS (Reichel, 2016). Although the direct identification of exogenous agents by LC-MS/MS was shown to be very efficient, the main disadvantage is that the method requires reference material, which is often available only from the pharmaceutical companies developing these products. The analytical task of the rHuEPO, especially for the first-generation compounds, is in the specificity of LC-MS/MS, as the differences with HuEPO are restricted to only a few sugar moieties. However, natural or artificial (darbepoetin alfa) changes in the amino acid sequence can be distinguished by mass spectrometry (Guan et al., 2009). This was successfully used in the detection of rHuEPO in horse plasma and urine specimens (Yu et al., 2010; Bailly-Chouriberry et al., 2012). Indeed, the method exploits the species-specific differences between human and horse EPO for detecting the presence of rHuEPO, NESP, and CERA in equine plasma where the specific task cannot be compared to the context of human doping control (Guan et al., 2010).

3.2. *Indirect detection methods*

3.2.1. *Athlete biological passport*

During the development of the IEF direct detection method for recombinant EPO, Parisotto et al. indirectly detected the drug using different blood markers of altered erythropoiesis (Parisotto et al., 2000; Parisotto et al., 2001). By combining several indirect hematological variables, two models (ON/OFF-models) were created to detect rHuEPO during the administration phase and the wash-out period. The ON-model took into account reticulocyte hematocrit (RetHct), s-EPO, serum soluble transferring receptor (sTFR), and Hct values as well as the macrocyte percentage (%Macro), while the OFF-model took into account RetHct, s-EPO, and Hct levels. With the availability of direct detection methods and the potential availability of indirect detection methods, the doping habits of endurance athletes shifted to low doses of rHuEPO (i.e.,"microdosing") and forced researchers to propose new models.

Consequently, Gore et al. introduced second-generation blood tests, in which the OFF-hr score was analyzed (Gore et al., 2003).

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This score derived from an algorithm ($Hb - 60\sqrt{(Ret\%)}$) is useful, because it can detect rHuEPO many weeks after the injections have stopped. Thereafter, researchers investigated the possibility of comparing an athlete's hematologic values against his/her own historical baseline, rather than a population-derived threshold to enhance the ability to detect rHuEPO (Sharpe et al., 2006; Robinson et al., 2002). This third-generation test aimed to distinguish the effects of rHuEPO abuse from the natural biological fluctuations of Hb and OFF-hr. In 2006, Sottas et al. updated the ABP concept by introducing wider set of data from a single blood profile of an athlete in a multiparametric score called the abnormal blood profile score (ABPS) (Sottas et al., 2006). The ABPS could incorporate 3 to 12 different blood markers influenced either by rHuEPO administration or blood transfusion.

In 2008, the Union Cycling International (UCI) was the first sports organization to implement the hematological module of the ABP to detect blood doping, followed 1 year later by the WADA. The hematological module involves the longitudinal monitoring of eight markers of erythropoiesis including Hct, Hb, RBC count, Ret%, Ret count (Ret#), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), from which OFF-hr and ABPS scores can be calculated (Saugy et al., 2014). The profile of each athlete is longitudinally evaluated using an adaptive model based on Bayesian inference techniques that considers previous values and identifies the pattern of blood manipulation (Saugy et al., 2014). A case is deemed suspicious if a particular value lies outside of the defined range, requiring review by a panel of experts, which may then lead to an anti-doping rule violation (WADA, 2016a, b). Finally, a longitudinal follow-up of the athlete's blood markers should demonstrate that the athlete is in a healthy physiological condition that is unaltered by performance-enhancing drug (Sottas et al., 2011).

The hematological module enables more sensitive and extended detection of EPO doping than non-targeted direct methods that often suffer from a short detection window (Fig. 5) (Reichel, 2011). Indeed, the ABP benefits from the fact that the effects of a drug on the metabolism last longer than its presence in the urine or blood. Furthermore, this new paradigm bears the advantage of already being sensitive to the marketing of any future compound that could potentially increase performance. Finally, the longitudinal monitoring of hematological parameters, Hb, Ret%, and the OFF-score is much more sensitive than the no-start rule such as 50% Hct introduced by the UCI in the mid-1990s.

