

## RESEARCH ARTICLE

# On the trail of blood doping—microRNA fingerprints to monitor autologous blood transfusions in vivo

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## Funding information

World Anti-Doping Agency, Grant/Award Number: ISF15E09MP; Technische Universitat Munchen

## Abstract

Autologous blood doping refers to the illegal re-transfusion of any quantities of blood or blood components with blood donor and recipient being the same person. The re-transfusion of stored erythrocyte concentrates is particularly attractive to high-performance athletes as this practice improves their oxygen capacity excessively. However, there is still no reliable detection method available. Analyzing circulating microRNA profiles of human subjects that underwent monitored autologous blood transfusions seems to be a highly promising approach to develop novel biomarkers for autologous blood doping. In this exploratory study, we randomly divided 30 healthy males into two different treatment groups and one control group and sampled whole blood at several time points at baseline, after whole blood donation and after transfusion of erythrocyte concentrates. Hematological variables were recorded and analyzed following the adaptive model of the Athlete Biological Passport. microRNA profiles were examined by small RNA sequencing and comprehensive multivariate data analyses, revealing microRNA fingerprints that reflect the sampling time point and transfusion volume. Neither individual microRNAs nor a signature of transfusion-dependent microRNAs reached superior sensitivity at 100% specificity compared to the Athlete Biological Passport ( $\leq 11\%$  6 h after transfusion versus  $\leq 44\%$  2 days after transfusion). However, the window of autologous blood doping detection was different. Due to the heterogenous nature of doping, with athletes frequently combining multiple medications in order to both gain a competitive advantage and interfere with known testing methods, the true applicability of the molecular signature remains to be validated in real anti-doping testings.

**Abbreviations:** ABP, Athlete Biological Passport; ABPS, Abnormal Blood Profile Score; ABT, autologous blood transfusion; ANOVA, analysis of variance; AUROC, area under the curve of receiver operating characteristics curve; BMI, body mass index; CI, confidence interval; CV, coefficient of variation; DGE, differential gene expression; EC, erythrocyte concentrate; EPO, erythropoietin; FC, fold change; HCT, hematocrit; HGB, hemoglobin; LMU, Ludwig Maximilians-University; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; miRNA, microRNA; mRNA, messenger RNA; OFFS, OFF-hr Score; PCA, principal component analysis; PLT, platelets; RBC, red blood cell count; RDW-CV, red cell distribution width (coefficient of variation); RET %, reticulocyte percentage; RIN, RNA integrity number; rRNA, ribosomal RNA; RT, room temperature; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; sPLS-DA, sparse Partial Least Square Discriminant Analysis; tRNA, transfer RNA; WADA, World Anti-Doping Agency; WBC, white blood cells.

## 1 | INTRODUCTION

Growth-promoting substances and performance-enhancing methods are prohibited in elite sports.<sup>1</sup> Due to missing detection, however, blood transfusions are misused by athletes to improve their oxygen transport capacity and muscle extraction by up to 15%–25%, as it was shown after the re-transfusion of stored erythrocyte concentrates (ECs) originating from two standard whole blood donations.<sup>2</sup> To date,

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homologous blood transfusions, in which blood donor and recipient are different persons, can be detected by flow cytometry differentiating red blood cell antigens distinct from the recipient's antigen proportion.<sup>3</sup> In contrast, directly tracing autologous blood transfusions (ABT), where donor and recipient are identical, is still not yet feasible, albeit ongoing and promising research projects focusing, inter alia, on plasticizer residues, "ironomics", or new biospecimens, such as dried blood spots.<sup>4-7</sup>

An initial attempt to screen for ABT was therefore introduced by way of the Athlete Biological Passport (ABP), which was approved by the World Anti-Doping Agency (WADA) in 2009.<sup>8-10</sup> Especially the hematological data longitudinally recorded therein is currently the gold standard methodology to capture non-physiological changes. The application of the ABP might seem to be effective in general doping detection, as depicted by more than 1300 Anti-Doping Rule Violations that emerged from adverse analytical findings.<sup>11,12</sup> However, a large number of undetected ABT cases, which could only be disclosed by a razzia at the executing physician ("Operation Aderlass" in 2019, Austria<sup>13</sup>), is just one example that points out limits of the ABP in revealing ABT. This emphasizes the urgent need to establish a comprehensive and legally valid biomarker signature to detect ABT in competitive athletes.

New hope to combat blood doping is based on indirect detection methods such as metabolomic, proteomic or transcriptomic analyses.<sup>14-16</sup> In this regard, especially microRNAs (miRNAs) emerged as extremely promising biomarker candidates due to their exceptional stability, highly conserved regions, and great regulatory impact on the majority of protein-coding genes (> 60%) on the post-transcriptional level.<sup>17-20</sup> Considering that some specific miRNAs also play key roles in hematopoiesis, and that erythrocytes are enriched with a highly diverse set of miRNAs, which is mirrored by the whole blood miRNA profile,<sup>21-24</sup> the utility of miRNAs to indirectly detect ABT seems auspicious and needs further investigation.

Moreover, our previous studies on the misuse of growth-promoting drugs already demonstrated the potential of miRNAs as sensitive and reliable biomarkers.<sup>25,26</sup> Likewise, the illicit use of drugs such as erythropoiesis-stimulating agents can be revealed via circulating miRNAs in plasma.<sup>27</sup> Further, the most decisive research on the promising biomarker potential of miRNAs in blood doping detection has been performed by Haberberger et al.<sup>28</sup> and Kannan et al.,<sup>29</sup> whose comprehensive analyses of the miRNA profile in stored ECs revealed highly significant expression changes.

The present study therefore aimed at monitoring ABT by screening (a) ABP markers implemented in the hematological module and (b) whole blood miRNA profiles after autologous re-transfusion of stored ECs in a cohort of 30 healthy males. Via an explorative untargeted approach using extensive bioinformatic analyses of small RNA sequencing data, we set out to identify an ABT-induced miRNA pattern that might be used to predict suspicious samples more precisely in the future.

## 2 | METHODS

### 2.1 | Study subjects and design

The present study was approved by the local ethics committee at the Ludwig Maximilians-University (LMU) of Munich, Germany, under the

protocol #359-14 and conducted in consistent compliance with the "Declaration of Helsinki"<sup>30</sup> and the "Belmont report".<sup>31</sup> In total, 30 healthy males who self-reported to engage in regular recreational activity and were not subjected to WADA or National Anti-Doping Agency Germany regulations were recruited (see Table S1 for detailed inclusion and exclusion criteria). After being briefed by medical doctors and transfusion specialists, written informed consent including the permission to publish was obtained.

The study design was based on a longitudinal randomized controlled trial. All individuals were randomly divided into three equal groups using the software "RandList", version 1.2 (DatInf, Germany): the first group with one standard whole blood donation (~ 500 mL) and EC re-transfusion (group 1), the second group with two regular donations for parallel re-transfusion of ECs (group 2), and a control group without any blood donation or transfusion (group 3) (Figure 1A). Refer to supplemental methods for power analysis as well as for the preparation and re-transfusion protocol of erythrocyte concentrates.

### 2.2 | Sample collection

The WADA guidelines were strictly followed for the collection of blood samples.<sup>32</sup> In this respect, blood samples for immediate analysis of hematological markers were taken via venipuncture, that is, *Vena mediana cubiti*, and collected into serum-monovettes or EDTA-monovettes (Sarstedt, Germany). For miRNA analysis, 2.5 mL whole blood was sampled into PAXgene Blood RNA tubes (PreAnalytix, Switzerland) according to the manufacturer's guidelines. After an over-night incubation at room temperature (RT), whole blood samples were stored at -20°C until further processing. In total, 17.1 mL of venous blood were sampled at each time point, corresponding to a total blood volume of 188.1 mL over 11 sampling time points.

### 2.3 | Measurement of hematological parameters

The following laboratory parameters that correspond to the hematological module of the ABP were investigated: the primary markers OFF-hr Score (OFFS) and hemoglobin (HGB; g/dL), the secondary markers Abnormal Blood Profile Score (ABPS) and reticulocyte percentage (RET%; %), moreover, hematocrit (HCT; %), mean corpuscular hemoglobin (MCH; pg), mean corpuscular hemoglobin concentration (MCHC; g/dL), mean corpuscular volume (MCV; fL), platelets (PLT;  $10^3/\mu\text{L}$ ), red blood cell/erythrocyte count (RBC;  $10^6/\mu\text{L}$ ), and white blood cells (WBC;  $10^3/\mu\text{L}$ ). In addition, red cell distribution width (coefficient of variation) (RDW-CV; %), erythropoietin (EPO; U/L), iron ( $\mu\text{g}/\text{dL}$ ), ferritin (ng/mL), transferrin (g/L), transferrin saturation (%), and transferrin receptor (mg/L) levels were examined to complement an individual's longitudinal blood profile. For the computation of OFFS and ABPS, as established by Gore et al.<sup>33</sup> and Sottas et al.,<sup>34</sup> respectively, the statistical environment of R, version 3.6.0, with the software package ABPS was utilized.<sup>35,36</sup> Routine clinical assays were performed by the department of laboratory medicine at the LMU Hospital Großhadern in Munich, Germany, and the

medical care center "Labor Dr. Limbach und Kollegen" in Heidelberg, Germany, according to highest quality standards. An extensive list of all used measurement instruments and blood compartments can be

accessed in Table S2. Contrary to the WADA guidelines,<sup>37</sup> hematological variables were measured only once as this is ordinary clinical practice.

