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GENE DOPING IN SPORT – PERSPECTIVES AND RISKS

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AUTHORS: Brzeziańska E[1], Domańska D[1], Jegier A[2]

[1] Department of Molecular Bases of Medicine, Medical University of Lodz
[2] Department of Sports Medicine, Medical University of Lodz

Reprint request to:
Anna Jegier
Department of Sports Medicine,
Medical University of Lodz,
90-647 Lodz, Plac Hallera 1, Poland
E-mail: anna.jegier@umed.lodz.pl

ABSTRACT: In the past few years considerable progress regarding the knowledge of the human genome map has been achieved. As a result, attempts to use gene therapy in patients' management are more and more often undertaken. The aim of gene therapy is to replace defective genes *in vivo* and/or to promote the long-term endogenous synthesis of deficient protein. *In vitro* studies improve the production of human recombinant proteins, such as insulin (INS), growth hormone (GH), insulin-like growth factor-1 (IGF-1) and erythropoietin (EPO), which could have therapeutic application. Unfortunately, genetic methods developed for therapeutic purposes are increasingly being used in competitive sports. Some new substances (e.g., antibodies against myostatin or myostatin blockers) might be used in gene doping in athletes. The use of these substances may cause an increase of body weight and muscle mass and a significant improvement of muscle strength. Although it is proven that uncontrolled manipulation of genetic material and/or the introduction of recombinant proteins may be associated with health risks, athletes are increasingly turning to banned gene doping. At the same time, anti-doping research is undertaken in many laboratories around the world to try to develop and refine ever newer techniques for gene doping detection in sport. Thanks to the World Anti-Doping Agency (WADA) and other sports organizations there is a hope for real protection of athletes from adverse health effects of gene doping, which at the same time gives a chance to sustain the idea of fair play in sport.

KEY WORDS: gene doping, methods of gene doping, methods of proteomic profiling, sport, WADA

Introduction

With the development of science, athletes enjoy the more modern methods and pharmacological agents supporting their physical fitness, muscle strength and improving athletic skills.

Doping, although banned by the International Olympic Committee (IOC) and the World Anti-Doping Agency (WADA), has been used since the early 1920s, in the form of, among others, anabolic steroids, erythropoietin, amphetamine and modafinil. Now, with the completion of the Human Genome Project (HUGO Project) and the development of gene therapy in medicine, there has been dynamic progress of research on gene doping and gene delivery technologies to improve athletic performance in various sports.

According to the published data, gene doping is associated with the introduction into the body of the transgene and/or recombinant protein in order to bring it to expression or to modulate the expression of an existing gene to achieve the further advantage of an athlete's physiological performance [1-7]. According to the list of prohibited substances published by WADA in 2008, gene doping has been defined as: "*nontherapeutic use of cells, genes, genetic elements, or modulation of gene expression, having the capacity to enhance athletic performance*" [8].

In 2013 WADA clarified the type of manipulation of genetic material prohibited in sport as the transfer of nucleic acids or their analogues into cells and the use of genetically modified cells [9].

Methods of gene delivery

Genetic material can be introduced into a cell either *in vivo* or *ex vivo*. The *in vivo* strategy is direct gene delivery into the human body, i.e., into main blood vessels or the target tissue/organ. In indirect DNA transfer strategy, i.e., *ex vivo* gene delivery, cells are collected from the body of the patient, and then, after genetic modification, breeding and selection, are reintroduced into the patient's body.

In gene therapy and, similarly, in gene doping the genetic material is delivered into cells and tissues using various carriers that can be viral or non-viral [10]. Using viral vectors (attenuated retroviruses, adenoviruses or lentiviruses) a transgene is released in target cells and is expressed using cell replication machinery. Some of these viruses, such as retroviruses, integrate their genetic material

with chromosomes of a human cell. Other viruses, such as adenoviruses, introduce the transgene into the cell nucleus without chromosomal integration [11].

