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Authors: Loria F, Manfredi M, Reverter-Branchat G, Segura J, Kuuranne T, Leuenberger N

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Abstract

Background: transcriptomic biomarkers originating from reticulocytes measured in dried blood spots (DBSs) may be reliable indicators of blood doping.

Methods/Results: here, we examined changes in the expression levels of the erythropoiesis-related 5'-aminolevulinate synthase 2 (*ALAS2*), carbonic anhydrase (*CA1*), and solute carrier family 4 member 1 (*SLC4A1*) genes in DBS samples from elite athletes and volunteers of clinical study with recombinant erythropoietin (rhEPO) injection.

Conclusion: by comparing the mean intra-day coefficients of variation (CVs) for *ALAS2L*, *ALASLC*, *CA1*, and *SLC4A1* between manual and automated RNA extractions, an average improvement of 5.2% units (from 11.5% to 6.3%) was observed, whereas the assessment of inter-day variability provided comparable results (mean CVs of 9.9% and 10.2%) for both manual and automated approaches. Moreover, obtains results illustrate the fact that mRNA biomarker could be interesting candidate to detect blood doping.

Key words: automation, transcriptomic biomarkers, dried blood spot, RNA extraction

Introduction

The World Anti-Doping Agency (WADA) recognizes venipuncture as the official method of collecting blood for the athlete biological passport (ABP). This process can be used to collect whole blood (EDTA tubes), plasma, or serum (SST tubes), and requires trained medical personnel. Transport and storage of blood matrices can be problematic owing to the space required for the tubes and the necessity to maintain them at cooled conditions during the transport and frozen during the long-term storage to ensure stability of the serum and plasma [1]. Consequently, the WADA and numerous research groups have endeavored to find and test new blood matrices. Since the 1960s, dried blood spots (DBSs) have been used particularly in pediatric sciences to detect disease in newborns [2]. The advantages of DBSs include e.g. the easier transport and store than required by traditional blood matrices [3]. In addition, trained medical personnel are not required because DBSs can be obtained by a finger-prick and the subsequent collection of a small volume (~20 µl) of blood on a filter paper matrix. As an alternative to a finger-prick, the TAP™ device developed by Seventh Sense Biosystems® (Boston, USA) to be attached on the upper arm and uses 30 microneedles to collect 100 µl of capillary blood in a painless way. The blood is introduced in a collection chamber containing lithium heparin to prevent coagulation. After collection via a finger-prick or the TAP™ device, DBSs are prepared by loading 20 µl of blood directly onto a filter paper matrix using a pipette [4, 5], which is dried and stored at room temperature or at 4°C.

The use of DBSs as a sample matrix to detect blood manipulation has been tested in multiple studies [6, 7], and it has been incorporated with direct methods of detecting recombinant human growth hormone and recombinant human erythropoietin (rhEPO) in DBSs [8, 9]. Moreover, DBSs have been applied also to indirect detection methods using protein or transcriptomic markers to detect blood doping [10, 11]. The transcriptomics strategy, which involves analyzing alterations in RNA transcript levels, has shown interesting prospects in number of research studies [10, 12-14]. The expression levels of some genes vary in response to the administration of doping agents [10]. Wang et al. [15] reported that the *5'-aminolevulinate synthase 2* (*ALAS2*), *solute carrier family 4 member 1* (*SLC4A1*), and *carbonic anhydrase 1* (*CA1*) genes are promising candidates to detect doping by rhEPO. Moreover, Salamin

et al. [14] reported that these genes could be also used to target blood transfusion. *ALAS2* is expressed at high levels during erythropoiesis and it encodes a protein involved in heme biosynthesis. Recently, Salamin et al. [10] found also that the levels of linear (*L*) and circular (*LC*) *ALAS2* RNAs increase following blood withdrawal or rhEPO administration. *SLC4A1* and *CA1* are also directly or indirectly involved in erythropoiesis. *SLC4A1* is a chloride-bicarbonate exchanger located on erythrocyte membranes that is essential for red blood cell function [16], whereas *CA1* is involved in many cellular processes, including cell respiration.