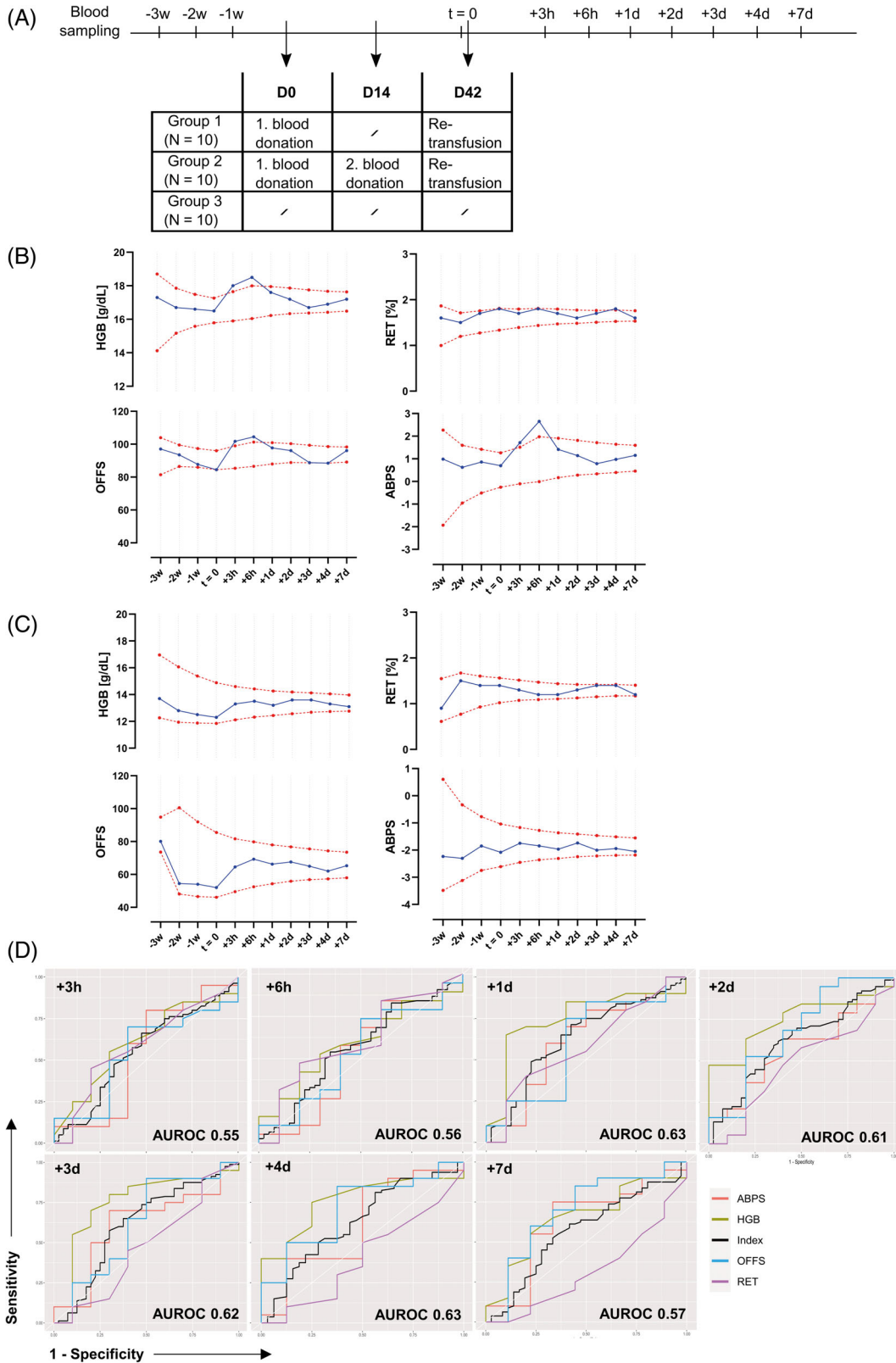


FIGURE 1 Legend on next page

## 2.4 | Evaluation of hematological data

Markers of the ABP hematological module were inspected using GraphPad Prism Software for Windows (version 8.0.1), La Jolla California USA, [www.graphpad.com](http://www.graphpad.com). Analysis of variance (ANOVA) for repeated measures, or mixed-effects model in case of missing values, with Benjamini-Hochberg's correction for multiple testing was conducted setting the level of statistical significance at adjusted  $P$  values  $<0.05$ . Coefficients of variations (CVs) were calculated based on baseline measurements ( $-3$  weeks,  $-2$  weeks,  $-1$  weeks) to assess inter-individual and intra-individual variability.

Hematological markers were further evaluated on the per-individual level based on an adaptive model as stated in the WADA technical document—TD2019BAR of the ABP operating guidelines,<sup>37</sup> which is considered the current gold standard. See supplementary methods for the detailed procedure. The classification performance of the main hematological markers was assessed based on sensitivity and specificity measures as well as the area under the curve of receiver operating characteristics curves (AUROC) using R, version 3.6.0, with the plotROC package.<sup>36</sup> For better comparability, an index was computed based on the primary and secondary ABP markers after centering and scaling for standardized units with the scale function.

## 2.5 | Total RNA isolation and small RNA sequencing

Total RNA was isolated from whole blood samples using the PAXgene Blood miRNA Kit (PreAnalytiX) according to the manufacturer's instructions. After RNA quality control, the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England BioLabs, USA) was applied according to the manufacturer's protocol using 200 ng of total RNA as input including a size selection step and library quality control prior to small RNA sequencing via 50 cycles of single-end sequencing on the HiSeq 2500 (Illumina, USA) with the HiSeq Rapid SBS Kit v2 (Illumina). Refer to supplementary methods for details.

## 2.6 | Sequencing data processing

Obtained small RNA sequencing data was pre-processed according to an in-house pipeline developed in R, version 3.6.0.<sup>36,38,39</sup> In brief, this pipeline included the following steps: quality control, adapter trimming, and mapping to human miRNA precursor sequences of the most recent miRBase (release 22).<sup>40</sup> Next, read count tables were generated and normalized in the statistical environment R, version 3.6.0<sup>36</sup> using the Bioconductor (version 3.9) package DESeq2 (version 1.24.0).<sup>41,42</sup> After removing lowly expressed miRNAs that are likely to be artifacts or not accurate in their expression,<sup>43,44</sup> obtained read counts were adjusted for known batch effects. See supplementary methods for more information.

## 2.7 | Biostatistical analyses of sequencing data

Differential gene expression (DGE) analysis was performed via DESeq2 to examine expression differences of individual miRNAs based on Wald test statistics.<sup>41</sup> Resulting  $P$  values were adjusted for multiple testing by Benjamini-Hochberg correction.<sup>45</sup> A value of  $\log_2$  fold change ( $|\log_2FC| \geq 0.585$ , which corresponds to  $|FC| \geq 1.5$ , and an adjusted  $P$  value  $\leq 0.05$  were set as filtering criteria. Overlaps in significantly differentially regulated miRNAs were pictured by Venn diagrams.<sup>46</sup>

To further investigate the entire batch-corrected sequencing data for an ABT-dependent miRNA expression pattern, sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was conducted using the mixOmics package<sup>47</sup> with default settings to select the most predictive features in a supervised classification allowing for a maximum of three features per component. To reduce overfitting, five-fold cross-validation was performed. Classification based on maximum distance was found to feature the lowest overall error rates at a maximum of four components and, thus, was retained for the final sPLS-DA models. Overlaps in discriminative miRNAs were visualized in UpSet plots using the intersect mode.<sup>48</sup> The discriminative abilities of the applied bioinformatic investigations (DGE and sPLS-DA) were assessed for every sampling time point individually, as multiple samplings of individual athletes within 1 week after a competition seemed

**FIGURE 1** Study design and Athlete Biological Passport (ABP) classification performance between autologous blood transfusion (ABT)-doped and non-doped subjects. (A), Study design. Baseline levels of hematological parameters as well as whole blood miRNA profiles were monitored via weekly sample collection for three consecutive weeks prior to the initial donation ( $-3$  weeks,  $-2$  weeks,  $-1$  weeks). In groups 1 and 2, the first whole blood bag was donated in the subsequent week (D0). A second whole blood bag was donated 14 days later exclusively by participants in group 2 (D14). The blood bags were instantly processed into erythrocyte concentrates and autologously re-transfused after 42 days of storage (D42), or 28 days, respectively. In order to observe transfusion-induced changes, blood samples for analysis of miRNA and hematological profiles were also taken shortly before ( $t = 0$ ), and at the third and sixth hour after transfusion ( $+3$  h,  $+6$  h) as well as on several days during the following week ( $+1$  day,  $+2$  days,  $+3$  days,  $+4$  days,  $+7$  days). (B), Application of the adaptive model implemented in the ABP for a study subject with a truly positive result (P33, group 1) and (C), a study subject with a falsely negative result (P18, group 1). The red lines indicate the individual's physiological range as determined by the 99% confidence interval, while the measured values are depicted in blue. Underlying raw data is available in Supplementary Data S1. See Figure S3 for further examples. (D), Classification performance of the main hematological ABP markers for every time point after ABT. The index calculated from them and its corresponding area under the receiver operating characteristics curve (AUROC) are summarized in black;  $n = 325$ . OFF-hr Score (OFFS), hemoglobin (HGB), Abnormal Blood Profile Score (ABPS), reticulocyte percentage (RET%)

unrealistic. In this regard, the most promising discriminative miRNAs were selected based on the largest congruency between group 1 and 2 to assure the use of the most robust set of miRNAs independent of the volume and time point of EC re-transfusion. To adhere to the WADA's ABP operating guidelines,<sup>37</sup> the 99% confidence interval (CI) of an individual's physiological range was generated from the selected discriminative miRNAs in form of ellipses in the two-dimensional environment of principal component analyses (PCAs), with every value outside of the ellipses being handled as a suspicious event. Overall classification performances of the most promising discriminative miRNAs were assessed the same way as for ABP hematological markers by using sensitivity and specificity measures as well as AUROCs, allowing for a direct comparison of the diagnostic potential of ABP vs miRNA pattern vs ABP index + miRNA pattern.

