TRENDS

Circulating miRNAs: a new generation of anti-doping biomarkers

Nicolas Leuenberger · Neil Robinson · Martial Saugy

Abstract MicroRNAs (miRNAs) are small non-coding RNAs that regulate a variety of biological processes. Cell-free miRNAs detected in blood plasma are used as specific and sensitive markers of physiological processes and some diseases. Circulating miRNAs are highly stable in body fluids, for example plasma. Therefore, profiles of circulating miRNAs have been investigated for potential use as novel, non-invasive anti-doping biomarkers. This review describes the biological mechanisms underlying the variation of circulating miRNAs, revealing that they have great potential as a new class of biomarker for detection of doping substances. The latest developments in extraction and profiling technology, and the technical design of experiments useful for anti-doping, are also discussed. Longitudinal measurements of circulating miRNAs in the context of the athlete biological passport are proposed as an efficient strategy for the use of these new markers. The review also emphasizes potential challenges for the translation of circulating miRNAs from research into practical anti-doping applications.

Keywords Doping · Circulating microRNAs · Biomarkers · Athlete biological passport

Introduction

A major challenge in anti-doping is identification of specific and sensitive non-invasive biomarkers that can be routinely

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measured in easily accessible samples. MicroRNAs (miRNAs) are a particularly promising class of biomarker. miRNAs are small (19–25 nucleotides), non-encoding RNAs that enable post-transcription regulation of gene expression by suppression of specific target messenger RNAs (mRNAs) [1]. Since their discovery in the early 1990s, these small molecules have been revealed to have important regulatory functions in a wide range of biological and pathological processes. To date, more than 1800 miRNAs have been identified in humans [2]. The number of miRNAs in our species therefore seems to be smaller than the number of conventional mRNAs, estimated to be 30,000 [3].

Since 2008, miRNAs have been detected in serum, plasma, urine, saliva, and other body fluids [4]. Although most RNA molecules are unstable, circulating miRNAs are highly stable and readily detectable. Cell-free miRNAs in body fluids are stable under harsh conditions, including boiling, low and high pH, extended storage, and multiple freeze–thaw cycles [5]. In contrast, synthetic or purified miRNAs added to serum or plasma are quickly degraded by high levels of RNase activity in plasma [6]. Thus, circulating miRNAs are not intrinsically resistant to endogenous RNase activity.

Stability of circulating miRNAs

The molecular basis of the high stability of circulating miRNAs has been investigated in different laboratories. One hypothesis suggests that circulating miRNAs are protected by microvesicles, for example exosomes and microparticles; alternatively, circulating miRNAs may be associated with specific protective proteins.

Recently, different groups have found that most circulating miRNAs co-fractionate with protein complexes [6, 7]. Arroyo et al. [6] found that miRNAs are sensitive to protease

treatment of plasma, indicating that protein complexes protect circulating miRNAs from plasma RNases. Investigation of the protective protein complexes revealed that Argonaute 2 (Ago2), a protein component of the RNA-induced silencing complex, is present in human plasma and elutes with plasma miRNAs in size-exclusion chromatography [6, 8]. The extracellular stability of Ago2–miRNA complexes has also been observed in cell culture. Both Ago2 protein and miRNAs remain stable for several weeks in cell lysates, even in the absence of protease inhibitors [7]. Immunoprecipitation assays performed on plasma revealed that Ago2 readily recovers non-vesicle-associated plasma miRNAs. In these studies, most miRNAs tested co-purified with the Ago2 ribonucleoprotein complex; however, only a minority of specific miRNAs were associated with vesicles [6]. These observations revealed the existence of two populations of circulating miRNAs, and suggested that circulating Ago2 complexes are most probably the reason for the stability of plasma miRNAs [6, 7]. These studies have important implications for the development of biomarker approaches based on sampling and analysis of circulating miRNAs.

Secretion mechanism and origin of circulating miRNAs

The presence of miRNAs in the extracellular environment has led researchers to develop two main hypotheses regarding systems for miRNA export from cells and tissues [9]. One hypothesis is that all types of circulating miRNA in biological fluid are by-products of cellular activity and cell death [7]. This "by-product release" hypothesis could explain multiple observations that, after toxicity occurs in some tissues, the levels of miRNAs specific for the affected tissue increase in the plasma [10–12]. The other hypothesis is that secretion of miRNAs is caused by an increase in cellular activity leading to the release of miRNAs, either in Ago2–miRNA complexes or, for a minority of miRNAs, packaged in microvesicles, for example exosomes.

