

“Atypical hemoglobin profile and erythroid-related miRNAs expressed following autologous blood transfusion: isolation of markers insensitive to physiological changes”

Fabio Manfredini, Roberto Gambari, Alessia Finotti, Monica Borgatti, Ilaria Lampronti, Lamberti Nicola, Gasparello Jessica Maurizio Govoni, Nicola Lamberti, Jessica Gasparello, Roberto Reverberi,
(Universita degli Studi di Ferrara)

Project Overview

In a funded WADA study recently completed (“Novel molecular biomarkers for detection of autologous blood transfusion in sport: fetal hemoglobin and microRNAs”) we tested the hypothesis that novel biomarkers as fetal hemoglobin (HbF) and related microRNAs might show changes of interest in a group of trained healthy volunteers exposed to ABT respect to controls. The first result of this study is the production of a validated WADA-UNIFE-Biobank constituted of around 2000 plasma samples from control and ABT trained subjects. It is established that hypoxic and hyperoxic stimuli and blood manipulation during procedures of withdrawal and reinfusion (distress, ageing, apoptosis/degradation of circulating blood cells, effects of preservative substances, etc.) might induce a predictable and an unpredictable series of changes of miRNAs expression. Therefore, we hypothesized that after significant blood collection and autologous reinfusion, the miRNA network in the athlete’s plasma is changed, allowing to generate integrated molecular profiles permitting to predict ABT. Therefore, the hypothesis is that a miRNA pathway might be much more informative than a single miRNA, even if demonstrated to be associated with ABT in a sub-set of athletes. To this end, a small sample of subjects enrolled for the cited study was recently spontaneously tested in our laboratory for a full genome analysis. Therefore, the aim of this study is the validation of the first release of ABT-miRNA list and the identification of novel miRNAs of putative interest in predicting ABT following global miRNA analyses.

Results and Conclusions:

This project was aimed at identifying novel molecular markers useful in the anti-doping field to detect autologous blood transfusion (ABT). The hypothesis was that both the phases of ABT, blood withdrawal and reinfusion, might be accompanied by changes in different parameters to be identified by OMICS approaches in the level of microRNAs (miRNAs), small non-coding RNAs that regulate gene expression. In a previous funded study “Novel molecular biomarkers for detection of autologous blood transfusion in sport: fetal hemoglobin and microRNAs” twenty-four healthy trained male subjects were enrolled and randomized into Transfusion (T) and a Control (C) groups. The T subjects underwent nine seriated blood samples before and after the procedures of withdrawal and reinfusion. Among erythroid-related miRNAs tested, following ABT a pool of 7 miRNAs associated with fetal hemoglobin and regulating transcriptional repressors of gamma-globin gene was found

stable in C and differently expressed in three out of six T subjects at 12 days time-point after re-infusion.

In the present study, using samples stored in the biobank, we aimed to validate the first release of ABT-miRNA list at other time-points (i.e. D-25 and D+3) to confirm the interest of this miRNA list in predicting ABT. ABT prediction was also demonstrated after global miRNA analyses, thus identifying novel ABT-informative miRNAs. The levels of selected informative microRNAs in relation to sex was determined and protocols/assays for ABT detection were developed. of specific interest. To this aim, microarray analysis and droplet digital RT-ddPCR were also performed for subjects of the T group and C groups for selected time-points.

The data obtained studying the global miRNome pattern demonstrated clustering of ABT-treated plasma samples at D+3 and D+15. These results support the concept that identification of ABT can be performed in one-step using the developed microarrays procedure for miRNA global analysis. The blood withdrawal was not found to be a "confounding factor". Secondly, the data obtained by RT-ddPCR performed in plasma samples from ABT treated subjects at D-25, D+3 and D+15 time points were first aimed at validation the "erythroid-associated miRNA list" (let-7a-5p, miR-126-3p, miR-144-3p, miR-191-3p, miR-197-3p, miR-486-3p, miR-486-5p and miR-92a-3p) representing ABT associated miRNAs involved in hypoxia, erythroid differentiation and fetal hemoglobin production. This set of 8 erythroid associated miRNAs were informative in detecting ABT in most (5/6) of the ABT-treated subjects at all the time-points considered. We then considered miRNA found specifically dysregulated at D-25, D+3 and D+15 time points according with the miRNA microarray analyses. This additional list was proposed in consideration of the miRNA fold changes, the availability of the assays for miRNA amplification and the necessity to have both up- and down-regulated miRNAs to test as possible ABT-associated biomarkers. A final list of six miRNAs was identified and selected for further experimental activity. While the data obtained for down-regulated miRNAs were not informative (no major differences with the control set), differential expression of miR-766-3p (D-25), miR-636 (D-25), miR-425-3p (D+3), miR-4284 (D+3), miR-3151-3p (D+15) and miR-5787 (D+15) was found. When the two miRNA lists (erythroid and global) were used, only one ABT subject was not identified as abnormal or potentially positive for ABT (score 11/12 = 92%). Despite a high heterogeneity within the male and female groups, minimal sex-biased miRNAs differences were observed. Interestingly several miRNAs found up-regulated at the D-25 time point, which is 10 days after the blood withdrawal, may represent "follow-me" markers to identify athletes at risk of ABT to be tested in the proximity of major agonistic events. The results of the present project allow to validate a list of 14 miRNA useful, by RT-ddPCR based analysis, to detect ABT in the majority (over 90%) of the ABT-treated subjects.