Viral vectors are efficient gene delivery carriers and they offer several advantages: large packaging capacity, cell-specific tropism, and/or long-term expression [12]. In some cases, however, irreversible side effects, such as unexpected endogenous virus recombination, may occur. It leads to the rapid transformation of normal cells *in vitro* as well as initiating tumours *in vivo* via amplification of the host proto-oncogene sequences in the viral genome [10-13]. Additionally, viral vectors can be recognized by the host immune system, resulting in an increased immune response. This effect reduces the effectiveness of the transfection efficiency by reducing the efficiency of the subsequent transgene delivery. The most important biological properties of the viral vectors used in gene therapy, including the treatment of sports injuries, are shown in Table 1.

Non-viral gene delivery techniques are less effective methods of introducing genetic material into human cells, though characterized by low cytotoxicity. These include physical methods, such as electroporation, "gene gun" [17] and chemical carriers using cationic liposomes, or biodegradable polymers (polyethylenimines; PEIs) [18]. Non-viral gene delivery systems may cause an increased immune response [19-20].

Physical methods of gene delivery allow DNA transfer into the cell cytoplasm or nucleus, through local and reversible damage of the cell membrane. The most common physical technique is electroporation, based on the application of a high voltage electrical pulse to the cells, leading to the formation of hydrophilic pores in the cell membrane, of several nanometres in diameter [15]. Electroporation is a very effective method, and one of its strengths is the protection of cells against the introduction of undesirable substances during the transgene delivery. Nowadays, electroporation is the most frequently used method to introduce DNA into skin cells or liver cells.

Biochemical methods involve the use of chemical carriers, which form complexes with nucleic acids to neutralize their negative charge. Such complexes are introduced into the cell by phagocytosis, and less frequently by fusion with the cell membrane. Some of the chemical carriers facilitate the release of nucleic acid into the cytoplasm from the endosome, and protect it from cellular nucleases [15].

The main difficulty in the application of gene transfer in gene doping is to achieve a long-lasting effect, as well as monitoring the changes induced in the genome. A long-lasting effect can be achieved by multiple (repeated) gene doping applications or by the integration of a transgene into the chromosome. However, it should be emphasized that the integration of gene transfer vectors is associated with a risk of undesirable side effects, including insertional mutagenesis. Integration of the transgene at the wrong site may lead to the development of cancerous cells [21].

TABLE I. BIOLOGICAL PROPERTIES OF VIRAL VECTORS USED FOR GENE DELIVERY INTO CELLS IN THE TREATMENT OF VARIOUS DISEASES AND/OR SPORTS INJURIES [9,14-16].

Viral vectors	Biological properties of vector
Adenoviruses	– efficiency: average, infects only mitotic cells – capacity: large – durability of expression: high – other features: cytotoxic/immunogenic in high doses,
Adeno-associated viruses (AAV)	– efficiency: depends on the type of transfected cells – capacity: small – durability of expression: high – other features: specifically cytotoxic/immunogenic; low efficiency of methods of AAV obtaining
Herpesviruses (e.g., HSV-1)	– efficiency: high, infects mitotic and post-mitotic cells – capacity: large – durability of expression: high – other features: cytotoxic/immunogenic in high doses
Oncoretroviruses, e.g., Rous sarcoma virus (RSV), Moloney murine leukaemia virus (MLV)	– efficiency: infects mitotic cells – capacity: small – durability of expression: high – other features: increased immune response; frequently causes insertional mutagenesis
Lentiviruses, e.g., simian immunodeficiency virus (SIV)	– efficiency: high, does not require dividing cells – capacity: large – durability of expression: high – other features: low cytotoxicity

Is gene doping already a reality?