Recently, more attention has been devoted to understanding the origin of circulating miRNAs and its effect on biomarker specificity [9, 13, 14]. Because blood cells are in extensive contact with plasma, they should be major contributors to the extracellular miRNA content of plasma [15, 16]. This idea is supported by observations that perturbations in blood-cell counts and haemolysis can alter plasma miRNAs; furthermore, ultracentrifugation steps that remove intact cells and debris reduce the level of miRNAs in plasma [16]. However, because tissue-specific mRNAs from liver, muscle, heart, brain, lungs, and placenta have been detected in plasma [10–13, 17], it is probable that other organs contribute to extracellular miRNA. Many groups have observed that tumours secrete miRNAs, and have detected cancer tissue-specific miRNAs in the circulatory system [18]. These observations support the theory that circulating blood cells are not the only source of circulating miRNAs.

Circulating miRNAs as disease biomarkers

In healthy individuals levels of cell-free miRNAs present in serum are stable, and the plasma and/or serum miRNA profile is similar to that of circulating blood cells. Altered serum miRNA levels may be indicative of physiological or pathological changes, and might therefore be used as surrogate biomarkers.

Lawrie et al. were the first to discover tumour-specific deregulation of circulating miRNAs [19]. Their study revealed that miR-21 is abundant in the sera of diffuse large B-cell lymphoma patients. Since then, many other investigations have revealed the diagnostic and prognostic value of measuring miRNAs in body fluids in the context of a variety of solid cancers [20, 21]. In 2008, Mitchell et al. reported that miRNAs derived from epithelial tumours are also rapidly released into the bloodstream [5]. Shen et al. [22] revealed that plasma miRNA expression profiles could be useful for discriminating non-small cell lung cancer (NSCLC) patients from healthy controls and patients with chronic obstructive pulmonary disease (COPD).

Regarding non-neoplastic changes, tissue-specific miRNAs have been analysed in the bloodstream as markers of myocardial [11] and liver injury [12]. For example, myocardial infarction induced an increase in the plasma level of miR-133a, a muscle-specific miRNA [23]. Several studies have revealed that liver injury is associated with increased plasma levels of miR-122 and miR-192 [12].

Physiological changes, for example pregnancy, increase the level of several miRNAs in plasma [13]. miR-517a and miR-526a undergo substantial changes that could be used to distinguish pregnant from non-pregnant women with high accuracy.

Circulating miRNAs as anti-doping biomarkers

In the same way that some biomarkers are used to detect the biological signs of disease, doping biomarkers have been used for some time in anti-doping tests to detect the biological signs of doping. The potential of circulating miRNAs for use as specific anti-doping biomarkers has been revealed by our laboratory and others. We found that plasma miRNA expression profiles can be useful in detecting erythropoiesis-stimulating agents (ESA) [24]. That study revealed that levels of a specific circulating miRNA, miR-144, increase in plasma samples after a single methoxy polyethylene glycol-epoetin

beta (Mircera) injection. This miRNA has been reported to be essential for erythropoiesis in a variety of organisms, and our finding revealed the potential of miRNAs as circulating biomarkers for ESA detection. Another study revealed that circulating miRNAs could be used as biomarkers to detect recombinant human growth hormone (rhGH) abuse: Kelly and colleagues identified and confirmed four miRNAs that were differently expressed in all individuals using therapeutic replacement doses of rhGH, relative to normal controls or individuals with naturally high levels of GH [25].

Recently, we found that circulating miRNAs could be used to detect autologous blood transfusion [26]. Transfusion of autologous blood induced an increase in the plasma levels of specific circulating miRNAs compared with a non-transfused control group. Because blood transfusion causes substantial changes in miRNA levels in the lung, the origin of these miRNAs was related to pulmonary tissue. We also observed partial suppression of erythropoietin (EPO) after transfusion. A combination of miRNAs and EPO measurement in a mathematical model increased the efficiency of detection of autologous transfusion via miRNA analysis.

