BACKGROUND

Blood doping is known to improve performance in elite athletes. Despite being prohibited by World Anti-Doping Agency (WADA), blood manipulations such as the administration of recombinant human erythropoietin (rHuEPO) and blood transfusions are allegedly frequently adopted by athletes to enhance their physical/sporting performance. rHuEPO is structurally similar to endogenous erythropoietin, hence direct detection of the drug is challenging. Moreover, there remains no direct detection method for autologous blood transfusions (ABT) and this also represents a significant limitation of the current anti-doping system. Historically, direct methods have been applied to detect doping agents such as using analytical testing of blood and urine. Despite some success, direct approaches suffer from numerous serious shortcomings such as coping with the short detection window of substances, the timing of the sample collection and the involvement of sophisticated doping. This situation led the introduction of indirect methods aimed at detecting the physiological/biochemical changes caused by the action of the doping substances and methods and for a period after their respective direct detection window. In 2009, WADA introduced the Athlete Biological Passport (ABP) method, which has further undergone significant development in the years since. The ABP is a longitudinal and individual monitoring technique that tests chemical and physiological parameters in order to identify atypical variability of the response(s) in the athlete, highlighting a potential doping violation. More specifically, the ABP could identify intra-individual abnormal variability over time for selected haematological and steroidal parameters to detect blood and exogenous steroid manipulation, respectively. The haematological adaptive model of the ABP estimates the probability of blood
doping based on previous individual test history and heterogeneous factors known to influence blood parameters. The model takes into account heterogeneous factors such as gender, ethnicity, altitude exposure, age and sports discipline, allowing the removal of variance created by these factors. Although the implementation of the ABP has been an encouraging step forward in the quest for clean/drug-free sport, this detection method remains imperfect. According to testimonies, with the specific aim to reduce the risk of being detected by the ABP, some athletes are now resorting to microdoses of blood doping to restrain abnormal fluctuations in the haematological parameters used as doping biomarkers, thereby reducing the sensitivity of the ABP. Additionally, transfusing smaller volumes of blood (less than three bags) reduced the sensitivity of ABP. Consequently, there is an urgent need to include new biomarkers in the ABP to improve the detection of blood manipulations and increase the sensitivity to detect microdosing with rHuEPO.

Recent studies are strongly encouraging a paradigm shift in anti-doping involving molecular markers, where a subset of genes are activated or deactivated in response to specific doping substance and/or method.

**GENE EXPRESSION MICROARRAY ANALYSIS IN ANTI-DOPING**

The human genome contains approximately 21,000 genes and at any given time point, each of our cells has some combination of these genes turned on, while others are turned off. Using gene expression profiling, a snapshot of actively expressed genes under various conditions can be provided. Microarray is defined as a high-throughput technology in the field of genetic research, which is currently employed by various research groups to measure the expression levels of large numbers of genes simultaneously. More specifically, microarrays are chips containing thousands of spots on a glass surface. Each spot is comprised of multiple copies of identified DNA sequences and represents one gene. The DNA sequence of each spot is unique. The first step in a microarray experiment is to isolate and amplify the target RNA and increase its number. The amplified target is subsequently converted to DNA, fragmented and fluorescently labelled. The labelled solution is hybridised to the glass surface of the chip, where the target binds to the DNA of each spot. The chip is washed to eliminate non-specific binding and is eventually scanned. The fluorescent intensity of each
spot correlates to the relative amount of the RNA target. Potential changes in the patterns of gene expression can then be identified by comparing the relative amount of RNA expressed between any number of experimental conditions.

Microarray technology has broadened our knowledge about the biology of several diseases, including breast cancer, and could potentially unravel the gene expression profiling of blood doping. It has been hypothesised that as blood doping alters erythropoiesis and haematological parameters, it may cause variations at the transcriptomic level, leaving a characteristic signature in the human organism. Recent studies from numerous laboratories, including ongoing studies, are confirming the potential of a transcriptomic microarray approach, which can assess distinct changes in gene expression after blood manipulations, to enhance the ABP. For example, a pilot study by Pottgiesser and colleagues examined the transcriptional response of T-lymphocytes in six males after reinfusion of autologous red blood cells stored at 4°C for approximately 35 days. They found 728 transcripts significantly altered after 72 hours post-transfusion, leading to a conclusion that ABT triggered a specific immune response to the recipient.

Varlet-Marie et al used a serial analysis of gene expression method and identified 95 genes differentially expressed after administration of high and microdoses of rHuEPO in two athletes. Thirty-three marker genes for rHuEPO administration were identified during the high dose regimen and five remained differentially expressed with microdoses. A more recent study successfully reported the whole-blood transcriptional signature of rHuEPO in two independent cohorts consisted of endurance-trained Caucasian males at sea level in Scotland and Kenyan endurance runners at moderate altitude (~2000 m). Participants received 50 IU/kg body mass subcutaneous rHuEPO injections for 4 weeks. Blood was obtained 2 weeks before, during and 4 weeks after administration. Relative to baseline (2 weeks before rHuEPO injections), hundreds of genes were differentially expressed during rHuEPO administration and remained differentially expressed up to 4 weeks after administration. A distinct pattern of several genes was found to be upregulated during rHuEPO administration (n=41) and subsequently downregulated up to 4 weeks after administration (n=10) in the Scottish cohort. This subset of genes was also reported after application of whole-transcriptome analysis in the Kenyan cohort. After validation of the results with a different platform, the authors confirmed a whole-blood 34-transcript signature of rHuEPO administration. This study provided the strongest evidence to date that genetic biomarkers have the potential to support the hypothesis that gene expression profiling may provide a sensitive method for the detection of rHuEPO and possibly blood doping.