Although the hematological module of the ABP is valuable, it is often criticized, requiring constant revision to accommodate the introduction of new markers of altered erythropoiesis. Ashenden et al. evaluated the sensitivity of the hematological module to flag microdoses of rHuEPO administered to athletes. They concluded that it was still possible for athletes to use rHuEPO and bypass abnormal changes in the blood variables currently monitored by the ABP (Ashenden et al., 2011). These limits were confirmed in the athlete that used HIF stabilizer FG-4592, whose hematological parameters demonstrated no profound alterations (Buisson et al., 2016). Moreover, it was recommended that seasonal changes in the hematological parameters due to training and competition should be taken into account (Banfi, 2011; Sanchis-Gomar et al., 2011). In addition to Hb and Ret%, a new marker of altered erythropoiesis caused by rHuEPO or blood transfusion, the total mass of hemoglobin (Hb_{mass}) was proposed especially for its independence of plasma fluctuations. Nevertheless, the variability observed over time when assessing Hb_{mass} was not justified in an anti-doping setting (Lundby and Robach, 2010), and the measurement of the variable based on carbon monoxide (CO) rebreathing method was too complicated to be implemented.

3.2.2. Ironomics

The production of RBC is tightly linked to the metabolism of iron, an element essential for Hb synthesis and erythroid proliferation. Furthermore, endogenous EPO regulates RBC production by inducing the proliferation and differentiation of erythroid progenitor cells and controlling cellular iron metabolism (Weiss et al., 1997). Hence, the exogenous administration of rHuEPO or ESAs greatly affects the physiology of iron metabolism and iron-related variables.

Transferrin (Tf) and ferritin are the main cargo proteins of blood iron in the organism. Serum Tf and ferritin are common biomarkers of iron-related disorders (Szoke and Panteghini, 2012). The two variables are inversely correlated; transferrin is the transport protein of iron in the circulation, while ferritin is the iron storage protein. Their production is regulated by the iron response element/iron response protein (IRE/IRP) regulatory system. Erythroblasts take up iron required for Hb synthesis via the transferrin receptor (TfR). The soluble form of TfR (sTfR) reflects the total cellular expression of TfR and represents an interesting candidate for blood doping, because it reflects the erythropoietic activity of the organism (Salamin et al., 2016a, b, c). Thus, the administration of ESAs results in an increase of sTfR through the activation of IRP-1 (Parisotto et al., 2000; Weiss et al., 1997; Dehnes and Hemmersbach, 2011). On the other hand, serum ferritin decreases after the administration of ESAs due to the mobilization of iron reserves for the production of new RBCs. It is another potential biomarker of altered erythropoiesis (Fig. 5). The re-infusion of an athlete's own blood also affects erythropoiesis and iron-related variables such as sTfR and serum ferritin. The monitoring of these two parameters could therefore assist in targeting this doping method for which no direct detection method is available (Salamin et al., 2016a, b, c).

Hepcidin, a peptidic hormone secreted by the liver, regulates the availability of iron for erythropoiesis by altering iron absorption and recirculation (Waldvogel-Abramowski et al., 2014). The peptide binds to the iron exporter ferroportin, and induces its internalization and degradation, thus blocking iron efflux (Robach et al., 2013). Erythroid cell proliferation dramatically alters iron metabolism to satisfy the high demand for iron during the synthesis of Hb, and thus influences the secretion of hepcidin by the liver. Indeed, rHuEPO decreases hepcidin expression 24 h after subcutaneous or intravenous injections (Robach et al., 2013; Laine et al., 2012). Other ESAs, such as HIF stabilizers or sotatercept, also decrease hepcidin expression (Buisson et al., 2016; Jelkmann, 2015) Therefore, hepcidin may be a new potential marker for the detection of ESAs (Fig. 5). The size of the peptide hormone (25 amino acids) allows for its quantitation in human plasma or serum using LC-MS/MS, a technique that is available in all accredited anti-doping laboratories. Furthermore, the hepcidin level strongly correlates with the ferritin level, and may serve as a marker of iron repletion that is required for erythropoiesis (Salamin et al., 2016a, b, c).

For some time, the factor responsible for the inhibition of hepcidin by erythropoietic signals in humans remained unknown. A previous study identified erythroferrone (ERFE) as the erythroid regulator of iron metabolism (Kautz et al., 2014). This protein, which is produced by erythroblasts upon hypoxia or EPO stimulation, suppresses hepcidin expression. ERFE may be a key regulatory protein in the release of stored iron release and appears to be an interesting candidate biomarker of blood manipulation. A recent study demonstrated an association between ERFE and biomarkers of erythropoiesis and iron metabolism after ESA treatment in patients on hemodialysis (Fig. 5) (Honda et al., 2016). The ERFE levels significantly increased from day 3 of treatment with darbepoetin alfa and CERA. Detailed comparative studies in healthy humans are currently ongoing.