### 3 | RESULTS

In the present study (Figure 1A), 30 male volunteers were recruited (age:  $27 \pm 4$  years, height:  $182 \pm 5$  cm, weight:  $79.9 \pm 6.6$  kg, BMI:  $24.0 \pm 1.9$  kg/m<sup>2</sup>, weekly physical activity:  $5.4 \pm 2.0$  h) and randomly divided into three groups as determined by prior power analysis estimating a type II (beta) error of 0.05 and a power of 95%. The treatment groups (group 1 and 2) donated whole blood with an average weight of 524.9 g ( $\pm 5.7$  g) and an average HCT percentage of 43.8% ( $\pm 3.1\%$ ). After processing into ECs, an average weight of 254.5 g ( $\pm 13.2$  g) accounting for around 232.2 mL ( $\pm 12.0$  mL) was obtained. The blood withdrawal and transfusion procedures were very well tolerated by all study subjects. In total, 325 of 330 possible samples were collected and subjected to hematological and miRNA analysis.

#### 3.1 | Longitudinal monitoring of hematological ABP markers shows a reasonable ABT detection rate

Mean blood levels of MCV, MCH, MCHC, PLT, WBC, RET%, RDW-CV, and transferrin remained largely unchanged with small but significant changes for RET%, MCH, WBC, RDW-CV, and transferrin (Table 1). Large and highly significant ABT-dependent effects were observed for OFFS, HGB, ABPS, HCT, RBC, EPO, iron, ferritin, transferrin saturation, and transferrin receptor levels (Table 1). While there was also a donation-dependent effect especially in group 2 for OFFS, HGB, ABPS, HCT, RBC, EPO, ferritin, and transferrin receptor levels, the enormous increase in iron and transferrin saturation levels 3 and 6 h post-ABT appeared to be related only to ABT and not to the donation itself (Table 1). Individual longitudinal profiles of hematological markers depicted varying baseline ranges and intensities by which the subjects responded to blood withdrawal and re-transfusion (Figure S1). Moreover, inter-individual and intra-individual variability was only lower than 10% for RBC, HCT, HGB, MCH, MCHC, MCV, RDW-CV, while it was lower than 30% for transferrin, PLT, transferrin receptor, OFFS, RET%, and WBC. For EPO, ferritin, iron, and transferrin saturation, CVs lower than 70% were calculated (Figure S2).

According to the WADA's ABP operating guidelines,<sup>37</sup> the adaptive model was applied to screen for outlier values exceeding an individual's critical range of the main hematological markers of the ABP (OFFS, HGB, RET%, ABPS) as exemplified for two participants (Figure 1B,C). While study subject P33 (group 1) was correctly identified to be truly ABT-doped by outlier values crossing the upper individual's threshold 3 and 6 h post-ABT (Figure 1B), study subject P18 (group 1), who had also undergone ABT, was falsely attributed to the control group, as all measured values appeared within the critical ranges (Figure 1C). After evaluating all study subjects, the highest sensitivities at 100% specificity were obtained one and 2 days after ABT in group 2 with 30% and 44%, respectively (Table S3, Figure S3), with only 10% in group 1. The ongoing impact of blood donation at  $t = 0$  was ascertained with sensitivities of 40% (group 1) and 60% (group 2). Overall classification performance of the main hematological markers after ABT was assessed by AUROCs per time point with highest rankings of categorization at time points 1–4 days after ABT, at which especially HGB provided superior classification (AUROC: 0.75–0.76) compared with other markers or an index thereof (Figure 1D).

#### 3.2 | Whole blood samples contain high-quality RNA with abundant miRNA

Consistent quality control steps during sample processing are fundamental requirements for reliable biomarker research.<sup>49</sup> Here, the purity of isolated RNA was spectrophotometrically confirmed via the indicative OD ratios:  $OD_{260\text{ nm}}/OD_{280\text{ nm}}$  2.1 ( $\pm 0.1$ ) and  $OD_{260\text{ nm}}/OD_{230\text{ nm}}$  1.3 ( $\pm 0.5$ ). Moreover, the average total RNA yield across all samples was 4.1  $\mu\text{g}$  ( $\pm 1.6$   $\mu\text{g}$ ). Further, excellent RNA integrity could be confirmed based on Bioanalyzer 2100-derived RNA integrity number (RIN) values (RIN:  $8.5 \pm 0.5$ ), providing optimal conditions for any quantitative downstream experiments.

On this basis, library preparation for small RNA sequencing was conducted next. By controlling for appropriate size selection in all eight library preparation batches, perfectly matching and reproducible size distributions with miRNA-specific cDNA library peaks at lengths of 147–149 base pairs could be verified (Figure S4A). Quality control of sequencing performance indicated an exceptionally high base call accuracy and per-base sequence quality with mean Phred scores consistently above 38.<sup>50</sup> Total library sizes of  $5.8 \pm 2.5 \times 10^6$  reads were obtained with length distributions peaking in the typical miRNA-specific size range of 21–23 nucleotides (Figure 2A). Moreover, an average of  $78 \pm 34\%$  of reads accounting for  $4.5 \pm 2.0 \times 10^6$  reads mapped to human miRNA precursor sequences (Figure 2B), spread across 327 distinct canonical miRNAs. Relative mean mapping distributions per study subject can be examined in Figure S4B.

#### 3.3 | ABT-dependent miRNA expression changes are highly diverse

The DESeq2-normalized sequencing data of the control group (group 3) was analyzed for differentially expressed genes to first elucidate

