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Perspective

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Practical steps to develop a transcriptomic test for blood doping

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Abstract: Blood doping remains a significant problem that threatens the integrity of sport. The current indirect method for detecting blood doping involves the longitudinal measurement of an athlete's haematological variables and identification of suspicious results that are indicative of doping (i.e., the athlete biological passport). While this has played a significant role in the protection of clean sport, improvements are needed. The development of a transcriptomic test, that can be used to detect the use of blood doping has been discussed and researched for decades and yet, an anti-doping test that can be adopted by the World Anti-Doping Agency (WADA) is yet to be established. With recent advancements in our understanding, as well as in methods of sample collection, the possibility of a transcriptomic test that can be used by WADA, is imminent. There are, however, several practical considerations that must first be made, that will be highlighted in this perspective article.

Keywords: anti-doping; testing; blood doping; EPO; transcriptomics

Introduction

In endurance sports, blood doping has the potential to significantly improve endurance performance, primarily by increasing the oxygen carrying capacity of the users' blood [1]. The use of rHuEpo to enhance athletic performance was prohibited by the International Olympic Committee (IOC) in 1990, however its detection to this day remains a challenge. In 1997, a "no start" rule was introduced by the Union Cycliste Internationale (UCI), with athletes providing a blood sample prior to competition [2] and if their Haematocrit (HCT) exceeded the predetermined limit, they were not permitted to race. The Athlete Biological Passport (ABP) was later introduced by the UCI in 2008 [3] and soon after adopted by WADA. Since then, it has been a critical element of anti-doping testing programmes.

The ABP measures an athlete's haematological variables longitudinally, and with the use of Bayesian statistics, generates an individual upper and lower limit [3]. If an athlete's haematological variables exceed the calculated upper or lower limit, it suggests that there is only a 1:100 chance that this is due to "natural" physiological variance [4] and can be used as evidence of doping and result in the sanctioning of an athlete [5]. It has, however, been demonstrated that it is possible to use low doses of Recombinant human erythropoietin (rHuEpo) and avoid detection by the ABP [6], and despite improvements to the ABP [7], questions remain about its reliability as a method of detecting doping and its efficacy as a deterrent, on a global scale [8].

A transcriptomic method of detecting rHuEpo abuse was described in 2001 [9], a year after the first direct test for rHuEpo in urine was established [10]. Recent research into transcriptomics for anti-doping has focused on identifying specific transcriptomic markers that are associated with rHuEpo abuse [11, 12], confounding variables such as altitude exposure [13] and reproducibility of transcriptomic markers in differing sample collection matrixes such as Dried Blood Spot (DBS) [14].

Despite significant research, a transcriptomic test, that is either standalone, or an addition to the current ABP has yet to be established. There are several theoretical and practical hurdles that must be overcome prior to the adoption of a transcriptomic test as an anti-doping tool. The summary of this article is presented in Figure 1.