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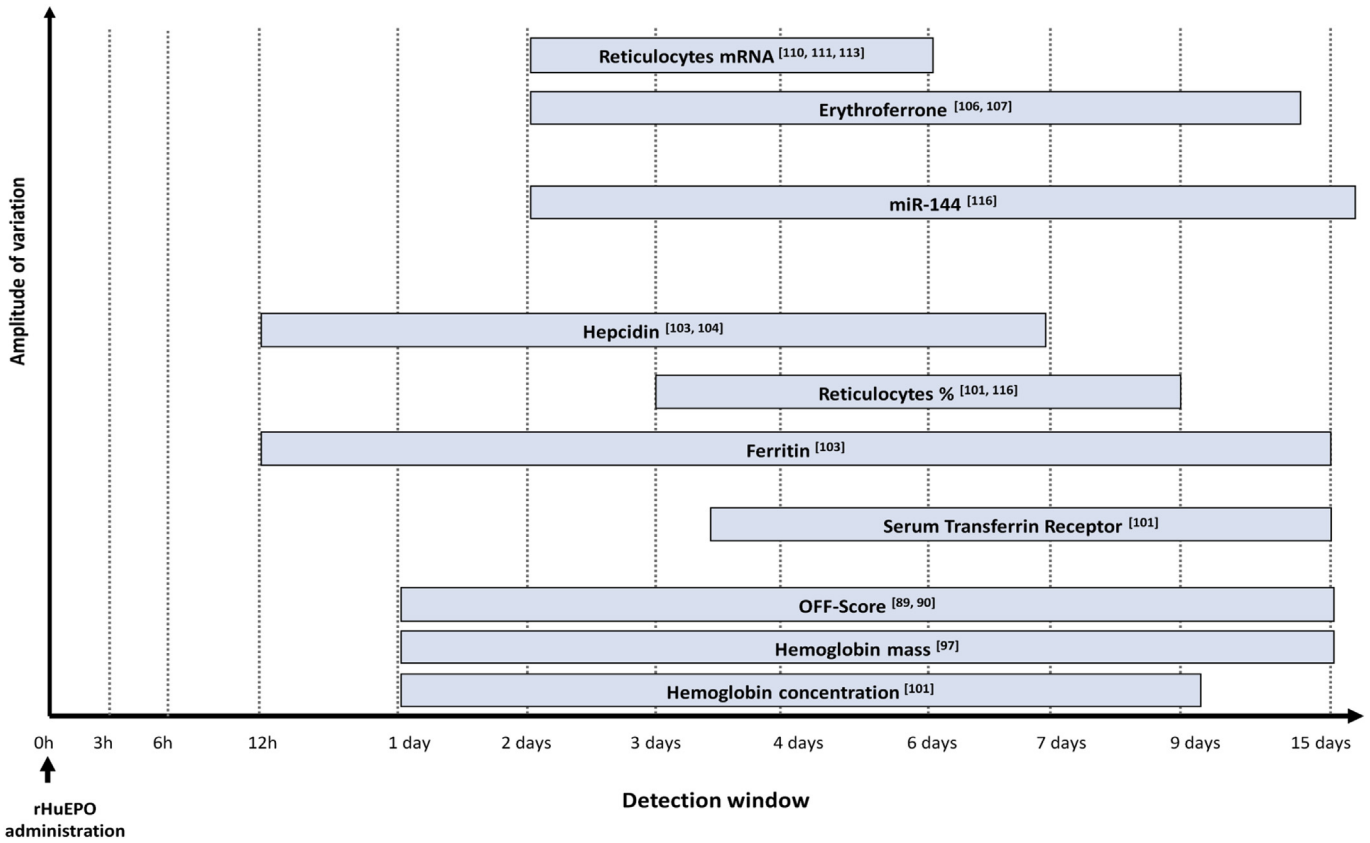

Fig. 5. Detection window of validated and emerging biomarkers of rHuEPO abuse. The different biomarkers are classified according to their fold-change and the duration of their detection window after rHuEPO administration.

Similar to the changes in hematological parameters after altered erythropoiesis, iron-related variables demonstrate high inter-individual variability, suggesting that individual longitudinal monitoring of these parameters by an "ironomics-based" approach may supplement the hematological module of the ABP for the detection of blood manipulation with ESAs. However, the effects of heterogenous factors, such as exercise, altitude-training, iron injections, or gender on these variables should be studied before any implementation of such a follow-up.