**TABLE 1** Hematological levels at baseline as well as before and after blood donation and re-transfusion

Marker	Baseline			After donation		After re-transfusion						
	-3 weeks	-2 weeks	-1 week	t = 0	+3 h	+6 h	+1 day	+2 days	+3 days	+4 days	+7 days	
OFFS	G	88.0 ± 16.3	84.9 ± 16.9	81.9 ± 16.0	76.3 ± 13.8*	84.5 ± 14.1	85.3 ± 13.9	85.8 ± 13.3†	85.8 ± 13.4†	82.2 ± 12.3	80.2 ± 13.9	83.1 ± 13.8
	1	87.0 ± 10.7	84.6 ± 11.9	81.9 ± 9.4	71.5 ± 9.6***	86.3 ± 10.4†††	85.3 ± 9.9†††	91.4 ± 6.3†††	93.0 ± 8.6*†††	88.7 ± 7.9†††	90.7 ± 11.0†††	92.1 ± 7.3**†††
	3	90.1 ± 10.5	88.3 ± 12.2	86.6 ± 9.2	84.9 ± 12.3	84.6 ± 11.3	83.8 ± 13.3	82.4 ± 14.6	79.8 ± 14.2	78.8 ± 15.8	73.2 ± 16.6*,†	78.0 ± 17.2
HGB (g/dL)	1	15.3 ± 1.5	15.2 ± 1.4	15.1 ± 1.4	14.3 ± 1.3**	15.1 ± 1.5†	15.2 ± 1.7	15.4 ± 1.4††	15.2 ± 1.3†	14.9 ± 1.2	14.9 ± 1.4	15.0 ± 1.5
	2	15.7 ± 0.7	15.6 ± 0.8	15.4 ± 0.8	14.3 ± 0.7***	15.7 ± 0.7†††	15.7 ± 0.7†††	16.2 ± 0.5***,†††	16.3 ± 0.6***,†††	15.8 ± 0.5†††	16.0 ± 0.7†††	15.5 ± 0.5†††
	3	15.5 ± 0.9	15.4 ± 0.9	15.2 ± 0.8	15.3 ± 0.7	15.2 ± 0.8	15.1 ± 0.9	15.1 ± 0.8	15.0 ± 0.9	14.8 ± 1.1	14.7 ± 0.7*,†	15.0 ± 0.7
ABPS	1	-0.8 ± 1.1	-0.8 ± 1.1	-0.9 ± 0.9	-1.5 ± 0.8	-1.1 ± 1.1	-0.9 ± 1.4	-1.0 ± 1.1	-0.9 ± 1.0	-1.1 ± 0.9	-1.1 ± 1.0	-0.8 ± 0.9†
	2	-0.6 ± 0.6	-0.6 ± 0.5	-0.8 ± 0.8	-1.6 ± 0.5***	-0.8 ± 0.6†	-0.8 ± 0.6†	-0.5 ± 0.6††	-0.6 ± 0.6††	-0.8 ± 0.8†	-0.7 ± 0.7††	-0.7 ± 0.6††
	3	-0.5 ± 0.8	-0.7 ± 0.9	-0.9 ± 0.7	-0.8 ± 0.7	-0.9 ± 0.8	-1.0 ± 0.8	-0.9 ± 0.7	-1.0 ± 0.6	-1.2 ± 0.8	-1.2 ± 0.9	-1.0 ± 0.7
RET% (%)	1	1.2 ± 0.4	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.4	1.3 ± 0.4	1.2 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.2
	2	1.4 ± 0.3	1.5 ± 0.4	1.4 ± 0.3	1.4 ± 0.2	1.4 ± 0.2	1.4 ± 0.2	1.04 ± 0.2	1.4 ± 0.2	1.3 ± 0.2	1.3 ± 0.3	1.1 ± 0.3***,††
	3	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.3 ± 0.4	1.3 ± 0.4	1.3 ± 0.4	1.3 ± 0.5	1.4 ± 0.4	1.4 ± 0.5	1.5 ± 0.6†	1.5 ± 0.5†
HCT (%)	1	44.8 ± 4.0	44.4 ± 4.1	44.3 ± 3.8	42.0 ± 3.3**	43.8 ± 3.7	44.0 ± 4.3	44.5 ± 3.3††	44.4 ± 3.3††	43.5 ± 3.1	43.4 ± 3.9	44.2 ± 3.5
	2	45.8 ± 2.2	45.5 ± 2.6	44.8 ± 2.9	41.7 ± 2.0**	45.6 ± 2.4	45.3 ± 2.4	47.1 ± 1.9††	47.4 ± 2.5††	46.4 ± 2.4	46.3 ± 2.3	45.4 ± 2.0†
	3	45.1 ± 2.4	44.8 ± 2.8	44.0 ± 1.7	44.0 ± 1.9	43.9 ± 2.3	43.0 ± 2.3	43.7 ± 2.5	43.3 ± 2.6	42.8 ± 3.1	42.1 ± 1.5*,†	43.4 ± 2.6
MCH (pg)	1	29.2 ± 1.2	29.2 ± 1.2	29.4 ± 0.5	29.2 ± 1.8	29.2 ± 1.7	29.4 ± 1.5	29.2 ± 1.6	29.1 ± 1.6	29.1 ± 1.4	29.2 ± 1.6	29.1 ± 1.8
	2	29.3 ± 1.1	29.4 ± 0.7	29.2 ± 0.7	29.4 ± 1.0	29.3 ± 0.9	29.5 ± 1.0	29.1 ± 0.8	29.1 ± 1.1	28.7 ± 1.0†	29.3 ± 1.1	29.1 ± 0.8
	3	30.2 ± 1.1	30.2 ± 1.2	30.3 ± 1.1	30.2 ± 1.1	29.9 ± 1.3	30.4 ± 1.0	29.9 ± 1.2	30.1 ± 1.1	29.9 ± 1.2	30.0 ± 0.8	29.9 ± 1.2
MCHC (g/dL)	1	34.2 ± 0.7	34.3 ± 0.9	34.0 ± 0.7	34.2 ± 1.1	34.5 ± 1.2	34.5 ± 0.9	34.5 ± 0.9	34.3 ± 1.2	34.3 ± 1.1	34.4 ± 1.1	33.9 ± 1.2
	2	34.3 ± 1.0	34.4 ± 0.6	34.3 ± 0.9	34.2 ± 0.8	34.5 ± 1.0	34.6 ± 0.6	34.4 ± 0.9	34.4 ± 1.0	34.0 ± 1.1	34.5 ± 0.8	34.2 ± 1.0
	3	34.5 ± 0.9	34.4 ± 0.6	34.5 ± 0.8	34.7 ± 0.9	34.6 ± 1.1	35.0 ± 0.8	34.6 ± 0.9	34.6 ± 0.7	34.7 ± 0.7	34.8 ± 0.6	34.6 ± 0.8
MCV (fL)	1	85.4 ± 2.7	85.2 ± 2.8	85.6 ± 2.4	85.3 ± 3.2	84.7 ± 2.9	85.3 ± 3.4	84.6 ± 3.4	84.8 ± 3.0	84.8 ± 3.0	84.8 ± 2.8	85.8 ± 3.0
	2	85.4 ± 2.5	85.5 ± 1.5	84.8 ± 1.8	85.8 ± 2.7	84.9 ± 2.2	85.1 ± 2.4	84.4 ± 2.1	84.6 ± 2.4	84.3 ± 1.9	84.8 ± 2.4	85.0 ± 1.8
	3	87.7 ± 2.9	87.7 ± 3.1	87.9 ± 3.5	87.1 ± 2.8	86.5 ± 2.8	86.8 ± 2.7	86.4 ± 2.2	87.0 ± 2.7	86.3 ± 2.9	86.1 ± 3.0	86.7 ± 3.1
PLT (10 <sup>3</sup> /μL)	1	212.2 ± 25.5	226.7 ± 26.5	211.4 ± 26.9	230.3 ± 29.0	227.8 ± 32.0	235.2 ± 23.3	225.3 ± 29.0	228.7 ± 34.0	226.2 ± 32.3	225.7 ± 27.9	223.2 ± 26.4
	2	220.1 ± 43.0	223.0 ± 44.4	224.5 ± 37.1	225.0 ± 44.0	227.0 ± 45.5	231.3 ± 38.2	230.2 ± 50.7	236.6 ± 43.2	227.0 ± 49.7	225.7 ± 49.6	213.0 ± 39.2

(Continues)

TABLE 1 (Continued)

Marker	Baseline		After donation								
	-3 weeks	-2 weeks	-1 week	t = 0	+3 h	+6 h	+1 day	+2 days	+3 days	+4 days	+7 days
G	247.6 ± 41.7	239.8 ± 21.9	246.5 ± 42.0	249.3 ± 35.0	261.0 ± 45.3	253.7 ± 40.8	246.1 ± 29.2	246.3 ± 31.3	245.1 ± 26.8	239.5 ± 18.3	259.6 ± 31.4
RBC (10 <sup>6</sup> /μL)	1 5.2 ± 0.4	5.2 ± 0.4	5.2 ± 0.4	4.9 ± 0.4*	5.2 ± 0.4	5.2 ± 0.4	5.3 ± 0.4 <sup>††</sup>	5.2 ± 0.3 <sup>††</sup>	5.1 ± 0.3	5.1 ± 0.4	5.1 ± 0.3
	2 5.4 ± 0.3	5.3 ± 0.3	5.3 ± 0.3	4.9 ± 0.3 <sup>***</sup>	5.4 ± 0.3 <sup>†††</sup>	5.3 ± 0.4 <sup>†††</sup>	5.6 ± 0.3 <sup>***,†††</sup>	5.6 ± 0.4 <sup>**†††</sup>	5.5 ± 0.3 <sup>**†††</sup>	5.5 ± 0.3 <sup>†††</sup>	5.3 ± 0.2 <sup>††</sup>
	3 5.1 ± 0.3	5.1 ± 0.4	5.0 ± 0.3	5.1 ± 0.3	5.1 ± 0.4	5.0 ± 0.4	5.1 ± 0.4	5.0 ± 0.4	5.0 ± 0.5	4.9 ± 0.3	5.0 ± 0.4
WBC (10 <sup>3</sup> /μL)	1 5.4 ± 1.7	5.0 ± 1.3	5.6 ± 1.5	5.2 ± 1.7	6.4 ± 1.8 <sup>*†</sup>	6.8 ± 2.0 <sup>*†</sup>	5.6 ± 1.6	5.6 ± 1.3	5.4 ± 1.8	5.1 ± 1.9	5.4 ± 1.7
	2 5.6 ± 0.6	6.1 ± 1.3	5.7 ± 1.0	5.7 ± 0.9	6.8 ± 1.0	6.9 ± 0.9	5.7 ± 0.9	5.8 ± 0.8	5.8 ± 0.9	5.6 ± 0.9	5.8 ± 0.5
	3 5.1 ± 1.2	4.4 ± 0.5	4.9 ± 1.1	4.9 ± 0.8	5.6 ± 1.0	6.1 ± 1.0 <sup>*†</sup>	5.4 ± 1.2	5.2 ± 1.4	5.0 ± 1.2	4.6 ± 0.7	5.1 ± 1.1
RDW-CV (%)	1 12.7 ± 0.8	12.7 ± 0.9	12.8 ± 0.9	12.8 ± 0.8	12.9 ± 0.9	12.9 ± 0.9	13.0 ± 0.9 <sup>†††</sup>	12.9 ± 0.9	12.8 ± 0.9	12.8 ± 0.9	12.7 ± 0.9
	2 12.3 ± 0.3	12.3 ± 0.4	12.3 ± 0.2	12.4 ± 0.5	12.7 ± 0.6 <sup>††</sup>	12.5 ± 0.5 <sup>†</sup>	12.7 ± 0.6 <sup>††</sup>	12.5 ± 0.5	12.5 ± 0.5	12.4 ± 0.5	12.3 ± 0.4
	3 12.3 ± 0.5	12.2 ± 0.4	12.3 ± 0.4	12.3 ± 0.4	12.1 ± 0.4	12.2 ± 0.4	12.2 ± 0.5	12.3 ± 0.4	12.2 ± 0.4	12.1 ± 0.5	12.3 ± 0.5
EPO (U/L)	1 10.7 ± 5.2	10.6 ± 4.8	12.0 ± 6.8	13.3 ± 10.1	14.8 ± 10.0	15.0 ± 10.2	11.2 ± 6.9	12.0 ± 6.9	11.7 ± 6.4	13.8 ± 7.0	11.3 ± 7.2
	2 8.9 ± 3.0	9.7 ± 2.7	9.9 ± 3.4	14.0 ± 6.2*	14.4 ± 5.6*	13.2 ± 5.9	7.6 ± 4.1 <sup>††</sup>	7.6 ± 3.7 <sup>††</sup>	7.5 ± 3.6 <sup>††</sup>	7.5 ± 2.7 <sup>††</sup>	8.5 ± 3.6 <sup>†</sup>
	3 9.7 ± 3.0	9.6 ± 2.7	10.5 ± 2.4	9.7 ± 2.6	11.5 ± 3.2 <sup>†</sup>	12.2 ± 2.9 <sup>†</sup>	11.5 ± 3.3	13.0 ± 3.6 <sup>*†</sup>	11.7 ± 3.9	12.4 ± 3.8	12.5 ± 3.9
Iron (μg/dL)	1 96.5 ± 40.9	106.1 ± 42.6	87.8 ± 42.1	97.5 ± 42.6	249.0 ± 52.2 <sup>***,†††</sup>	262.9 ± 37.7 <sup>***,†††</sup>	82.6 ± 50.2	79.5 ± 27.1	90.5 ± 45.3	94.9 ± 41.4	87.9 ± 40.3
	2 122.0 ± 44.4	101.0 ± 38.9	112.4 ± 37.2	90.2 ± 49.4	346.5 ± 48.4 <sup>***,†††</sup>	360.7 ± 55.9 <sup>***,†††</sup>	170.6 ± 112.3	114.6 ± 31.5	130.8 ± 39.7	115.7 ± 37.4	104.2 ± 28.4
	3 101.5 ± 46.6	136.1 ± 49.7	97.6 ± 28.1	105.4 ± 28.8	95.5 ± 28.9	85.8 ± 30.2	95.7 ± 48.1	113.9 ± 44.0	90.0 ± 31.5	98.1 ± 31.2	91.0 ± 26.5
Ferritin (ng/mL)	1 126.1 ± 93.3	118.1 ± 91.6	109.4 ± 87.7	66.1 ± 48.7*	71.0 ± 58.6*	76.7 ± 65.9*	100.9 ± 76.7 <sup>†</sup>	95.4 ± 68.1 <sup>†</sup>	88.2 ± 62.9 <sup>*†</sup>	83.7 ± 62.6 <sup>*†</sup>	78.2 ± 56.1 <sup>*†</sup>
	2 127.4 ± 61.1	124.8 ± 71.1	122.0 ± 74.3	34.7 ± 29.2 <sup>***</sup>	36.9 ± 31.0 <sup>***</sup>	42.9 ± 34.9 <sup>**††</sup>	87.4 ± 30.6 <sup>†††</sup>	88.9 ± 35.8 <sup>†††</sup>	81.7 ± 38.2 <sup>†††</sup>	79.0 ± 37.4 <sup>†††</sup>	77.8 ± 43.6 <sup>**†††</sup>
	3 98.0 ± 47.0	97.9 ± 42.2	90.0 ± 28.3	100.4 ± 54.0	100.4 ± 56.4	96.6 ± 52.2	95.5 ± 53.4	93.0 ± 48.0	94.6 ± 52.3	105.3 ± 52.6	87.3 ± 52.3
Transferrin (g/L)	1 2.6 ± 0.4	2.5 ± 0.4	2.6 ± 0.4	2.6 ± 0.4	2.6 ± 0.3	2.6 ± 0.3	2.6 ± 0.4	2.7 ± 0.4	2.6 ± 0.3	2.5 ± 0.3	2.6 ± 0.3
	2 2.7 ± 0.4	2.7 ± 0.4	2.7 ± 0.4	3.0 ± 0.4 <sup>**</sup>	2.9 ± 0.4*	2.9 ± 0.4	3.0 ± 0.3*	3.0 ± 0.5 <sup>**</sup>	3.9 ± 0.3 <sup>**</sup>	2.9 ± 0.4*	2.9 ± 0.4
	3 2.9 ± 0.4	2.8 ± 0.3	2.8 ± 0.3	2.8 ± 0.3	2.8 ± 0.3	2.8 ± 0.3	2.7 ± 0.3	2.7 ± 0.3	2.7 ± 0.4	2.7 ± 0.4	2.8 ± 0.3
Transferrin saturation (%)	1 30.3 ± 14.4	34.4 ± 14.6	28.2 ± 15.0	30.6 ± 14.0	78.9 ± 20.5 <sup>***,†††</sup>	83.3 ± 17.9 <sup>***,†††</sup>	26.1 ± 17.8	24.1 ± 9.1	29.3 ± 17.4	30.5 ± 13.7	27.5 ± 13.3
	2 36.0 ± 13.9	29.2 ± 10.2	33.1 ± 8.9	25.0 ± 14.4	95.2 ± 3.3 <sup>***,†††</sup>	98.0 ± 2.8 <sup>***,†††</sup>	43.9 ± 26.0	30.3 ± 8.5	36.0 ± 13.7	31.4 ± 9.8	28.7 ± 8.7