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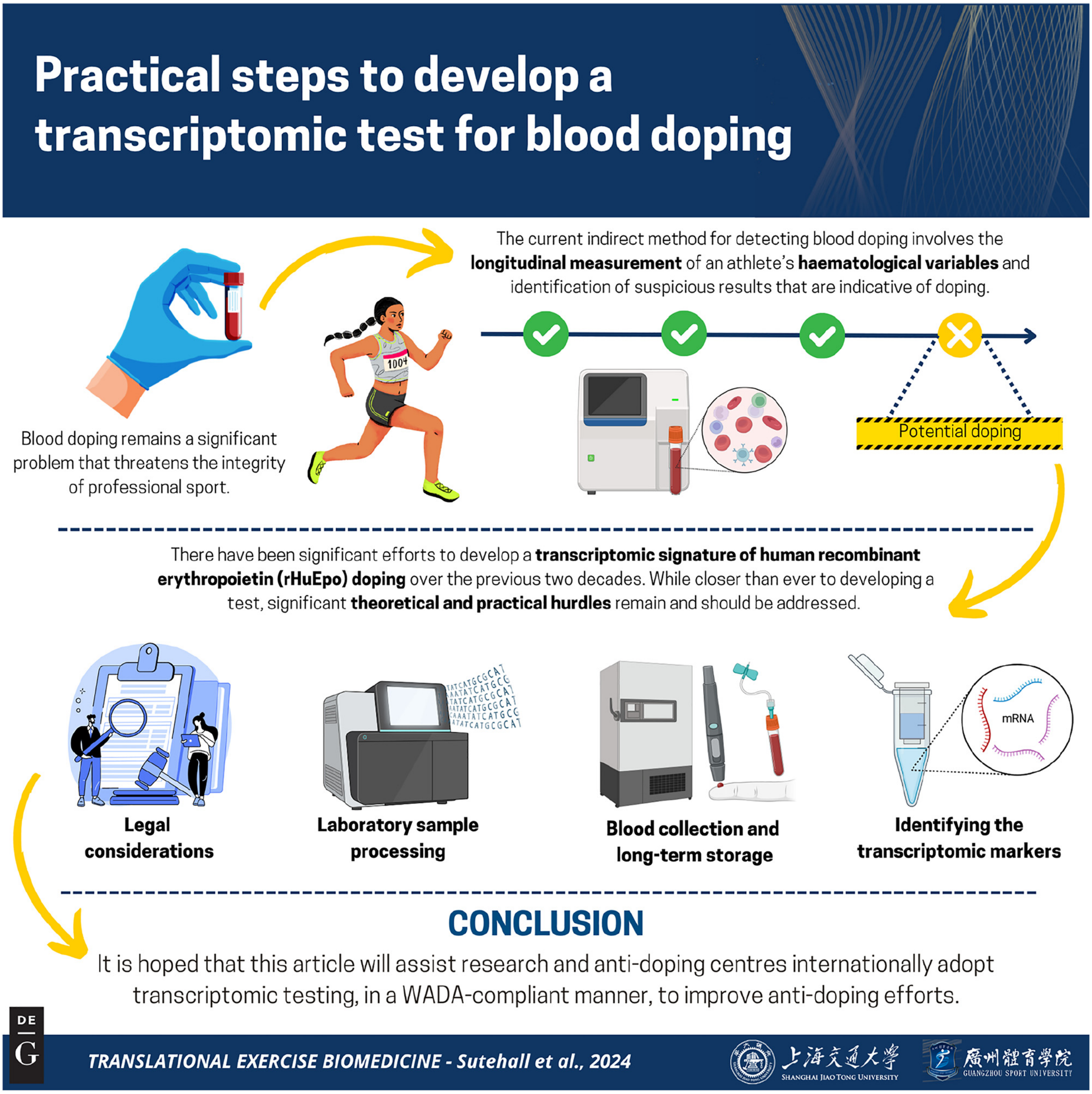

Figure 1: Graphical representation of this article. *Figure created with BioRender*.

Identifying transcriptomic markers

Numerous studies have characterised the transcriptomic response to blood doping in whole blood (e.g., Table 1, [15]) and DBS [16], along with conditions that may confound results, such as altitude exposure [13] and iron injections [17]. There is yet to be consensus on what markers should be used to indicate blood doping, or which confounding factors should be accounted for in an anti-doping test.

There is growing evidence for specific transcriptomic markers as candidates for a transcriptomic test. For example, the transcript 5′-Aminolevulinate Synthase 2 (ALAS2) has been shown to be differentially expressed following both high [11] and low [12] doses of rHuEpo in whole blood in addition to being identifiable following high doses of rHuEpo in DBS [18]. Although ALAS2 appears to also be differentially expressed to altitude exposure [13], which might reduce its sensitivity to detecting blood doping, a recent pilot study has demonstrated that the administration of rHuEpo at altitude, remains detectable, using this marker [19].

There is currently no consensus on how to integrate gene expression data into a test. Broadly, it appears that there are two likely methods. Firstly, the integration of transcriptomic biomarkers into the existing ABP, which show evidence that their differential gene expression indicates doping [20]. For this method, upper and lower limits should be determined and if exceeded, the data sent to a panel for review. Secondly, a standalone test, that uses numerous transcripts to create a signature of doping, which will require a regulatory approach more similar to a direct test of rHuEpo in urine [10]. It is not yet obvious which method will prove most useful to anti-doping efforts, with more research required once a more substantial number of suitable transcriptomic markers has been established.

Future developments in identifying transcriptomic markers

As Artificial Intelligence (AI) develops, it could be harnessed to review the substantial data outputs from Next generation sequencing (NGS) [21] and create a "signature" of blood doping, highlighting unique signatures over and above current methods of identifying transcriptomic markers and/or create a "profiling" approach as has been theorised [22]. The better the quality data used, the better the generative AI prediction. We would expect the prediction of doping to improve as the AI learns from doping cases. However, there are AI-specific challenges that should be addressed, including the requirement to have large training datasets to create robust algorithms. More importantly, the development of explainable AI algorithms is crucial, as these would allow researchers and regulators to understand the decision-making process of the AI, ensuring transparency and trust in its findings [23].