3.2.3. *Transcriptomics (messenger RNA (mRNA), microRNA (miRNA))*

Anti-doping authorities are continuously forced to revise the existing assays and develop new tests to reach the athletes that successfully bypass anti-doping detection methods. The genome is the genetic material of the organism, and includes genes and non-coding DNA. Genes code for proteins and are first transcribed into RNA. The transcriptome represents the set of all RNA transcripts, including non-coding RNA, and the quantification of the total RNA complement in a cell, tissue, or organism is defined as transcriptomics. Although the genome is unchanging, the transcriptome is constantly in a state of flux and is highly reflective of the cell milieu and metabolic activity (Rupert, 2009). Therefore, considering that doping agents affect messenger RNA expression, the transcriptional profiling of erythroid target genes may be a promising tool for the discovery of new biomarkers for the detection of blood doping (Fig. 5).

ESAs stimulate the production of RBCs and immature RBCs known as reticulocytes. After these immature cells expel their nucleus, they still contain functional residual nucleic acid material that can be analyzed (Lee et al., 2014). Varlet-Marie et al. investigated changes in the expression of reticulocyte genes after rHuEPO administration (Varlet-Marie et al., 2004). Using a serial analysis of gene expression (SAGE) library of human reticulocytes, three erythroid gene markers (hemoglobin subunit beta (*HBB*), ferritin light chain (*FTL*) and ornithine decarboxylase antizyme (*OAZ*)) were selected and their levels were quantified by real time-polymerase chain reaction (RT-PCR) in whole blood during and 3 weeks after drug administration. The selected genes showed a characteristic profile in subjects using rHuEPO compared to the placebo group. The *OAZ* mRNA expression profile was strongly indicative of rHuEPO treatment. Thereafter, 95 genes differentially expressed after the administration of high and microdoses of rHuEPO were identified using three SAGE libraries and confirmed by quantitative RT-PCR (Varlet-Marie et al., 2009). The microarray-based significance analysis identified 33 markers genes for the detection of rHuEPO among which five remained differentially expressed during the microdose regimen. A similar approach was applied for the long-term detection of rHuEPO abuse in horseracing (Bailly-Chouriberry et al., 2010).

Recently, Durussel et al. identified, replicated, and validated the whole blood transcriptional signature of rHuEPO in two distinct populations comprising Caucasian endurance-trained males at sea level and Kenyan endurance runners at moderate altitude, all of whom received rHuEPO injections for 4 weeks (Durussel et al., 2016). Transcriptional profiling showed that hundreds of transcripts were altered by rHuEPO in both cohorts. The expression pattern was characterized by a rebound effect, with a profound upregulation during rHuEPO injections and a subsequent down-regulation for up to 4 weeks after administration. A transcriptomic-

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based approach was also applied to autologous blood transfusion (ABT) with some success. Salamin et al. measured the expression of a subset of genes involved in RBC metabolism using digital multiplex mRNA counting technology after ABT and demonstrated that changes in transcript number were more significant than those in hematological parameters (Salamin et al., 2016a, b, c). Another pilot study demonstrated that blood reinfusion altered the expression profile of T lymphocytes. This was based on the hypothesis that exposure of cell detritus derived from stored blood can induce a cellular and molecular immune response (Pottgiesser et al., 2009).

Non-coding RNAs can also serve as potential biomarkers of doping methods. MicroRNAs (miRNAs) are small non-protein coding RNAs that function in the post-transcriptional modulation of gene expression. These molecules can be detected in the blood plasma or serum, and they have emerged as sensitive markers of various pathophysiological conditions. The usefulness of the circulating miRNAs as markers of altered erythropoiesis was first investigated by Leuenberger et al. (Leuenberger et al., 2011a, b). Using miRNA microarrays and quantitative RT-PCR, a significant change was observed in the plasma levels of several miRNAs in subjects receiving CERA injections. In particular, a specific miRNA, miR-144, exhibited a significant increase that lasted 27 days after CERA injection (Fig. 5). Different studies demonstrated that circulating miRNAs could also be used as biomarkers of ABT (Leuenberger et al., 2013), testosterone administration (Salamin et al., 2016a, b, c), and growth hormone treatment (Kelly et al., 2014).

A longitudinal monitoring of these transcriptomic biomarkers can complement the hematological parameters of the ABP, although further studies are needed to address the effects of possible confounding factors. The combination of transcriptomic, iron-related, and hematological biomarkers could reflect the general effects of ESAs on athletes and provide a longer detection window than direct detection methods (Fig. 5).