RNA extraction from DBSs is a fundamental step to analyze transcriptomic biomarkers. Typically, the process is performed manually, as described in a previous study by Salamin et al. [10]. However, automated RNA extraction is also possible, as reported for the extraction of RNA from viruses and microbes [17-19]. The potential benefit of automated RNAis in the improvement of the accuracy of transcriptomics analyses by avoiding human error and sample contamination due to repeated manipulations. Moreover, automation can reduce the variability of sample extraction caused by different technicians and allow standardization across laboratories [20, 21] as well as improve the accuracy of RT-qPCR [22].

In this study, we examined the response (i.e. expression levels) of new emerging biomarkers (*SLC4A1*, *CA1*, and *ALAS2*) to rhEPO administration, using DBSs as the sample matrix. Moreover, we compared the expression levels of these genes in RNA samples extracted using automated and manual methods, and compared the results obtained from finger-prick DBSs with those obtained from TAP™ DBSs.

Materials and methods

Manual DBS extraction

The complete DBS was cut out from the collection card and transferred into a 2 ml conical polypropylene microcentrifuge tube. The whole RNA was extracted using the miRNeasy® Mini Kit (Qiagen, Germany), following the manufacturer's instructions with minor modifications [10]. First, the cells were lysed with 1 ml of QIAzol lysis reagent (Qiagen, Germany) and agitated (450 rpm) for 15

min at 37°C. Subsequently, the tube was sonicated for 15 min and then incubated for 15 min at 37°C with agitation (450 rpm). In the next step, chloroform (250 µl) was added to the tube, the sample was vortex-mixed two times, and incubated at room temperature for 5 min. The tube was then centrifuged at 12,000 g for 15 min. Finally, the aqueous phase (525 µl) was transferred to a new 2 ml polypropylene microcentrifuge tube and mixed with 800 µl of ethanol, before being transferred into an RNeasy Mini spin column to perform the washing phases. The extracted RNA was eluted with 50 µl of RNase-free water.

Automated DBS extraction

Automated extraction was performed using the Maxwell® RSC Instrument (Promega, USA). The extraction procedure has first manual part following the same steps as manual extraction until the first centrifugation (15 min, 12,000 g), at which point the aqueous phase was processed using the Maxwell® RSC miRNA Plasma and Serum Kit (Promega). The contents of the kit were insert into the Maxwell® RSC Instrument and extraction was performed automatically in 70 min. The instrument was controlled using Maxwell® RSC software (version 3.0) and enabled the simultaneous extraction of 16 RNA samples. After extraction, the obtained RNA was eluted in 50 µl of RNase-free water.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The extracted RNA was reverse transcribed into complementary DNA (cDNA) using the Transcriptor First Strand cDNA Synthesis Kit (Roche Life Science, Switzerland). The final volume of cDNA obtained was approximately 20 µl. To perform the qPCR analysis, a 1:10 dilution of each cDNA was prepared and 4 µl samples were loaded manually into each well of a 384-well plate (Roche Life Science). Subsequently, 6 µl of the primer mix was added to each well and sum up to the 4 µl of cDNA for a total of 10 µl in each well. The primer mix was prepared using 240 µl of SYBR Green Master Mix (Qiagen, Germany) and 48 µl of primers targeting the reference or target genes (i.e., a 1:6 dilution of primers). The primers were prepared by MicroSynth based on sequences used by Salamin et al. [10, 14]. All the reactions were performed in triplicates. The target genes were *ALAS2LC*, *ALAS2L*, *SLC4A1*, and *CA1*. Based on a previous study by Salamin et al. [10], three reference genes were used:

glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and the linear and circular versions of

regulator of cell cycle (*RGCC L* and *RGCC C*). Once loaded, the plate was centrifuged at 2,000 rpm for 2 min prior to the qPCR analysis. Thermal cycling was performed using the LightCycler® 480 System (Roche Life Science). Finally, the results were normalized and analyzed using LightCycler software (version 1.5.0.39).