Methods for analysis of circulating miRNAs

Three matrices have been studied in the context of miRNA profiling in blood: serum, plasma, and whole blood. The choice of matrix has been reported to have a significant effect on miRNA concentration. Serum has lower levels of circulating miRNAs than plasma [27]. Because of the large number of blood analyses required for the athlete biological passport (ABP), EDTA–plasma is the matrix most widely used in anti-doping. Because blood variables and miRNAs are measured in the same matrix, a single EDTA–plasma sample would be sufficient to perform the test, and plasma would therefore seem to be the best material for miRNA analysis in anti-doping laboratories. However, plasma samples containing heparin as an anticoagulant should not be used because this substance can interfere with subsequent assays, for example the reverse transcription reaction of RT–PCR.

One critical factor that might affect the results of miRNA quantification is the presence of haemolysis. Several studies have observed that mature erythrocytes contain abundant and diverse miRNA. In a recent study, Blondal et al. suggested different procedures and tests for assessment of haemolysis [28]; one simple and cost-effective way is to use a spectrophotometer and measure oxyhaemoglobin absorbance at $\lambda = 414$ nm. Other methods, based on delta calculation between erythrocyte-enriched miR-451 and miR-23a, which is not affected by haemolysis, have been proposed as indicators of haemolysis.

Because of the small amount of circulating miRNA and the large amount of proteins and lipids, miRNA extraction from blood samples is technically challenging. Moreover, plasma

and serum contain large amounts of reverse transcription and PCR inhibitors. To overcome these challenges, several phenol–chloroform-based procedures are available for extracting miRNA from biological fluid [29].

Most of these methods combine phenol–guanidine-based lysis of samples and silica-membrane-based purification of total RNA (Fig. 1). The lysis reagent is a solution of phenol and guanidine thiocyanate, designed to facilitate lysis, to denature protein complexes and RNases, and to remove most of the residual DNA and proteins from the lysate via organic extraction. After addition of chloroform, the lysate is separated into aqueous and organic phases by means of centrifugation. The aqueous phase is extracted and the sample is then applied to the silica membrane-based spin column, where the RNA binds to the membrane but phenol and other contaminants are efficiently washed away. To increase the efficiency of phenol–chloroform extraction, Andreasen et al. revealed that addition of a small carrier RNA before total RNA extraction improved the yield and reproducibility of separate RNA extractions [29].

Commercially available kits without acid-phase separation can also be used for isolation of miRNA from body fluids (Fig. 1). The general principle of extraction is similar to that of phenol–chloroform extraction, but the non-phenol lysis solution contains only guanidine thiocyanate, and salt solution is added to denature and precipitate proteins. The phenol–chloroform procedure yields more miRNA, reduces the variance between assays [27], and could be fully automated, and this procedure will therefore usually be the best method for extracting miRNAs from biofluids. Nevertheless, different extraction methods should be tested for each study to establish the optimum procedure.

One of the main problems associated with circulating miRNA extraction and comparison of collected samples is quantification of the miRNA. Because the miRNA content of plasma is low, it can only just be determined by use of spectrophotometers. Efficiency of extraction of circulating miRNA could be assessed by spiking plasma with known quantities of non-human miRNAs (e.g. that of *Caenorhabditis elegans*). Synthetic spike-in miRNAs should be added to the denaturation buffer (e.g. Trizol or lysis buffer). These miRNAs go through the entire extraction process and are measured in the final miRNA eluate by use of qRT–PCR, providing an internal reference against which to assess the efficiency of extraction. Addition of synthetic spike-in miRNA can also be used to assess the reverse transcription reaction.

Profiling new circulating miRNA anti-doping biomarkers

When searching for circulating miRNA-based doping biomarkers, different profiling methods can be used. Profiling enables us not only to discover changes in miRNA levels after treatment, but also to find stable miRNAs that could be used as