4. Adverse effects of ESAs

The public often forgets that ESAs are first intended to ameliorate the quality of life in patients suffering from a particular syndrome. Molecules designed to increase erythropoiesis are mainly used to treat anemia in patients with CKD or undergoing chemotherapy. Under normoxic physiological conditions, Hb levels are endogenously regulated by the blood oxygen level via the HIF system. ESA injections shock the system, and increase the erythrocyte number and the transport capacity of oxygen, which increases blood viscosity and the probability of thromboembolic events. Although professional athletes gravitate toward microdosing regimens that require medical supervision, amateur athletes may use high doses of these compounds and often according to anecdotal information. Besides increasing blood viscosity, long-term use of ESAs can result in various side effects such as red cell aplasia and heart failure (Tsitsimpikou et al., 2011; Locatelli and Del Vecchio, 2003). In individuals with an iron deficiency, epoetin can elevate thrombocyte counts and increase the risk of cardiovascular problems, including cardiac arrest, seizures, arrhythmia, hypertension, congestive heart failure, vascular thrombosis, myocardial infarction, and edema (Franz, 2009; Tsitsimpikou et al., 2011; Streja et al., 2008). Moreover, EPO is also involved in angiogenesis (Hardee et al., 2007), and EPO withdrawal may lead to neocytolysis (Trial et al., 2001). Furthermore, the combination microdoses of rHuEPO with other substances, such as testosterone or transfused blood, can have harmful consequences for the individual. The administration of ESAs to subjects with naturally high endogenous levels of EPO can also cause serious adverse effects. Finally, endogenous EPO is depleted upon the administration of rHuEPO through a negative feedback mechanism and this inhibition can be irreversible after long-term treatment (Tsitsimpikou et al., 2011).

Another major concern is the availability of non-approved compounds on the black market for which clinical studies are not completed. The use of these drugs in healthy individuals may show unexpected side effects (Franz, 2009). For example, HIF stabilizers may possess tumorigenic potential, because HIFs activate hundreds of genes coding proteins involved in carcinogenesis and neovascularization. The administration of sotatercept may also induce adverse effects in healthy individuals, because the mechanism of action for erythropoiesis is still not completely understood.

Last but not least, the source of the drugs used by athletes may raise some concerns. Indeed, epoetins produced from non-controlled sources lack sufficient quality control and may contain impurities such as bacterial endotoxins (Tsitsimpikou et al., 2011). In some compounds, aggregated proteins were detected, which may trigger the onset of anti-EPO antibody-induced pure red cell aplasia (PRCA) (Jelkmann, 2015). Moreover, subcutaneous injections are not recommended for several biosimilar epoetins and the lack of information on these compounds may lead to serious health risks.

Human nature is marked by the will to win, often without regard for the risks involved and their consequences on health. It is fitting to assume that athletes can become addicted to performance-enhancing drugs (Gil et al., 2016). From a psychological point of view, an athlete performing well in a doped-state will be tempted to repeat the doping in future competitions. It is therefore important to educate athletes on the risks of performance-enhancing drugs.

5. Conclusions and perspectives

Increasing the Hb level is one of the most effective ways of enhancing performance in endurance sports. rHuEPOs can markedly enhance oxygen availability to muscles. However, they constitute a major challenge for anti-doping authorities. The availability of a direct test to detect rHuEPO has revolutionized the fight against this dangerous substance and provoked a change in the doping habits of cheating athletes. Currently, athletes gravitate toward microdosing to decrease the detection window and to avoid large fluctuations in their hematological parameters. The development of novel ESAs has provided new ways of boosting the Hb level, which has been met with the development of new direct detection methods, such as LC-MS/MS in addition to immunological analyses. Several ESAs that are still in clinical trials have been detected in athletes indicating that collaboration between pharmaceutical companies and anti-doping laboratories is key. The companies should inform anti-doping laboratories about potential performance-enhancing products in clinical trials and provide access to the molecules prior to marketing, which may support new doping research.

The introduction of the hematological module of the ABP has offered a new approach for the indirect detection of blood doping and ESAs. The individual longitudinal monitoring of the different parameters ensures that athletes compete in a non-doped state. The proper functioning of the ABP requires close cooperation between the Athlete Passport Management Unit (APMU), which associates with a WADA-accredited laboratory or a national anti-doping organization (NADO), and anti-doping organizations (ADOs), international sports federations (IFs), and the WADA. The APMU reviews the passports in the adaptive model and advises the ADO on intelligent testing strategies (Saugy et al., 2014). The main asset of this tool is that it is independent from new doping drugs on the market. This promising tool is pushed to the limits of sensitivity in the detection of rHuEPO use in microdoses. Additional data

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would therefore supplement the ABP and offer new evidence of ESA use by athlete. Several markers originating from studies using omics technologies such as ironomics or transcriptomics could be introduced in an individual subject-based model to complement the ABP for the detection of ESA misuse.

Conflict of interest

The authors disclose no conflict of interest.

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