TABLE 1 (Continued)

Marker	Baseline			After re-transfusion							
	-3 weeks	-2 weeks	-1 week	t = 0	+3 h	+6 h	+1 day	+2 days	+3 days	+4 days	+7 days
G	28.9	39.2	28.2 ± 9.9	30.1 ± 9.0	27.2 ± 8.9	24.6 ± 9.1	27.7 ± 13.1	32.8 ± 13.0	26.7 ± 10.4	28.8 ± 9.0	26.0 ± 7.9
		± 14.6									
Transferrin receptor (mg/L)	1	1.4 ± 0.4	1.2 ± 0.3	1.3 ± 0.3	1.5 ± 0.4	1.5 ± 0.4	1.5 ± 0.4	1.5 ± 0.3	1.4 ± 0.3	1.4 ± 0.3	1.4 ± 0.4
	2	1.3 ± 0.2	1.3 ± 0.1	1.3 ± 0.2	<b>1.9 ± 0.2***</b>	<b>1.9 ± 0.3***</b>	<b>2.0 ± 0.3***</b>	<b>1.9 ± 0.3***</b>	<b>1.8 ± 0.2***</b>	<b>1.8 ± 0.3**</b>	<b>1.7 ± 0.2***, ††</b>
	3	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.2	1.2 ± 0.2	1.1 ± 0.3	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.2	1.2 ± 0.2

Abbreviations: ABPS, abnormal blood profile score; EPO, erythropoietin; G, Group; HCT, hematocrit; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; OFFS, OFF-hr score; PLT, platelets; RBC, red blood cell count; RDW-CV, red cell distribution width (coefficient of variation); RET, reticulocyte percentage; WBC, white blood cells.

Note: Results are shown as mean ± standard deviation; n = 10 per group. ANOVA-obtained levels of statistical significance adjusted for multiple comparisons (bold): \*P < .05, \*\*P < .01, \*\*\*P < .001 vs baseline levels (mean of -3 weeks, -2 weeks, -1 week); †P < .05, ††P < .001 vs t = 0 levels. Underlying raw data is available in Supplementary Data S1.

any ABT-independent miRNA regulation. Indeed, 21 miRNAs were detected to be significantly differentially regulated upon multiple blood sampling in at least one of the pairwise comparisons when comparing each single time point to the combined baseline levels (-3 weeks, -2 weeks, -1 week) (five up-regulated and 16 down-regulated; Table S4). Thus, the respective miRNAs were excluded from further analyses.

Next, pairwise comparisons within each treatment group (group 1 and group 2), juxtaposing expression levels at the different time points after ABT with averaged baseline levels, revealed significant regulation of 40 miRNAs (Table S5). In group 2, a large proportion of those miRNAs (22/40 miRNAs, 55%) was indicative of a previously performed whole blood donation as they demonstrated significant changes when comparing t = 0 with baseline. Fifty percent (20/40 miRNAs) were differentially regulated in at least one group at least at one sampling time point post-ABT.

Since a complete recovery from blood donation prior to re-transfusion could not be achieved, as suspected by the hematological profiles and the differentially regulated miRNAs at t = 0 vs baseline levels, the pairwise comparisons of all time points post-ABT were also conducted vs t = 0, emerging with a total of 61 differentially regulated miRNAs (49 up-regulated, 12 down-regulated; Table S6). Interestingly, only one miRNA (miR-320d) was significantly down-regulated in both treatment groups 6 h post-ABT, while miR-4646-3p was significantly down-regulated 3 and 6 h post-ABT vs baseline and vs t = 0 only in group 1. Five miRNAs (miR-3158-3p, miR-181a-5p, miR-4443, miR-1260a, miR-1260b) were differentially regulated only in group 2 when compared to baseline and to t = 0 (Figure S5A). Although miR-320d was congruently down-regulated in both treatment groups, and could therefore also be of legal relevance in the doping scene, the overall classification performance, as determined by AUROCs between 0.39 and 0.61, was not superior to ABP at any of the sampling time points post-ABT (Figure S5B). Accordingly, the underlying individual longitudinal expression profiles appeared quite volatile (Figure S5C) with intra-individual and inter-individual variabilities of 7%–55% and 32%–49%, respectively.