Research on gene doping, which has been carried out mainly in animal models, but also more and more often as gene therapy in humans, has brought many successes. It was reported that injection of a plasmid with a vascular endothelial growth factor (*VEGFA*) gene into the muscle of patients with chronic critical limb ischaemia led to improved distal flow [22]. Injection of the peroxisome proliferator-activated receptor-delta/beta (*Ppard/β*) gene into mouse zygotes improved the endurance and running capacity of mice on a treadmill, and simultaneously resulted in resistance to obesity, even in the absence of exercise and with a high-fat diet [23]. In rats, the introduction of the insulin-like growth factor-1 (*Igf1*) gene in a recombinant viral vector led to an increase in muscle mass and strength and to increase in endurance [24]. Transfer of the phosphoenolpyruvate carboxykinase (*Pck1*) gene resulted in increased activity of the transgenic mice, increased strength and speed during a race, and additionally, the mice were characterized by lower mass and fat content as compared to control mice [25].

In other studies on animals, gene therapy was used to increase the production of growth hormone (GH). By intramuscular injection of a plasmid containing the somatoliberin (*Ghrh*) gene, under the control of a muscle-specific gene promoter, increased concentrations of GH and IGF-1, and improvement of anabolic and haematological parameters were achieved. Moreover, the obtained results persisted for over one year [26].

The results of such studies are a major cause for concern over the direct threat of the spread of gene doping in competitive sports.

Another problem – which is a priority for sport organizations – is the difficulty in detecting gene doping. So far, the attempts to standardize the ideal test that could be used to detect gene doping have failed [5-6]. It should be emphasized, however, that several intensive studies on a number of promising strategies are being carried out (e.g., detection of a transgenic protein or vector that is the carrier of the genetic material in the site of intramuscular or tissue injection, monitoring the immune response after the use of a viral vector, or gene expression profiling) [5-6,27]. Lack of tests to detect gene doping is associated with the fact that the protein produced by the foreign gene or genetically manipulated cells will be structurally and functionally very similar to the endogenous proteins. Most transgenic proteins, especially those that enhance muscle strength, are produced locally in the injected muscle and may be undetectable in blood or urine. The only reliable method would require a muscle biopsy, but such an approach is virtually impossible to use in sport. Furthermore, gene expression can be modulated as desired using the appropriate pharmacotherapy. At present, according to the opinion of the United States Anti-Doping Agency (USADA), it is not possible to detect gene doping with current technology.

Gene doping makes it possible to create a "super athlete", but at the cost of breaking the rules of sporting ethics and undermining the principles of fair play in sport. It is also associated with a high risk of danger to the health of athletes.

Gene doping and its side effects

The main candidates for gene doping are: *EPO*, *IGF1*, *VEGFA*, *GH*, hypoxia-inducible factors (*HIFs)*, *PPARD*, *PCK1*, myostatins (*MSTN*), and some of their recombinant protein products (rEPO, rhGH) [2,5,6]. Data suggest that *IGF1*, *GH*, *MSTN* and *rhGH* may play a major role in strength sports while *EPO*, *VEGFA*, *HIF-1*, *PPARD*, *PCK1* and *rEPO* are essential in endurance sports. Of course, the full list is much longer.

Functional protein products of those genes are related to specific increase of endurance, physical strength, redistribution of fat or increase of muscle mass. Some of them control the distribution of oxygen to the tissues, or regulate the growth and/or regeneration of muscle tissue. In addition, gene doping takes into account the genes encoding the peptides that relieve pain (e.g., endorphins and enkephalins) – they can be used as prohibited analgesics [28].

Erythropoietin (EPO)

The *EPO* gene encodes a glycoprotein hormone that increases the number of red blood cells and the amount of oxygen in the blood, thereby increasing the oxygen supply to the muscles [29,43]. The expected effect of the physiological expression of the EPO gene would be increased endurance. For gene doping, an additional copy of the *EPO* gene may be introduced into the athlete's body using a viral vector, thus leading to the overexpression of *EPO*, increased production of red blood cells in the liver and kidneys, and to increased oxygen binding capacity of the blood. Physiologically dangerous side effects of doping with *EPO* transfer are primarily an increase in haematocrit, which may enhance the likelihood of stroke, myocardial infarction, thrombosis and an increase in total peripheral vascular resistance [29].