As transcriptomic markers are identified and validated, there exists an opportunity to develop hybrid testing models, combining the outputs of transcriptomic tests with outputs from other, complimentary tests to provide a more comprehensive understanding of an athlete's physiology. There are numerous methods of doping detection that could be used as part of a hybrid model of anti-doping testing, many of which have promising areas of development [24]. The development of hybrid model of anti-doping seems most likely to incorporate aspects of the "OMICS" cascade, incorporating information from genomic, transcriptomic, metabolomic and proteomic analysis [25]. A hybrid testing approach will take a considerable time to develop, as the influence of confounding factors and individual variation for each aspect of the test must be validated prior to incorporation into anti-doping testing. While the development of transcriptomic markers in whole blood and other sample matrixes is yielding potential new biomarkers, other matrixes should also be explored in the OMICS cascade. For example, investigation of metabolomics and proteomics in cell-free matrixes such as plasma and serum may provide another avenue for developing anti-doping biomarkers. Similarly, the investigation of spatial transcriptomics may yield additional biomarkers, however a significant amount of further research is required before it will be of practical use for anti-doping efforts.

Blood collection and long-term storage

In order to integrate a transcriptomic test within the current anti-doping system, methods of collection, transport and long-term storage should align closely with those described in the WADA International Standard for Testing and Investigations (ISTI) [26] and sample collection guidelines [27]. For example, there are a series of conditions/situations that an athlete should declare when a sample is collected (e.g., recent significant blood loss or exposure extreme environmental conditions), however, the effect of these on transcriptomic markers has not yet been established.

Studies that have collected samples for transcriptomic analysis have predominantly collected whole blood in Tempus™ Blood RNA tubes (Life Technologies, Carlsbad, CA, USA) which contain reagents to stabilise Ribonucleic acid (RNA). These are not currently approved for use by WADA, with K_2EDTA tubes used for the collection of blood for analysis of gene doping [27]. An initial study has demonstrated the stability of transcriptomic material using K_2EDTA collection tubes [28], suggesting integration with existing sample collection guidelines will be possible.

Recently, the use of DBS as a sample collection method was approved and adopted by WADA [26]. The use of DBS in anti-doping offers unique advantages beyond traditional venepuncture collection including greater convenience for athletes [29] and improved sample stability at room temperature [30]. Research has demonstrated that DBS is a suitable matrix for monitoring transcriptomic markers [14, 31].

If using an ABP-like strategy, a single A-sample, which can be discarded within a month should be sufficient for transcriptomic testing. However, to create a transcriptomic "signature" of blood doping, both an A- and B- sample would be necessary to be compliant with WADA's analytical testing procedures. Long-term storage would provide the opportunity to store blood for new analyses when new RNA biomarkers are discovered that better characterise doping.

Laboratory sample processing

WADA has established the standards related to the laboratory procedures for the analysis of anti-doping samples within the International Standard for Laboratories (ISL) [32] and any methods developed for the analysis of a transcriptomic markers should adhere to these.

As with all blood tests, a degree of processing is required once a sample arrives in the laboratory. While RNA extraction, for transcriptomic analysis is no longer considered a complicated process, there are several methodologies available and, to establish international harmonisation, a single method should be selected that is quick, easy to perform, cost-effective and reliable in "real-world" settings.

Following on from RNA extraction, the level of expression of a transcriptomic marker must be established. The most widely available method, Real Time Quantitative Polymerase Chain Reaction (RT-qPCR), was developed ~ 40 years ago [33] and its use was prolific during the COVID-19 pandemic [34]. There exist guidelines for laboratories to use RT-qPCR in WADA-accredited laboratories as a method of detecting gene doping [35]. While this method is based on replication of DNA, the methodological approach that is needed to measure transcripts, is largely similar. As with RNA extraction, the subsequent analysis would require international harmonisation and therefore, decisions related to the type of real-time chemistry (e.g. TaqMan vs. SYBR), reverse transcription method and assay design and quantitation, would need to be made. Particular attention should be paid to developing methods to minimise the variation that is often introduced into results of transcriptomic analysis (i.e. [36]), with methods harmonised across countries.