### GENE EXPRESSION MICROARRAY ANALYSIS OF AUTOLOGOUS BLOOD TRANSFUSION

The encouraging gene expression microarray results from previous blood doping studies posed the question whether similar discrimination is possible when a microarray approach is applied to identify a transcriptomic signature of ABT. In order to investigate this, a study was designed to test the application of whole transcriptome analysis in the detection of ABT. Fifteen healthy Caucasian males (20 to 35 years, body mass index ≤30) were recruited and participated in a control phase followed by an ABT intervention. Participants received a saline injection for the control phase and then donated one full bag of blood (500 ml) 14 days later. After processing, the volume of red blood cells was approximately 280 ml, which was stored at 4°C for 36 days before reinfusion to the donor. For the autologous transfusion phase, whole-blood samples were collected in Tempus Blood RNA tubes from Life Technologies (Thermo Fisher Scientific) at baseline (4 and 1 days before blood reinfusion) and after blood reinfusion at 3 hours, 6 hours, 12 hours, 1 day, 2 days, 3 days, 6 days, 9 days and 15
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days, respectively\(^6\). For the control phase, whole blood samples were collected at the same time points, except for 15 days\(^6\). RNA was extracted from the whole blood and stored at -80°C until further analysis. To investigate the gene expression signature of ABT, a microarray analysis was employed using the Affymetrix GeneChip® Human Transcriptome Array 2.0. Briefly, 100 ng/ul of total RNA was profiled using the Affymetrix® GeneChip® WT PLUS Reagent Kit following the manufacturer’s instructions. The RNA was first reverse transcribed into double-stranded cDNA (complimentary DNA). This cDNA was transcribed in vitro to generate cRNA (complimentary RNA). Single-stranded cDNA (ss-cDNA) was synthesised by the reverse transcription of cRNA. After purification of the ss-cDNA, 176 ng/ul were fragmented and labelled prior to hybridisation to the Human Transcriptome Array 2.0. Next, the arrays were washed, stained and scanned. A transcriptome analysis software was used for the visualisation of expression changes at the gene level and statistical analysis.

While this research continues, preliminary results presented at the 2016 FIMS World Congress, in Ljubljana, Slovenia, are summarised here. Prior to multiple testing correction, transcriptional profiling revealed that hundreds of transcripts were altered by ABT. Compared to baseline, the main expression patterns of these genes are an upregulation during the first 3 days after reinfusion and a downregulation at day 6, 9 and 15 days after reinfusion. The range of fold changes in gene expression varied from 1.5 to 2.3 across time points. Genes differentially expressed during the first 3 days after ABT are linked with iron homeostasis, anti-inflammatory activity and host defence pathways and genes expressed at 6 and 9 days after blood reinfusion are related to haeme biosynthesis, chemokine signalling pathway and immune response. The downregulation of genes linked to haeme biosynthesis could be related to the alteration of erythropoiesis confirmed by previous studies\(^5,6\). In addition, reticulocyte percentage was significantly decreased 3, 6, 9 and 15 days after blood reinfusion compared to baseline, in line with the observed gene expression changes. Although preliminary, these results are encouraging and would appear to support the detection of ABT using whole genome expression.

A potential confounder that could limit the application of a gene expression solution to the detection of ABT is the effect of prior exercise training. It has previously been shown that exercise significantly influences gene expression profiles of peripheral blood mononuclear cells and white blood cells\(^13,14\). Therefore, it is essential to define the molecular effects of exercise in order to provide a set of robust candidate genes that can be used for the detection of ABT. This is being achieved through an international collaboration with the Gene SMART (Skeletal Muscle Adaptive Response to Training) study. The Gene SMART study aims to identify the gene variants that predict the skeletal muscle response to both a single bout and 4 weeks of high-intensity interval training in two different training centres, one in Victoria University, Melbourne and another at Bond University, Gold Coast, Australia. Using transcriptional microarray analysis, whole blood samples are being processed before and immediately following 3 hours high-intensity exercise, and following 4 weeks of high-intensity interval training in healthy physically active Caucasian males\(^9\). To date only a relatively small number of subjects have completed the study (n=22), nevertheless, the robust gene expression profiles identified are restored after 30 minutes thereby arguing against exercise/training being a significant confounder and limiting the application of a gene expression solution to the detection of ABT.
CONCLUSION

In summary, the ultimate goal in the fight against doping is to protect the clean athlete and prevent any method of cheating. WADA has introduced the ABP method to detect any suspicious forms of blood doping, but it remains imperfect. Rapid progress in omics applications has promoted the investigation of changes in gene expression due to blood manipulations. Recent studies provided evidence that a transcriptomic approach may have the potential to significantly improve current anti-doping strategies. However, future studies are needed to evaluate the effects of major confounders – such as the effects of altitude exposure, gender or exercise – on the discovered transcriptomic signature of ABT. Once the transcriptomic signature of ABT is validated and replicated, the proteomic (proteins) and metabolomic (metabolites) signature of ABT should be determined. These omics approaches could introduce a distinguishing signature of ABT independently or in combination with the ABP approach.

References