In 2002, the British pharmaceutical company Oxford BioMedica developed Repoxygen as a potential drug for the treatment of anaemia associated with chemotherapy used in kidney cancer. The drug is administered intramuscularly, and consists of a viral vector transferring the modified human *EPO* gene under the control of genes encoding proteins of oxygen homeostasis (e.g., HIF-1, HIF-2). EPO transgene is expressed in response to low levels of oxygen, and is turned off when the oxygen concentration reaches the correct value. In 2006, Repoxygen attracted the attention of the world of sports, when in Germany it began to be administered to young female runners to maintain constant expression of EPO in muscle cells. Repoxygen is prohibited under the World Anti-Doping Code 2009 Prohibited List.

Recombinant EPO (rEPO) is widely used to treat anaemia caused by chronic renal disease. Erythropoietin was the first recombinant haematopoietic growth factor produced and available commercially as a recombinant protein drug [30]. Several types of rEPO are now commercially available, including: epoetin alpha (Eprex, Janssen-Cilag), epoetin beta (Neorecormon, Roche), and darbepoetin alfa (Nespo, Dompe`) [30-31]. It is estimated that doping with rEPO is used by 3–7% of the best athletes of endurance sports [32].

The Sydney 2000 Olympics marked the beginning of the use of effective methods to detect injected rEPO.

Insulin-like growth factor 1 (IGF1) and growth hormone (GH)

Potentially, gene doping using *IGF1* gene transfer could provide the desired, stable and high levels of IGF1 protein. This method would be relatively safe, because the effect of its actions would be limited to the target muscles. It has been shown that overexpression of *IGF1* and its protein product combined with increased resistance training induced greater muscle hypertrophy [24]. Additionally, studies have shown that *IGF1* gene transfer enabled the regeneration of skeletal muscle following injury and was more efficient than systemic administration of its protein product [33]. *IGF1* expression is associated with increased muscle size and weight, thereby increasing muscle strength [2]. However, *IGF1* delivery may lead to profound hypoglycaemia, similar to the administration of insulin. Moreover, *IGF1* expression is also associated with cell cycle progression and apoptosis inhibition, by interacting with signalling pathways, such as *IGF-1*/*PI3K*/*Akt*/*AP-1* or *IGF-1*/*Shc*/*Ras*/*MAPK*, that are activated during carcinogenesis (e.g., in colon, breast or prostate cancer) [34-36].

IGF1 protein, also known as somatomedin C, belongs to the group of polypeptide hormones, which are essential for proper development of the fetus. In the mature organism it is involved in the regeneration of tissues, especially connective tissue, and also exhibits insulin-like activity, e.g., inducing hypoglycaemia [37]. IGF1 mediates some anabolic processes of growth hormone. One of the main functions of GH – mediated also by IGF1 – is the stimulation of body growth and body weight. GH also affects carbohydrate metabolism (stimulation of glycogenolysis and increased glucose release from the liver), fat metabolism (increased lipolysis and decreased lipogenesis) and protein metabolism (increased protein synthesis) [38]. There are only a few published reports confirming the enhancing effects of GH on muscle strength and cardiovascular and respiratory functions in trained healthy individuals [39-40]. On the other hand, evidence of the health risks associated with the use of GH (e.g., insulin resistance, impaired glucose tolerance and limited efficiency of the cardiovascular and respiratory systems) is accumulating. GH overexpression is associated with intracranial hypertension, headache, peripheral oedema, carpal tunnel syndrome, joint and muscle pain, or cardiomegaly in trained persons [41]. However, there is anecdotal evidence that recombinant GH (rGH) is commonly abused by athletes.