Fig. 1 Schematic outline of two miRNA extraction procedures. The commercial kit does not use phenol–chloroform to degrade protein from plasma samples
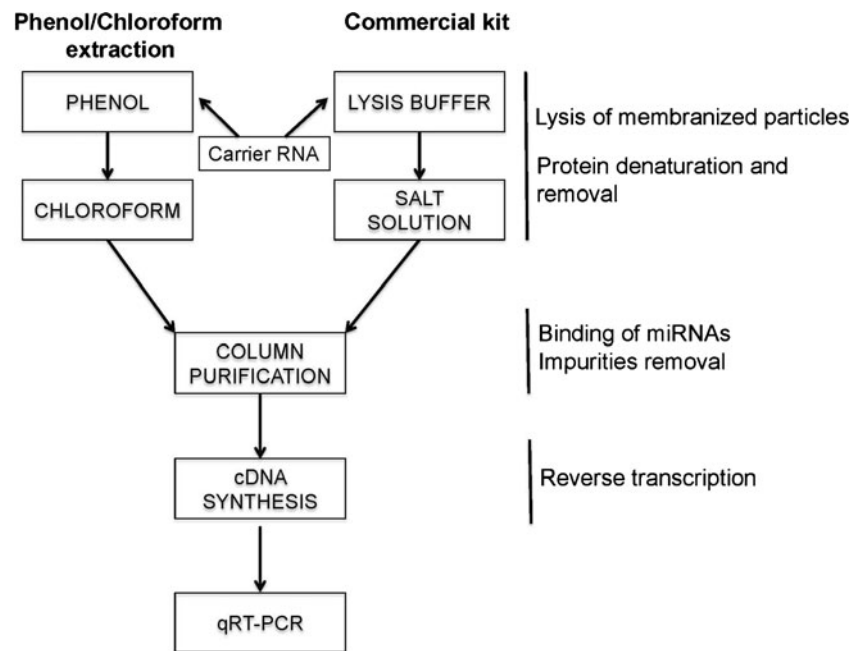
endogenous controls. Technological advances have resulted in many miRNA-profiling techniques.

High-throughput quantitative reverse transcription (qRT)–PCR remains the most sensitive and reliable method for detecting circulating miRNAs. A positive aspect of this approach is its ease of incorporation into the workflow of laboratories that are already familiar with real-time PCR. For large-scale miRNA profiling (which involves hundreds of miRNAs) by use of qRT–PCR, commercially available pre-plated PCR primers, typically distributed across multi-well dishes, are available [3]. To prevent variation of primer hybridisation among the hundreds of PCR assays, locked nucleic acids (LNA) are incorporated into primers [30].

Microarrays were among the first methods to be used for parallel analysis of large numbers of miRNAs, and several variations of the approach have been developed [3]. miRNA microarrays have the advantage of being less expensive than other profiling methods. However, several limitations have been described, including a restricted linear range of quantification, low sensitivity, and imperfect specificity for some miRNAs.

A deep-sequencing approach has been investigated in several studies with the objective of evaluating the potential use of circulating miRNAs as disease biomarkers [13]. The major advantages of next-generation sequencing for miRNA profiling are the detection of both novel and already known miRNAs and the precise identification of miRNA sequences. Potential limitations of next-generation sequencing include high cost, although this is dropping with the introduction of new instruments and the use of DNA "barcoding". For example, use of barcoded adapters enables multiplexing of 20 samples in a single sequencing reaction, dramatically reducing the cost of sequencing five million reads per sample to 150 USD [13].

Strategies for identifying circulating miRNA-based anti-doping biomarkers

In the process of searching for blood miRNA-based doping biomarkers, a high-throughput RT–qPCR strategy was used to identify plasma miRNA-based biomarkers for detecting autologous blood transfusion [26]. In this strategy, multiplex real-time RT–qPCR is used to identify a set of specific circulating miRNAs. RT–qPCR was chosen because it is a faster, simpler, and more sensitive technique than sequencing or use of microarrays.

As depicted in Fig. 2, the strategy was separated into several steps:

1. profiling with pre-plated PCR primers, using subjects who have undergone treatment including blood transfusion, to find a potential profile of circulating miRNAs and endogenous controls;
2. individual RT–qPCR validation of selected miRNAs obtained at every time point before and after doping intervention (from three days before treatment, D − 3, to 10 days afterward, D + 10);
3. comparison with variations in blood properties; and
4. pre-analytical and analytical characterisation of selected miRNAs.

In general, initial screening by use of the high-throughput technique is intended to identify differently expressed plasma miRNAs in artificially doped volunteers. However, once we know the specific miRNAs linked to doping, we can directly determine their levels by use of individual qRT–PCR.