Clinical study: rhEPO injection

A pilot clinical study was conducted by the Catalonian Anti-Doping Laboratory at the Fundaciò Institut Mar D'Investigaciones Mèdiques (IMIM), with the approval of the local Ethics Committee and the Spanish Medicine Agency. The study was performed in accordance with national and international law and procedure, and with the principles of the Declaration of Helsinki. As described by Reverter-Branchat et al. [9], two healthy male volunteers were recruited for the study. Each volunteer received three subcutaneous therapeutic doses (3500 IU, ~50 IU/kg) of first generation rhEPO, epoetin alpha (Eprex®, Janssen-Cilag, USA), at 48 h intervals. Blood collection was performed 72 h before the first rhEPO administration (Day -3) and then every day from the day of the first rhEPO administration (Day 0, Day 1, Day 2, Day 3, and Day 4). On Day 4, four blood samples were collected (hours 96 (h96), h97, h100, and h104). A final sample was also collected on Day 7. Blood was collected using two methods: in the first method, capillary blood (~20 µl) was sampled by a finger-prick and spotted directly onto DMPK-C cards (Whatman®, GE Healthcare), and in the second method, venous blood was collected in an EDTA tube and 20 µl of the sample was spotted onto DMPK-C cards. The blood spots were left to dry for a minimum of 4 h. DBSs generated from finger-pricks were stored at 4°C and those generated from venous blood were stored at room temperature.

To assess the suitability of the detection method for longitudinal follow-up and to compare gene expression levels in blood samples collected by finger-prick or the TAP™ device, and in RNA samples generated using manual and automated extraction methods, blood samples were collected from one healthy male and one healthy female. Blood was collected once per week for 5 weeks via a finger-prick and the TAP™ device, and 20 µl aliquots were spotted onto DMPK-C cards. The blood spots were then

left to dry and stored at room temperature. Samples collected on different days were analyzed at the same time after manual or automated RNA extraction.

Athlete samples

To compare manual and automated RNA extraction methods, samples were collected from 40 anonymous male consented athletes. DBSs were generated by spotting 20 µl of anonymized routine laboratory EDTA blood samples onto DMPK-C cards (Whatman®, GE Healthcare, USA). The cards were then left to dry and stored at 4°C.

Results and discussion

Our first step was to evaluate changes in the expression levels of the *ALAS2*, *CA1*, and *SLC4A1* genes in human blood samples after therapeutic administration of rhEPO. For this purpose, we used finger-prick DBSs generated during a previous study involving two male volunteers [9, 10] (Figure 1 and Supporting data).. RNA was extracted from the samples using the manual method. Consistent with the results obtained previously using DBS samples from the same volunteers [10], for both of the volunteers, the expression levels of *ALAS2LC* and *ALASL* started to increase 2 days after the first injection and reached a peak at Day 7. Moreover, the observed increase in the expression level of *ALAS2* (linear and circular) was higher than that of the percentage reticulocytes (%RET), one of the two key markers in the hematological module of the ABP. These results confirm the validity of *ALAS2* as a marker to detect rhEPO doping. There was also a clear increase in the expression level of *CA1* in blood samples from both volunteers after rhEPO administration, and the increase was higher than that of the %RET. The expression level of *SLC4A1* was also increased in the two volunteers after rhEPO administration, although the increase was similar to that of the %RET in one volunteer. Like *ALAS2*, *CA1* and *SLC4A1* showed the highest response 7 days after the first rhEPO injection.

Next, we compared the expression levels of *ALASL*, *ALAS2LC*, *CA1*, and *SLC4A1* in RNA samples extracted from DBSs using a manual or automated (Maxwell® RSC Instrument) method. For this purpose, 40 DBS samples from athletes were analyzed and the correlation between the expression level

of each gene in the paired RNA samples was calculated using Spearman's correlation (Figure 2). A strong significant correlation was observed for all three genes (*ALAS2LC*, $r = 0.92$, $p < 0.0001$; *ALASL*, $r = 0.93$, $p < 0.0001$; *CA1*, $r = 0.93$, $p < 0.0001$; *SLC4A1*, $r = 0.83$, $p < 0.001$), suggesting that both automated and manual RNA extraction methods are robust and sensitive enough to enable quantitation of reticulocyte-related mRNAs in DBS samples.