Hypoxia-inducible factor-1 (HIF-1)

The *HIF-1* gene encodes proteins involved in the process of hypoxia, angiogenesis and erythropoiesis activation or regulation of glucose metabolism. Doping associated with stimulation of *HIF-1* expression under normal conditions of oxygen supply, e.g., using chemical agents or gene transfer into cells, may improve athletic endurance. On the other hand, it affects mitochondrial oxygen metabolism, while also stimulating genes associated with metabolic adaptation of cells (e.g., *GLUT1*, *GLUT3*, *GPI*, *ENO1*), angiogenesis, apoptosis, or carcinogenesis (e.g., *VEGFA*, *IGF-1*, *IGF-2*, *TGFβ*, *Ang1*, *MMP*, *ADM*) [42]. These molecular changes in the cells may result in myocardial infarction, stroke or cancer. HIF-1 regulates oxygen homeostasis, thus facilitating the cell's adaptation to low oxygen conditions. HIF-1 also affects erythropoiesis, iron metabolism, pH regulation, apoptosis, cell proliferation and intracellular interactions. Hypoxia itself regulates the expression of genes involved, among others, in cell energy metabolism, glucose transport and angiogenesis [42]. Thus, gene therapy using the HIF-1 gene or protein may result in physiological changes at many levels in the body. Research is being conducted with the use of *HIF-1* in the treatment of cardiovascular diseases. Based on the animal studies and early clinical trials in humans, it is believed that *HIF-1* administered as gene therapy effectively induces neovascularization in ischaemic tissues [43-45].

Peroxisome proliferator-activated receptor (PPAR)

The family of *PPARs* consists of the following genes*: PPARA (α)*, *PPARD (δ)*, and *PPARG (γ). PPAR-α* influences carbohydrate-lipid metabolism, thus regulating homeostasis and body mass [46]. Experiments have shown that the activation of *PPARD* reduces weight gain, increases skeletal muscle metabolic rate and endurance, and improves insulin sensitivity. It was further found that the increase in *PPARD* expression suppresses atherogenic inflammation [47]. The results of some studies suggest that polymorphisms in exon 4 (+15 C/T) and exon 7 (+65 A/G) of the *PPARD* gene correlate with changes in the capacity of the cardiovascular system and the concentration of lipids and glucose in healthy subjects in response to regular exercise [48,49]. Nuclear hormone receptor protein is associated with *de novo* formation of skeletal muscle fibres of type I (slow-twitch fibres) and their transformation from type II fibres (fast-twitch fibres), which determine the athlete's endurance and speed. In addition, *PPARD* also plays an important role in the differentiation and maturation of adipocytes. It controls the body's energy balance. Thus, this protein plays an important role in the control of body weight [50]. So far, no studies have been conducted on gene doping with *PPARD* in humans or in animal models.

Peroxisome proliferator-activated receptor D (PPARD) agonist (GW1516; GW501516)

Animal studies showed that administration of GW1516 for a period of five weeks to mice subjected to training increased the exercise tolerance by 60-70%, as compared to mice in the control group. It is recognized that GW1516 improves the exercise capacity of trained animals [51]. However, there are no published data on the ergogenic effects of GW1516 in healthy and trained people. GW1516 is an experimental drug that has been used in the treatment of obesity, metabolic syndrome, and type 2 diabetes in some clinical trials [52]. This molecule is included in the WADA prohibition list and there are reports that some athletes have already been caught using such doping.

Adenosine monophosphate analogue (AMP)-(AICAR)

It is an analogue of adenosine monophosphate (AMP), the activator of AMP-dependent kinase (AMPK). Studies have shown that activated AMPK enzyme may reduce the level of anabolic processes, including synthesis of fatty acids and proteins, and increase the level of catabolic pathways such as glycolysis and fatty acid oxidation [53]. It has been proven that after 4 weeks of AICAR administration to mice subjected to training their speed and strength increased by 20-40%. It is believed that the ergogenic effect is achieved by the mutual interaction between AICAR and training, and their effect on the activation of many genes, determining the exertion efficiency [51,54]. So far, however, there have not been published any data on AICAR ergogenic effects in healthy and trained people. AICAR is also an experimental drug and is included in the WADA prohibition list. The method to detect this molecules in humans by anti-doping laboratories was described by Narkar et al. [51].

Myostatin (MSTN)

The protein product of the *MSTN* gene belongs to the transforming growth factor β (TGF-β) superfamily, and is considered as a negative regulator of muscle growth and biogenesis [55].