The detection window for the panel of differently expressed miRNAs from the screening study was determined

Fig. 2 Suggested strategy for investigating use of circulating miRNA-based blood biomarkers to detect abuse of doping compounds
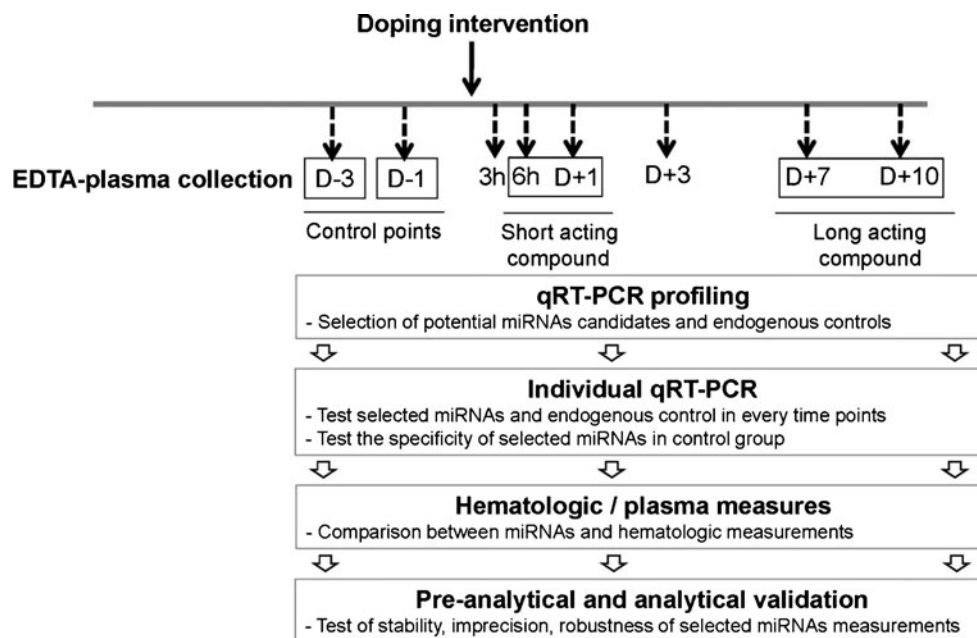
by longitudinal measurement using a maximum number of time points. The stabilities of non-differently expressed miRNAs at every time point were also investigated, and were used as endogenous controls. The screened endogenous controls and artificial spike-in miRNAs were used for normalisation.

For decades, haematological variables have been commonly used as reference values in clinical and anti-doping studies. For example, haemoglobin has been tested to detect autologous blood transfusion [31]. Correlation between blood-marker variables and circulating miRNAs could increase the efficiency of result interpretation. In our study the combination of circulating miRNAs and other variables, including EPO, resulted in greater discriminative power for the detection of autologous blood transfusion [26].

Providing proof of principle for a reliable miRNA doping test is a long process. Many pre-analytical and analytical variables that might affect the measurement of miRNAs in anti-doping applications have yet to be studied in detail. McDonald et al. proposed a method for validating circulating miRNAs used in clinical diagnosis [27]. The relative contribution to assay imprecision from components including intra and inter-assay imprecision, miRNA extraction, reverse transcription, real-time PCR, and normalisation to spiked or internal-control miRNAs has yet to be established.

Potential use of circulating miRNAs in the athlete biological passport

The fight against doping is mainly based on direct detection of a prohibited substance in an athlete's biological sample. Some methods, including the ABP, also use indirect markers [32].

The ABP is a new tool, which has immense potential in the current climate of rapidly advancing biomarker discovery [32]. Doping induces physiological changes that enhance performance. In the same way that disease-related biomarkers are invaluable tools that assist physicians in the diagnosis of pathology, specific biomarkers can be used to detect doping.

In 2008, the haematological module of the ABP was the first to be implemented by the International Cycling Union [33]. Biomarkers related to the haematopoietic system (haemoglobin concentration, reticulocytes) are monitored over time and analysed by use of mathematical models that quantify individual variation to identify patterns that raise suspicion of blood doping (e.g. ESA and autologous blood transfusion) [31].

The underlying principle of the ABP is the use of information from biological tests as indirect evidence to detect doping.