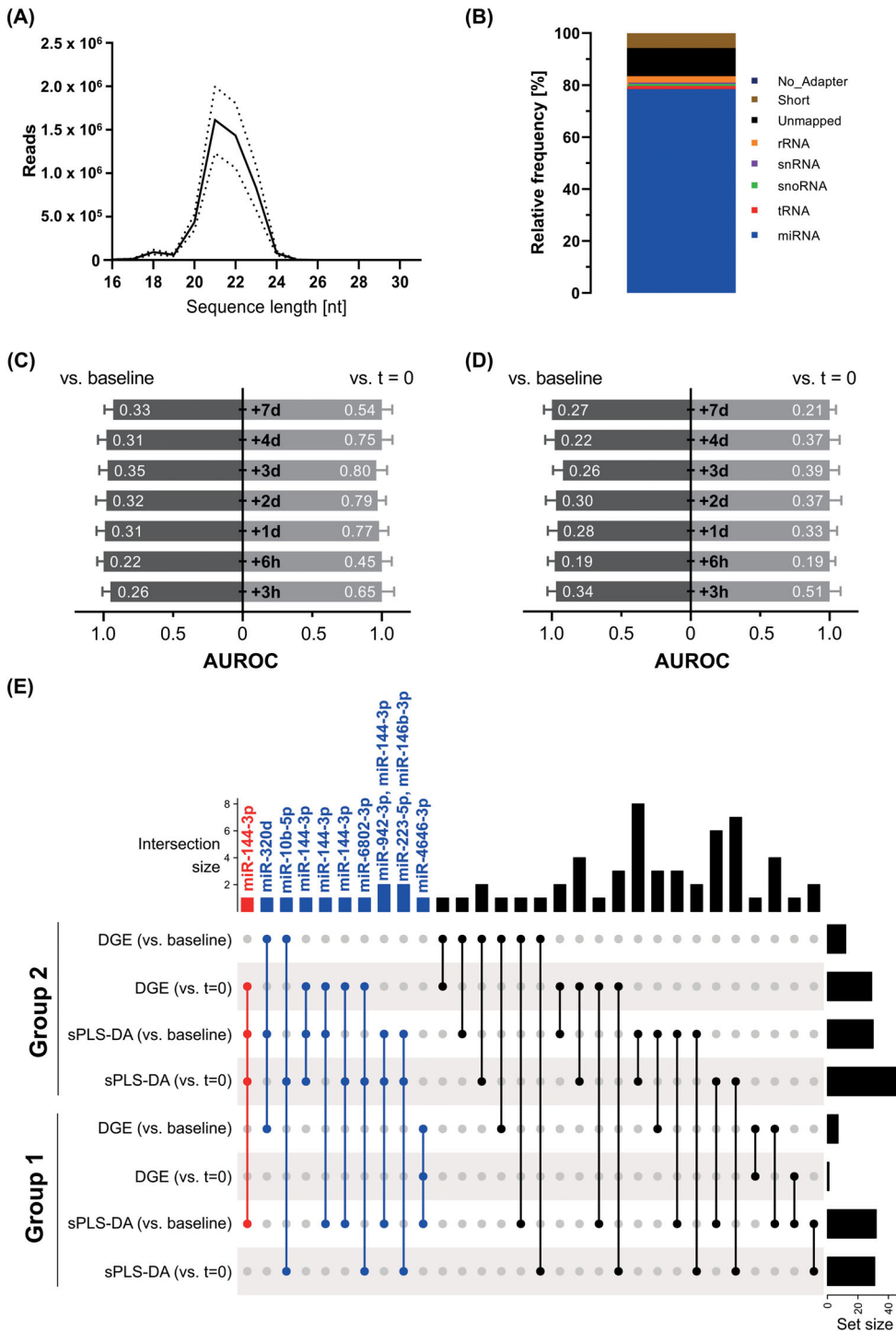
Based on the observed heterogeneity in miR-320d expression levels and its low classification performance, deemphasizing the use of just a single miRNA in ABT detection, multivariate data analyses using sPLS-DA were conducted to determine a more robust combination of miRNAs discriminative for ABT. Reasonable accuracy rates could be achieved with AUROCs >92%, however, overall error rates reached high levels of up to 0.80, especially in group 1 when comparing post-ABT sampling time points with t = 0 (Figure 2C,D). Accordingly, after visual inspection of the discriminative ability of the respective classifier miRNAs by PCA, only a poor separation of ABT-doped and non-doped clusters was observed (Table S7, Figures S6 and S7). With decreasing error rates, only a slight trend of improved separation was recognizable, particularly in group 2 as evidenced by a shift of ABT-dependent clusters within the principal components (baseline vs +6 h; Figure S7A). Remarkably, classifier miRNAs were different for every pairwise comparison and appeared to be group-specific and time point-specific (Table S7).



### 3.4 | ABT detection with a universal miRNA pattern is inferior to the ABP

To screen for the most dominant and universal features within the discovered disparate miRNA patterns, which would potentially allow for ABT detection less affected by sampling time point or re-transfused EC volume, an UpSet plot was generated (Figure 2E). The largest overlap was detected for miR-144-3p in four of the eight applied comparison strategies, followed by another seven miRNAs (miR-320d, miR-10b-5p, miR-6802-3p, miR-942-3p, miR-223-5p,

miR-146b-3p, and miR-4646-3p) that were discriminative in three of the eight comparisons. On that note, miR-320d and miR-144-3p (set 1) were nominated as the most promising miRNAs, as determined by DGE and sPLS-DA, and evaluated for their ABT categorization ability. To this end, the concept of the adaptive model of the ABP was transformed into the two-dimensional environment by creating the individuals' critical range, with every value outside of the 99% ellipses being considered suspicious. In the control group, all data points were within the individuals' 99% ellipses, as exemplified by study subject P13, indicating the correct classification as non ABT-doped (Figure 3A). Time

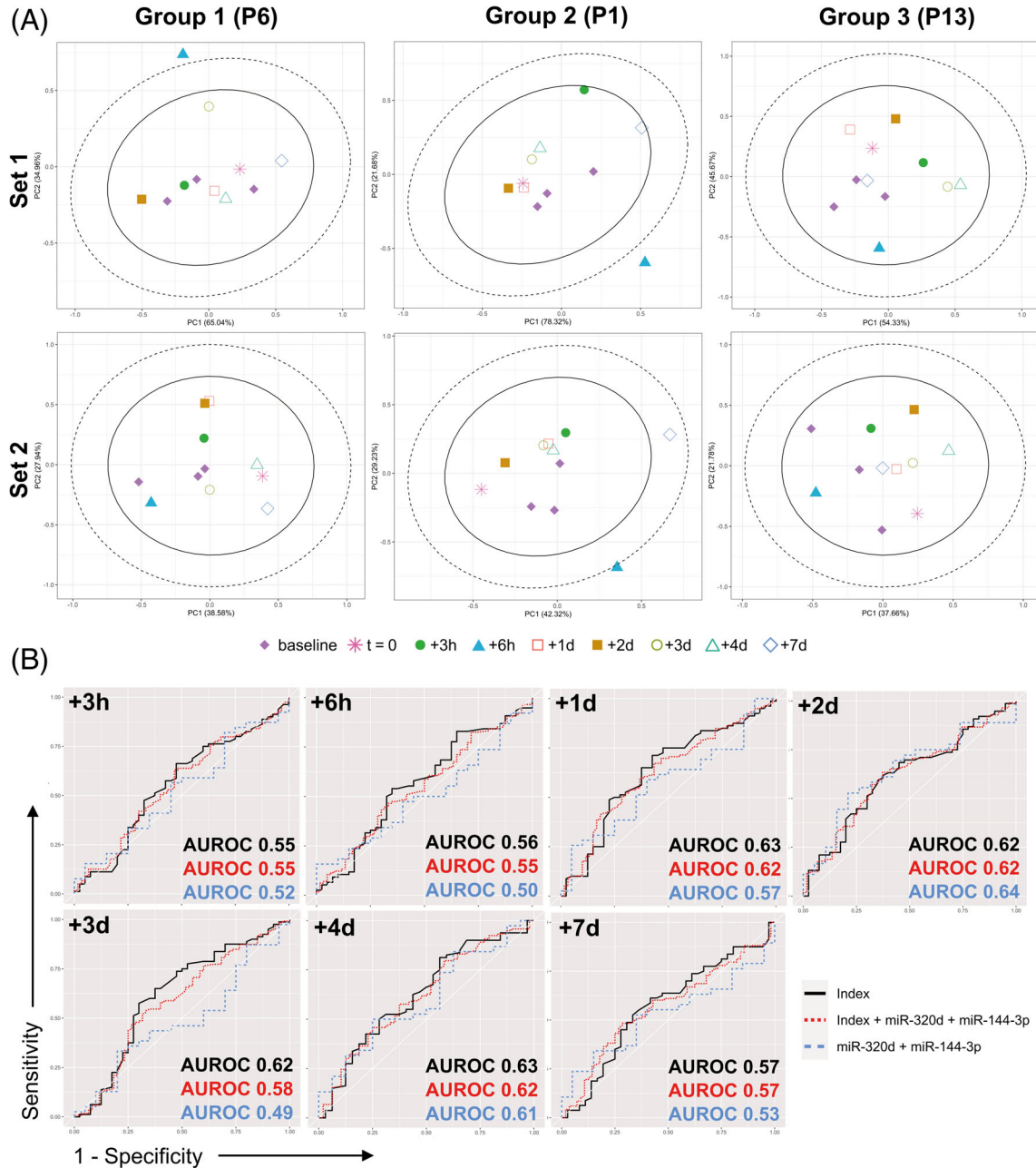


**FIGURE 2** Summary of small RNA sequencing quality and multivariate data analysis.

(A) Overview of the mean length distribution (solid line) ± standard deviation (dotted line); n = 325; and (B) relative mean mapping distribution of all samples; n = 325. Nucleotides (nt), microRNA (miRNA), transfer RNA (tRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), ribosomal RNA (rRNA), short: sequences <16 nucleotides. (C) Classification performance of sparse partial-least square analysis (sPLS-DA) at a maximum of four components with three features each, as depicted by the area under the curve of receiver operating characteristics curve (AUROC) ± standard deviation at every time point post-ABT versus (vs) baseline or versus t = 0 in group 1 and (D) group 2. Values at the end of the bars indicate the overall error rate. (E) Overlaps of significantly differentially regulated miRNAs identified by differential gene expression (DGE) analyses and discriminative miRNAs identified by sPLS-DA are illustrated using an UpSet plot with the intersect mode. Intersection size indicates the number and identity of miRNAs that are shared in the different comparisons, which are listed on the left size. Set size depicts the total number of classifier miRNAs at each performed comparison, summed up for all time points. Red highlights the largest overlap across four comparison groups, while blue highlights the second largest overlap across three comparison groups; underlying data is available in Table S7

point +6 h post-ABT appeared with highest detection rates of suspicious values (illustrated by P1 and P6, Figure 3A). In each of the treatment groups, one data point obtained at baseline was falsely categorized as suspicious, as they were outside of the respective critical ranges (Table S8). However, the majority of post-ABT time points

remained inconspicuous resulting in overall low sensitivity (0%–11%). Thus, the second-best performing miRNAs were aggregated (set 2: miR-144-3p, miR-320d, miR-10b-5p, miR-6802-3p, miR-942-3p, miR-223-5p, miR-146b-3p, and miR-4646-3p) to test for a potential improvement in ABT detection when adding even more miRNAs to