In an animal model, it has been proven that suppression of the gene leads to reduced muscle growth restriction, and thus to increased muscle mass and strength [56]. Studies have demonstrated that the introduction of small interfering RNA (siRNA) into the body may negatively regulate *MSTN* [57]. It has been shown that suppression of the myostatin gene or protein triggers non-physiological – in terms of the number and size of cells – development of the muscle tissue. In some pathological conditions, the increase of muscle mass in a short period of time can promote hypertonic cardiomyopathy, subsequently resulting in a heart attack. The excessive growth of muscle mass also leads to overloading of the musculoskeletal system, increasing the susceptibility to bone and tendon injuries.

Phosphoenolpyruvate carboxykinase (PCK1, PEPCK-C)

It is a key enzyme regulating gluconeogenesis. This enzyme is considered crucial in glucose homeostasis and is involved in the Krebs cycle [46]. Studies in mice have shown that its expression is associated with increased muscle endurance in animals [25].

So far, there are no published literature data confirming the occurrence of side effects associated with transfer of the PCK1 gene or protein used as doping.

Gene transfer as a method of strengthening the desired physical and physiological characteristics or improving the natural athlete phenotype is an attractive way to achieve success in sport for many athletes [17]. For this reason, intensive investigations on the potential use of gene doping in many sports are nowadays increasing in number.

Table 2 shows the genes that could be potential targets for doping in sport and indicates the potential risk to the athlete's health in case of a possible application of this type of doping.

Methods of gene doping detection

Nowadays, the detection of gene doping is a priority for many sports organizations, because of the proven effects of its use in experimental animals and the progress achieved in over 1000 clinical trials of gene therapy in humans [3-4].

Scientists supported by the WADA are looking for effective methods and tests for the detection of gene doping used currently in sport. The first developed and officially approved test to detect gene doping is a test for the presence of GW1516. It is known that GW1516 affects muscle strength and endurance, and also increases the cell's ability to burn fat [9,52,66].

However, there are still problems associated with the development of a credible and effective test to detect gene doping. This type of problem includes a plurality of protein isoforms encoded by a single gene and the similarity of genetically modified proteins to their endogenous counterparts. In addition, most transgenic proteins – especially those that enhance muscle strength – are synthesized locally in the muscle into which they are injected. Therefore they may be undetectable in the blood and urine. It appears that the only reliable method of their detection would require muscle biopsy, but this approach is practically impossible to use in sport.

The search for methods of gene doping detection in sport is based on the identification of both the carriers, such as a vectors, as well as the detection of the introduced genes (QPCR, real-time PCR) or their protein products (protein profiling method: mass spectrometry, phosphoproteomics, glycoproteomics, SELDI-TOF method).

Detection of viral vectors and monitoring of the host immune response

Detection of the carrier used in gene doping, usually a vector (used to transduce the gene), is possible in the site of intramuscular injection or tissues within weeks, and often months, after the application of doping. However, the collection of samples for testing would require information about the exact site of injection, and finally muscle biopsy. However, both approaches are inapplicable in a sport setting [67]. An additional difficulty in detecting delivery vectors in body fluids is the need for sampling in a relatively short time after doping administration. This requires regular testing of athletes out of competition. Another problem is the collection of samples, their storage and further analysis. These steps should be conducted according to the standardized, validated protocols, including snap freezing of samples in liquid nitrogen.

It seems that the evaluation of the host immune response to the viral vector is also an effective approach, but it requires refinement. There is a possibility that the tested athlete could have been infected by the virus via non-doping routes (such as viral infection or reactivation of latent viral infection by a pathogen similar to the used viral vector). Therefore the test confirming the presence of antibodies against the virus in the blood will not constitute irrefutable evidence of the use of doping. It is also possible to produce genetically engineered viral vectors which are less immunogenic, thereby minimizing

TABLE 2. POTENTIAL GENES THAT CAN BE USED IN DOPING, TARGET TISSUES/SYSTEMS AND POTENTIAL RISK TO THE ATHLETE'S HEALTH [29-65].