Table 1 Advantages of using circulating miRNAs in the athlete biological passport

Step	Advantages of circulating miRNAs
Collection	Similar to guidelines used for the haematological module of the ABP
Transport	High stability during transport
Analysis	Long time period for detection
	Correlation with blood-property variables
	qPCR technology enables multiplexing analysis
	Multiple quality controls could be easily added
Storage	High stability during storage in EDTA–plasma
	Not sensitive to unregulated room-temperature storage (up to 10 years)
	Stability in plasma subjected to multiple freeze–thaw cycles

For this reason, a stringent process of sample collection, transport, and data analysis has been put into place to guarantee objective and reliable use of this tool [34]. This is of particular importance because blood is a living tissue that undergoes permanent changes over time; these changes must be limited as much as possible, either by using standardised procedures or by taking these changes into account in evaluation of the data.

Circulating miRNAs have been investigated as potential biomarkers for detection of blood doping, and could therefore be incorporated into the adaptive model of the ABP. Among the advantages of miRNAs as biomarkers is their high stability in blood (Table 1), which enables detection over a longer time period. Furthermore, in contrast with haematological variables, miRNAs are not affected by environmental factors, for example inadequate storage during transport of the blood samples [35]. Thus, use of miRNAs could reduce difficulties caused by the extensive documentation regarding transport and storage of samples. cDNA samples are also highly stable under different storage conditions [36].

Blood samples included in haematological modules of the ABP are analysed in WADA-accredited laboratories, where strict quality-control criteria are applied to provide analysis of forensic quality. Blood-variable analyses are performed by use of automated blood-cell counters, and haematological variables could be correlated with miRNA measurements. Circulating miRNAs could be analysed by use of qPCR. miRNA qPCR is a well-established, robust, and reproducible method with several important advantages, including high sensitivity and specificity, potential for target multiplexing, and low RNA input requirements, all of which facilitate expression analysis, even for anti-doping samples with a limited amount of material [28]. This method has already been implemented in many diagnostic laboratories, and routine procedures are well established. Quality control could easily be included at every step to monitor sample haemolysis and extraction, reverse transcription, and qPCR efficiency, and such controls could be used as normalisation factors [28]. Incorporation of quality controls eliminates within-subject variation, increasing the efficiency of the evaluation of biological data.

To validate and implement new biomarkers in the ABP, a rigorous procedure must be followed. For the haematological module of the ABP it has been possible to take advantage of experience gained over many years with biomarkers including haematocrit and haemoglobin. Schumacher et al. revealed that haemoglobin concentration has significant diurnal and exercise-related variation [37]. The same group observed that a percentage of reticulocytes is modified by both long-term and short-term exercise [38]. All these observations are already used for interpreting athlete blood-profile analyses. For circulating miRNA, few data exist regarding external and environmental factors that could interfere with miRNA measurements in blood. Nevertheless, Baggish et al. revealed that exercise can affect some types of circulating miRNA, including those involved in inflammation (miR-146). Interestingly, miRNAs involved in heart and skeletal muscle physiology (miR-133a) and adaptation to hypoxia and ischaemia (miR-210) did not vary in response to exercise [39].

Other confounding factors in the interpretation of miRNA measurements should be investigated. As an example, the effect of altitude and of use of hypoxic chambers on circulating miRNA levels should be studied, and the effect of the circadian cycle on miRNA measurements should be investigated. Unlike haemoglobin, circulating miRNAs were discovered in 2008. Therefore additional studies from different disciplines, for example diagnosis of diseases for which circulating miRNAs have been tested as blood-related biomarkers, should be used in the interpretation of longitudinal measurements of miRNAs in the context of the ABP.

Outlook

Many elements contribute to the enormous potential of circulating miRNAs as a class of ideal anti-doping biomarkers. Circulating miRNAs are very stable molecules, which are well preserved under harsh conditions and resistant to RNase activity. They are easily accessible, can be sampled in a relatively non-invasive manner, and can be readily measured by use of simple qPCR technology. Longitudinal measurement of circulating miRNAs in the context of the ABP should be a suitable way of using these new biomarkers in anti-doping. Circulating miRNA research is still in its early stages, and further work must be done to characterise the potential confounding factors affecting these biomarkers. As a result of the plethora of data supporting the clinical use of miRNAs as biomarkers for diseases, robust and reproducible tests for use in patient treatment decisions are currently being developed. The increasing clinical knowledge of these next-generation biomarkers could be easily extrapolated to anti-doping.

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