**FIGURE 3** Discriminative performance measures. (A), Exemplary illustration of categorization performance of the most promising discriminative miRNAs on a per-individual basis. Set 1(miR-320d and miR-144-3p); Set 2(miR-144-3p, miR-320d, miR-10b-5p, miR-6802-3p, miR-146b-3p, miR-4646-3p, miR-942-3p, miR-223-5p). Ellipses indicate the 95% (solid line) and 99% (dashed line) confidence interval. (B), Performance of the most promising miRNAs (Set 1: miR-320d and miR-144-3p) in the classification of autologous blood transfusion (ABT)-doped and non-doped study subjects compared to and in combination with the main hematological Athlete Biological Passport (ABP) markers (OFF-hr Score, OFFs; hemoglobin, HGB; Abnormal Blood Profile Score, ABPS; reticulocyte percentage, RET%) for every time point after ABT. The index calculated from ABP markers and its corresponding area under the receiver operating characteristics curve (AUROC) are summarized in black while the miRNA classification performance is indicated by the dashed blue line, and the combination is highlighted by the dotted red line; n = 325

the analysis. However, this did not result in a better diagnostic potential (Figure 3A, Table S8), so that only set 1 was investigated further. When analyzing the overall classification performance, the miRNA fingerprint of set 1 was not superior to the Index obtained from the main hematological markers of the ABP, neither alone nor in combination with the Index (Figure 3B). Although using set 1 resulted in an AUROC of 0.64 at +2 days post-ABT, which was higher compared to the Index, its sensitivity rate was still negligible compared to the use of HGB alone.

## 4 | DISCUSSION

The actual field of legal application of ABT was to provide sufficient blood supply in medical surgeries while reducing the risk of immunosuppression, transfusion reactions and transmission of infections.<sup>51</sup> Unfortunately, it is misused and illegally applied in competitive sports for ergogenic purposes regardless of any adverse side effects and consequences of anti-doping rule violations. Therefore, the present study performed monitored re-transfusions of stored ECs aiming at identifying ABT-dependent miRNA expression patterns and examining their applicability in the detection of ABT.

### 4.1 | General considerations on the study design

Based on existing literature and guidelines, reasonable recovery from blood donations within 4–6 weeks, with inter-individual differences regarding the recovery speed, could have been expected.<sup>2,16,52–56</sup> Our results, however, indicated an overall insufficient recovery from blood withdrawal after 4 weeks (group 2) and, to a lesser extent, after 6 weeks (group 1) (Table 1), which was comparable to the outcomes reported by Bejder et al.<sup>57</sup> As a matter of fact, only some study subjects completely returned to their baseline physiological ranges (Figure S1). In ABT-focused anti-doping studies, the use of frozen or cryopreserved instead of refrigerated ECs might circumvent such deficient restoration of baseline levels. In fact, it is a common assumption in the anti-doping scene that the majority of transfusions utilize frozen ECs, and this was at least partly confirmed by the huge amount of frozen material that was found during the “Operation Aderlass”.<sup>13</sup> Indeed, there are some advantages of frozen or cryopreserved compared to refrigerated storage of ECs, such as (a) a prolonged storage time, which allows the donor to fully recover from prior blood withdrawal and re-transfuse the blood bags even years later<sup>58</sup>; (b) a larger increase in circulating hemoglobin, even compensating the higher loss during storage<sup>59</sup>; and (c) the absence of storage lesions, as described for refrigerated ECs (reviewed by Yoshida et al.<sup>60</sup>). But freezing or cryopreserving ECs also has its drawbacks, including a more complicated protocol and the need for specialized equipment, high costs, and changes in erythrocyte structure and function.<sup>59,61</sup> Furthermore, the production of cryopreserved ECs is considered a special preparation in Germany, which is only used in very exceptional cases, such as for patients with multiple transfusion-relevant antibodies or

antibodies against high frequency antigens.<sup>56</sup> Based on these crucial limitations, the refrigerated storage of ECs was used in the present study.

Apart from the assumptions that the anti-doping scene makes about the type of EC storage, there are also speculations about the volumes, which could range from low (150–200 mL EC) to high (300–900 mL EC) dosages, and specific time points of transfusion, which are suspected to be performed several hours before a competition.<sup>62–64</sup> However, there are conflicting study results on the efficacy of said tactics. Regarding the time point of transfusion, the performance-enhancing effects might be of rapid-onset but short-term lasting only a few days,<sup>65</sup> but there is also the assumption that stored erythrocytes first have to restore their ability to oxygenate the microcirculation, which might take several hours.<sup>66,67</sup> Studies on dose–response relationships revealed that low-volume transfusions might already result in detectable performance enhancement.<sup>57</sup> However, due to a poorly placed baseline measurement, this finding has to be considered carefully.<sup>68</sup> Moreover, although correlations between transfusion volume and associated increase or decrease in hematological variables were observed,<sup>2,16,52–55</sup> a conclusive connection with aerobic capacity remains to be established.<sup>57,65</sup> Since an increase in miRNA changes with increasing transfusion volume cannot be excluded either and a non-negligible amount of blood of almost 190 mL is collected during multiple samplings post-ABT, which might reduce the ABT-dependent effect size in the present exploratory study, a second standard-volume blood donation of about 500 mL with subsequent EC re-transfusion after 28 days of storage was conducted in group 2 to increase the ergogenic aid and achieve a more distinctive magnitude of change.

Consequently, there are two main aspects that should be considered in the evaluation of the present study's results: (a) An individual's critical range might have become extended due to the donation-dependent changes in hematological markers. Even though this impact was reduced the more additional sampling time points were included, ABT identification might have been hampered at first. (b) The transcriptomic changes after ABT might represent a mixture of effects that result from ongoing recovery processes and physiological reactions to EC re-transfusion. So, blood donations, which are *per se* permitted by the WADA, might lead to suspicious events in the ABP and could thus provide a sound basis for follow-up and more frequent out-of-competition testing of conspicuous athletes.

### 4.2 | The ABP as the gold standard method in ABT detection

Individual hematological profiles were evaluated according to the adaptive model implemented within the ABP, achieving reasonable classification performances with sensitivities between 0% and 60% at specificity rates between 50% and 100% depending on the sampling time point. This was comparable to a study simulating real conditions during a 42-week cycling season (sensitivity 36%, specificity 90%).<sup>11</sup> However, it has to be mentioned that the true ABP-based detection

rate might be underestimated in the present study. According to the WADA's ABP operating guidelines,<sup>37</sup> a WADA-approved expert panel is consulted to review an individual's hematological profile in any case of suspicious events. Here, it was the authors who assessed outlier values based on profound hematological knowledge and a comprehensive set of variables. Nevertheless, this cannot be equated to an expert panel, which could have interpreted our results differently. Furthermore, four study subjects pertaining to the control group showed outlier values exceeding the critical range (P13, +3 days; P19, +4 days; P21, +4 days; and P24, +4 days; Table S3). However, the group assignment was unknown to the authors as they were blinded for it prior to ABP data interpretation. After reconsideration based on known group allocations, the downward trend in longitudinal profiles, which could have also been indicative for a blood donation, might have resulted from accumulated adaptations to daily samplings. Since daily samplings seem to be unrealistic in anti-doping, these artificially introduced false positives could be disregarded, leading to higher specificity rates at the respective sampling time points. Additionally, multiple samplings within 1 week after ABT are unable to yield an appropriate reflection of real longitudinal profiles, and the individual's critical range will adapt accordingly to high-frequency sampling. As a result, the probability that values exceed the critical range is reduced, which could have diminished the sensitivity rate in the present study.

Interestingly, the diagnostic potential of the ABP appeared to be higher for group 2 as compared to group 1. Especially at time point  $t = 0$ , 20% more study subjects were identified with suspicious outlier values in group 2 compared to group 1 (Table S3), which was most likely a consequence of the second blood donation 4 weeks prior. Since donating blood is not an anti-doping rule violation, the ABP's promising detection rate should rather be used in more frequent out-of-competition testing, as mentioned above, to identify athletes with the possible intention to perform blood doping, even months later. Moreover, even though the ABT-dependent effect might have been weakened by an incomplete recovery from donation, our results point towards a dose-response relationship with respect to hematological variables. Of note, the magnitude and duration of response differed a lot between study subjects (Figure S1). It is already known that the hydration status can influence HGB and OFFS levels,<sup>69,70</sup> which could be the reason for the noted inter-individual differences, as the fluid intake of the study population was not controlled. However, inter-individual as well as intra-individual differences in the levels of hematological variables were also recognized by others,<sup>71,72</sup> indicating the putative presence of low-responders, high-responders and even non-responders to ABT.