Potential genes	Target tissue/system	Risks to health	1. Physiological function 2. Expected phenotypic performance
EPO Locus: 7q22	Blood system	– Increased blood viscosity, – Difficult laminar blood flow through the vessels, – Severe immune response	1. Increased number of red blood cells and increased blood oxygenation 2. Increased endurance
IGF1/ GH *Locus:* 12q23.2/ 17q22–q24	Endocrine and muscle system	– Intracranial hypertension, – Abnormal vision, – Headache, nausea, vomiting, – Peripheral oedema, – Carpal tunnel syndrome, – Pain in the joints and muscles, – Overgrowth of the cartilage of the nose and jaw, – Cardiomyopathy, – Insulin resistance and diabetes, – Neoplastic disease	1. Excessive growth of bones and tissue mass, muscle hypertrophy and hyperplasia, and stimulation by muscle regeneration (IGF1), – stimulation of glycogenolysis and increased release of glucose from liver, increased lipolysis and reduced lipogenesis, increased protein synthesis (GH) 2. Increased endurance, efficiency, increased muscle mass and strength (IGF1, GH)
HIF-1 *Locus:* 14q23	Blood and immune system	– Increased blood viscosity, – Hypertension – Neoplastic disease	1. Increased number of red blood cells and increased blood oxygenation (indirectly by affecting, among others, EPO gene or genes encoding glycolytic enzymes) 2. Increased muscle strength and endurance
PPARD *Locus:* 6p21.2	Muscular system	– Overexpression of sex hormones, – Colon cancer	1. Acceleration of skeletal muscle cell metabolism, increased insulin sensitivity, increased lipolysis 2. Increased endurance and speed. Probably involved in the control of body weight
MSTN *Locus:* 2q32.2	Muscular system	– Damage of the ligaments, tendons and bones	1. Hypertrophy and hyperplasia of muscle mass 2. Increased muscle mass and strength
ACTN2 and *ACTN3* *Locus:* 1q42–q43 / 11q13.1	Muscular system (actin filaments within the myofibrils of the striated muscle, fast-twitch fibres ACTN3 (type II fibres).	– No data on the negative effects of gene doping using ACTN2 and ACTN3	1. Increased rate of glucose metabolism in response to training (ACTN3), – Compensation for loss of function of ACTN3 gene by ACTN2 gene 2. Increased endurance, muscle strength and speed of muscle; increased efficiency in sprinters
VEGFA *Locus:* 6p12	Vascular endothelium	– Neoplastic disease, – Immune response	1. Induction of new blood vessel formation (angiogenesis) 2. Increased endurance
POMC/ PENK precursors Endorphin/ enkephalins *Locus:* 2p23.3/ 8q23–q24	Central nervous system	– Increased risk of overloading the musculoskeletal system and cardiovascular system, – Stress and increased cardiac workload, – Sudden death	1. Modulation of pain perception threshold 2. Increased endurance
ACE *Locus:* 17q23.3	Skeletal muscle	– Angioedema	1. Adjusting blood pressure by acting on angiotensin II (increase in blood pressure), and participation in the inactivation of bradykinin (decrease in blood pressure), increasing the proportion of slow-twitch muscle fibres (type I) 2. Increased endurance and/or sprint efficiency
PCK1 *Locus:* 20q13.31	Skeletal muscle	No data on the negative effects of gene doping using PCK1 in athletes	1. Adjusting the metabolic processes including gluconeogenesis, involved in the Krebs cycle 2. Increased muscle endurance

a host immune response [5,67]. Systematic and periodic testing of athletes – in order to detect the antibodies against viral vectors – would be required to monitor the level of anti-viral antibodies, which could potentially be used as a method for the detection of gene doping.

Methods of gene and proteomic profiling

Profiling methods are based on the monitoring of the secondary changes which arise as a result of gene doping. Transgene expression will lead to alterations in expression of other genes and their protein products.