Other methods are available that could be used as part of a transcriptomic anti-doping test, such as short- and long-read sequencing [37] or microarray analysis [38]. The complexity of these technologies currently hinders their use in anti-doping laboratories, however continual advances in the simplification of such technologies, make this a possibility in the future. Similarly, advances in robotic technologies provide the possibility of automating a significant portion of the laboratory process. By removing the human interaction in the sample handling and analysis, rates of human errors and contamination that may affect the analysis should decrease. For example, an automated transcriptomics analysis method can be developed, from sample to report (Figure 2). The process begins with the collection of blood samples from test subjects, which are then transferred, using robotics, from tubes to 96-well plates. Once loaded, an automated nucleic acid extractor extracts total RNA from the blood samples. Following this, the samples undergo quality control checks and normalization procedures, ensuring the quality and quantity of RNA is suitable for sequencing. The extracted RNA is then enriched, library prepared, and further sample preparation completed before sequencing, all of which can also be completed by an automated sample preparation system. Once the samples are loaded into flow cells, which are in turn, loaded into the sequencer, sample analysis begins. The raw data outputs are then sent to a data centre that integrates a laboratory information management system, bioinformatics accelerator, and high-performance data storage. For transcriptomics analysis, a comprehensive gene expression report along with a report detailing Single nucleotide polymorphisms (SNPs) and insertions/deletions is created. To ensure a fully automated and unmanned operation, a robot transfers samples and consumables in and out of each automated instrument, with an automatic scheduling system managing and controlling the entire experimental process.

Using a pipeline illustrated in Figure 2, it is possible to run four flow-cells at one time, each containing 232 samples, resulting in 928 samples analysed in 4–5 days. The data from the analysis can then be integrated into an ABP-style system, or a more complex system that identifies "signatures" of doping.

Legal considerations

Strict international harmonisation in collection, storage and analysis will be necessary to legally defend a transcriptomic biomarker. When compared to the haematological biomarkers, the development of transcriptomic markers is in

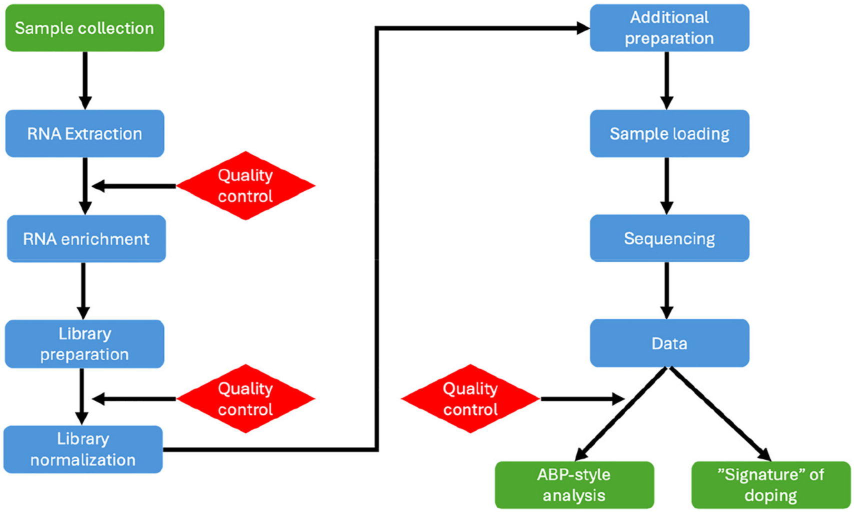

Figure 2: Automated high throughput sequencing pipeline, from sample to analysis.

relatively infancy. Therefore, there are no reference values available, which contrasts with the haematological markers used in the ABP, such as for haemoglobin which have been measured for over 100 years. Additionally, the confounding factors are not yet well known, but there is growing literature on this topic.

Currently, outliers in an athletes' ABP are evaluated by a group of experts. However, it would be challenging to find enough experts who are able to identify doping using transcriptomic markers. Further research is urgently needed to generate the knowledge required in the field. Integration of transcriptomic markers in routine testing could prove beneficial as the data generated could be used as additional evidence and simultaneously develop the necessary knowledge and experience over time. However, ethical and legal concerns related to the secondary use of samples for research would need to be addressed.

Conclusions

With the advancement of doping practices, more sophisticated methods of doping detection are required. The growing number of studies identifying transcriptomic markers characterising blood doping makes the development of an effective transcriptomic anti-doping test ever more likely. This perspective article has outlined some limitations of a transcriptomic anti-doping test and described the most pressing steps needed to develop and integrate it within an effective testing programme, following guidance issued within the ISTI and ISL. Further collaborative work is needed between sport scientists, bioinformaticians, anti-doping organisations and regulators to develop transcriptomic approaches to anti-doping and further investigate the integration of AI into routine testing.

Declarations

Opinions expressed in this publication are the author's own and do not necessarily represent their institutions. None of the authors have any conflicts of interest, other than those detailed within competing interest section.

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