### 4.3 | The potential of whole blood miRNA profiles in ABT detection

In terms of whole blood miRNA analysis, quality control steps of the experimental setting revealed the presence of high-integrity RNA with ample miRNA proportions and successful small RNA sequencing, thereby providing a solid basis for downstream investigations. Via

DGE analysis, various significantly differentially regulated miRNAs were detected in pairwise comparisons. Remarkably, some even emerged when comparing sampling time point  $t = 0$  with baseline levels in group 2. In accordance with the hematological data, this would indicate insufficient and still ongoing recovery from the second blood donation. This assumption is supported by the donation-dependent decrease and immediate re-transfusion-induced compensation of miR-144-3p and miR-144-5p expression levels, both of which are among the most abundant miRNAs in erythrocytes, and key players in erythroid differentiation.<sup>24,73,74</sup> Moreover, these findings strengthen the hypothesis that miRNAs derived from stored ECs could have a huge and instant influence on the circulating miRNA profile upon re-transfusion. Therefore, it might well be assumed that miRNAs that were identified to be highly significantly upregulated upon long-term storage of ECs would also appear instantly and in significant concentrations in the circulation after re-transfusion. However, no such event was ascertained in the present study. Intriguingly, five of the six miRNAs that were upregulated during long-term EC storage (miR-1260a, miR-1260b, miR-5100, miR-4443, and miR-4695-3p), as detected by Haberberger et al.,<sup>28</sup> were identified in the present study to be slightly but significantly downregulated at 1 or 7 days after ABT. In addition to the volume of infused ECs being highly diluted in the whole-body blood volume, up to a quarter of re-transfused erythrocytes is already broken down within the first day.<sup>75</sup> Thus, both dilution and erythrocyte breakdown could be the reason why the storage-dependent changes in miRNA profiles cannot be detected sufficiently *in vivo* after re-transfusion. Although the extent to which the expression levels of previously identified storage-dependent miRNAs were changed might have been too low to be rediscovered in the circulation, it might have been high enough to induce some feedback mechanisms within the span of a few days.

While the miRNA profile of group 2 seemed to resemble a mixture of transcriptomic effects in response to both blood donation and EC re-transfusion, the significantly differentially regulated miRNAs observed in group 1 might represent the more genuine response to ABT, as there were seven miRNAs detected to be only regulated upon ABT and not upon blood withdrawal. Nevertheless, one miRNA (miR-320d) featured significantly different expression levels compared to baseline in both treatment groups 6 h post-ABT without any obvious reaction to blood withdrawal. For this reason, as well as the already established association of the miR-320 family with erythroid differentiation,<sup>76,77</sup> this specific miRNA seemed to represent a robust indicator for ABT in anti-doping. However, further investigation of its classification performance revealed a negligible diagnostic potential in any of the sampling time points, which could result from highly heterogeneous expression levels between individuals but also within one individual, as has already been investigated elsewhere.<sup>78,79</sup> This further implied that the combined use of miRNAs might offer a greater value in doping detection than just focusing on a few highly regulated but heterogeneously responding individual miRNAs, as already indicated by Gasparello et al.<sup>16</sup> Prompted by these findings, we turned to multivariate data analysis using sPLS-DA, which is a routinely used method for the identification of discriminative patterns.<sup>47</sup> Remarkably,

distinct miRNA patterns were identified in each of the different pairwise comparisons in group 1 and 2. This is most probably an additive effect resulting from several factors, some of which have already been mentioned: (a) ongoing recovery from blood donation and parallel reaction to EC re-transfusion; (b) high inter-individual and intra-individual variability; (c) individual differences in starting time point and duration of reactions to ABT; and (d) early and delayed onset of biological processes in response to EC re-transfusion. The latter factor would also be in line with earlier findings that report an early increase in HGB and HCT and a noticeable increase in RET% after one week.<sup>53</sup>

To account for the various determinants just mentioned, the occurrence of miRNAs that were universally present in the many pairwise comparisons was investigated aiming at identifying the most robust combination thereof. Of the most common eight miRNAs, as depicted in Figure 2E, especially miR-144-3p and miR-320d seemed promising. However, neither the combination of those two nor the addition of the remaining six promising miRNAs resulted in ABT detection sensitivities greater than 11%. Computing confidence ellipses based on baseline miRNA profiles might result in both higher detection sensitivity and a broader window of detection. However, this approach would not be applicable in reality, since the doping status of an athlete is unknown at the time point of testing. Nevertheless, with an increasing number of samples in a real longitudinal profiling, the clusters of normal physiological ranges might also get tighter, allowing for the detection of more suspicious samples, although this is an assumption that remains to be tested in future.

So far, there is only a small number of studies on miRNA profiles in connection with ABT: Gasparello et al.<sup>16</sup> identified seven miRNAs (miR-126-3p, miR-144-3p, miR-191-3p, miR-197-3p, miR-486-3p, miR-486-5p, and miR-92a-3p) that were dysregulated 15 days after autologous re-transfusion of ECs that had been stored for 35 days. However, the post-ABT time point for fold change calculation might have been chosen inappropriately, as it appears more likely that competitive athletes are tested within 48 hours after a competition rather than 15 days later. Leuenberger et al.<sup>80</sup> identified three miRNAs (miR-30b, miR-30c, and miR-26b) that were differentially regulated up to 1 day post-ABT after 42 days of EC storage. While assessing miRNA changes within a more relevant window of detection shortly after ABT, this study missed the opportunity to include a pre-donation measurement. Thus, it is impossible to evaluate whether the study subjects had already fully recovered to baseline levels on the day before ABT, which was used as the reference time point for statistical analyses. Interestingly, these two studies did not identify any overlap of ABT-impacted miRNAs. Of the miRNAs mentioned above, only miR-144-3p was confirmed in the present study to be ABT-associated. Apart from the aforementioned differences in the study design and statistical analyses, this discrepancy might be explained by the respective choice of blood compartment and quantification techniques to study miRNA profiles.<sup>81-83</sup> While the research approach of the abovementioned articles was based on targeted analysis of individual miRNAs in plasma, the present study investigated the whole blood miRNA profile using RNA sequencing without requiring any *a*

*priori* knowledge. Also, in order to verify the validity of RNA sequencing results, reproducibility was representatively ascertained by real-time PCR (see supplemental methods and Figure S8). In fact, the majority of miRNAs that were defined in the present study as most valuable in reliable ABT detection, was already brought up in the context of hematopoiesis.<sup>22,84</sup> Additionally, miR-144-3p was not only associated with erythroid differentiation, but was also described as useful long-term marker in EPO abuse.<sup>27,74</sup> Further, *in silico* analysis using TargetScan (v7.2; www.targetscan.org)<sup>85</sup> established the EPO receptor as target of the miR-320 family, miR-4646-3p, and miR-223-5p, while miR-146b-3p seems to target EPO directly. This indicates the vast biological relevance of these miRNAs in blood doping. However, further research is required to validate the true biomarker value of all highlighted miRNAs.

#### 4.4 | Conclusion on the applicability of miRNA fingerprints in ABT detection

Apart from an unambiguous detection of cheating athletes, one of the main aims of anti-doping systems should also be the protection of clean athletes by correctly identifying them as such, since anti-doping rule violations may under certain circumstances have serious consequences in an athlete's career. Based on our results, the incidence of false positive allegations was low to absent for both the ABP and the miRNA fingerprints. However, the detection rate of truly ABT-doped study subjects by miRNA fingerprints only adds little value to overall ABT detection compared to the ABP alone or combined with miRNAs. Although a differential miRNA pattern 6 h post-ABT seems certainly reasonable, the detection window might be only minimally extended by adding miRNA to hematological profiles. Even if higher sensitivity rates were to be achieved by assessing the profiles of all the different classifier miRNAs ascertained by sPLS-DA, implementing more than a dozen of miRNAs in field testings would be inconvenient, cost-intensive, and thus somewhat inapplicable. It has also to be mentioned that the present study was conducted in a controlled environment with information on the absence of other medications. However, since little is known about the modus operandi of cheating athletes, this and other variables cannot be controlled in an experimental setting. A likely scenario to evade detection could be the use of multiple medications to mask the re-transfusion of ECs and/or to impede hematological data interpretation. For instance, iron supplementation, recombinant human EPO, plasma expanders, or micro-dose injections might be misused. However, investigating the influence that these and other confounders might have on ABT-associated miRNA profiles was beyond the scope of this study investigating the general feasibility of miRNAs in ABT-detection. As there is no uniform doping protocol that athletes follow, the best possibility to evaluate the true applicability of miRNAs, including our most promising candidates miR-144-3p and miR-320d, in anti-doping could be to implement them as routine analysis in every doping control and to retrospectively investigate profiles of convicted athletes.

## ACKNOWLEDGMENTS

The authors thank the staff members of the Department for Transfusion Medicine, Cell therapeutics and Haemostaseology for representatively supervising blood donations and transfusions. We thank Benedikt Kirchner for mapping sequencing data and Dominik Buschmann for critically proof-reading the manuscript. Special thanks go to the subjects who volunteered for this study. This work was supported by grants from the World Anti-Doping Agency (WADA) (Grant No. ISF15E09MP) (<https://www.wada-ama.org>). The content of this publication does not necessarily reflect the views of the WADA. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Open access funding enabled and organized by Projekt DEAL.

## CONFLICT OF INTEREST

The authors declare that no conflict of interest.

## AUTHOR CONTRIBUTIONS

V.M., G.W., and M.W.P. designed the study; V.M. recruited study subjects and cared of them; V.M. and G.W. conducted the study; G.W. and M.W.P. provided essential equipment; V.M. performed experiments, analyzed results, and made the figures; V.M. wrote the manuscript; all authors reviewed the manuscript and approved the final version for submission.

## DATA AVAILABILITY STATEMENT

The small RNA dataset of raw trimmed sequence reads is available online in the European Nucleotide Archive (study accession number PRJEB38354, <http://www.ebi.ac.uk/ena/data/view/PRJEB38354>). All other relevant data are within the paper or its supporting information files.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Mussack V, Wittmann G, Pfaffl MW. On the trail of blood doping—microRNA fingerprints to monitor autologous blood transfusions in vivo. *Am J Hematol.* 2021;96:338–353. <https://doi.org/10.1002/ajh.26078>