Gene expression profiling assesses the expression profile of endogenous genes that may be modified following the expression of the introduced gene [5,27,67]. For this purpose DNA microarrays – which simultaneously compare mRNA expression patterns of thousands of genes – are used. In this method mRNA is isolated from cells of the athlete who used doping and cells from normal individuals from a control group. Then, mRNA is transcribed into cDNA,

which is radioactively or fluorescently labelled. The principle of the method is based on the complementary binding of synthesized cDNA with oligonucleotides (probes) immobilized on glass or silicon plates (chips). Next, a laser is used to scan the chip to visualize the fluorescent signal given by the cDNA bound to the complementary probes. Changes in fluorescent signal intensity reflect increase/decrease of expression of the studied genes [68-69].

Based on this method, there is a possibility to develop microarray chips for expression analysis of the panel of genes that can be used in gene doping. Development of such chips gives the potential of expression analysis of genes used in doping as well as genes regulated by the transgene. For example, the development of a microarray chip for the *EPO* gene makes it possible to monitor the modified expression of about 100 *EPO*-dependent genes [27]. Expression analysis of these genes – in relation to the reference gene – might be an indirect method of doping detection.

Another strategy is proteomic profiling. This technique is based on the detection of minor structural differences between the recombinant proteins – which result from the expression of transgenes – and their endogenous counterparts. Investigation of global alterations in protein biomarkers upon doping can be done using the SELDI-TOF (surface enhanced laser desorption/ionization time-of-flight mass spectrometry) method, which combines chromatography and mass spectrometry for protein profiling [21,70]. This technique is particularly applicable to indirect identification of GH, which is used in doping, by detecting the presence of the alpha chain of haemoglobin in blood serum [71].

Another method of protein expression profiling is searching for transgenic proteins based, among others, on differences in their glycosylation or differences in the evoked host immune response. Such research has already been applied in the case of the EPO protein [72].

Both gene and proteomic profiling require extensive research, in order to establish the reference ranges for the general population and individual athletes. Specific reference ranges should be established with regard to gender, population and sport.

Summary

Progress of research on gene therapy and clinical trials in this area significantly increased the possibilities of gene doping in sport. Simultaneously, the prospect carried by this new method of doping – the creation of a "super athlete" and lucrative professional sports – further complicates the situation.

It is believed that new methods, including *ex vivo* gene transfer to allogeneic stem cells, would considerably accelerate the possibility of the practical application of gene doping in sports. It should be noted, however, that these manipulations involve a lot of risks. The transmission of foreign genes into the genome can cause numerous – as yet unrecognized – interactions between genes and the internal and environmental modulators.

In contrast to gene therapy, which is carried out under strictly controlled conditions, gene doping can be performed without the use of security and protective measures. Vectors for gene transfer produced in uncontrolled laboratory conditions can be contaminated e.g., by chemical and/or biological agents, thereby endangering the health and life of athletes.

However, despite the documented and unpredictable risks associated with gene doping, some athletes ignore safety issues.

An additional problem is still not completed work on the standardization of reliable tests to detect gene doping. The scientific and medical communities should support the activities of the World Anti-Doping Agency (WADA) in developing new methods of gene doping detection and updating the lists of banned agents. In addition to educational programmes for athletes, and development of tests for gene doping detection, an individual method of gene doping control should be introduced, in which each athlete would be the self-reference baseline. In the case of such an approach it would be necessary to collect in an individual athlete database the results of his/her tests (biochemical and haematological), and possibly the expression profile of genes that can be potentially used for gene doping, to monitor it over time. UCI (*Union Cycliste Internationale)*, WADA and IAAF (*International Association of Athletics Federations)* have already introduced a project known as the Athlete Passport to gather individual athlete testing data (WADA 2009–2013). In the future, such a personalized method of doping control may be the main method of combating this complex problem. In summary, to prevent the development of gene doping, international sports organizations should conduct numerous educational campaigns among athletes, pointing to the risks and ethical problems associated with its use.

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