Subsequently, DBSs from three individuals with different expression levels of *ALAS2L, ALASLC, CA1*, and *SLC4A1* were analyzed in triplicate on the same day after automated and manual extraction of RNA. When manual extraction was performed, the mean intra-day coefficients of variation (CVs) for *ALAS2L*, *ALASLC*, *CA1*, and *SLC4A1* were 10.1%, 12.3%, 12.5%, and 11.2%, respectively. When automated extraction was performed, the mean intra-day CVs were 4.3%, 8.3%, 4.1%, and 8.5%, respectively. To assess inter-day variability, DBSs were analyzed in quintuplicate over 5 days. The mean CVs for manual extraction were 7.4% (*ALAS2L*), 9.7% (*ALAS2LC*), 12.8% (*CA1*), and 9.5% (*SLC4A1*), and for automated extraction were 9.5% (*ALAS2L*), 10.8% (*ALAS2LC*), 11.8% (*CA1*), and 8.5% (*SLC4A1*). Taken together, these data indicate that the manual and automated extraction procedures produced similar results.

To assess their suitability for longitudinal follow-up, the variabilities in the expression levels of *ALAS2L, ALASLC, CA1*, and *SLC4A1* in DBSs were assessed using samples from two healthy volunteers (one male and one female), collected once per week for 5 weeks. DBS samples were collected using finger-pricks and the TAP™ device, and RNA was extracted from both sample types using the manual or automated protocol. For the samples processed via manual RNA extraction, the inter-individual variabilities of finger-prick samples were similar to those of samples collected using the TAP™ device. These results are consistent with those reported previously [10]. Likewise, the inter-individual variabilities of the samples processed via automated RNA extraction were similar for both DBS collection protocols (Table 1). These data confirm that automated RNA extraction could be used for longitudinal follow-up of transcriptomic biomarkers in DBSs.

To assess the sensitivity of automated RNA extraction the analysis was made in DBSs spiked with venous EDTA blood samples, generated during a previous clinical study [9]. As described above, in this study, two volunteers received three rhEPO injections over 4 days. In the RNA samples extracted using the automated method, *ALAS2* expression increased progressively after the second rhEPO injection and was maximal 7 days after the first injection (3 days after the third injection) (Figure 3). These results are consistent with those obtained from the same volunteers using DBS samples that were extracted manually and collected via capillary finger-pricks (Figure 1 and [10]). Similarly, the expression levels of *CA1* and *SLC4A1* exhibited a significant maximum increase at 7 days after rhEPO injection. These results corroborate and support the data shown in Figure 1.

DBSs offer the advantage of facilitated sample collection and may complement the test menu of whole blood samples collected for the ABP. As demonstrated previously [9], four to five DBSs on the same card can be used to detect rhEPO. Moreover, other indirect biomarkers could be used to support suspicious test results, such as detection of the immature reticulocyte-specific protein CD71 [6] and the measurement of hemoglobin, as demonstrated by other groups [23, 24]. Altogether, these data could increase the specificity of detection of erythropoiesis stimulating agent abuse. In addition, analysis of the circular forms has improved the detection of RNA biomarkers in body fluids in forensic sciences [25, 26]. Similarly, analysis of the circular form of *ALAS2* enhances the detection of stimulated erythropoiesis in the anti-doping field [10]. In this study, an automated method was successfully applied to the measurement of transcriptomic biomarkers in RNA samples extracted from DBSs. The sensitivity of detection of alterations in the levels of erythropoiesis-related genes was found comparable between RNA samples extracted using a manual or automated method.

For future studies, the analysis of the circular forms of *SLC4A1* and *CA1* could be included to evaluate their applicability to improving the detection of blood doping.

In order to extend the knowledge on the confounding factors, it would also be interesting to investigate the detection of RNA-based biomarkers in a cohort of female volunteers, although the data for the

female volunteer shown in Table 1 suggest that hormonal cycles do not have a significant impact on the expression levels of *ALAS2*, *CA1*, and *SLC4A1*. Furthermore, it would be useful to examine the impact of hypoxia context (hypoxic chamber or altitude training) on the expreesion levels of the RNA biomarkers described here.

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Figure legends

Figure 1. Relative expression levels of *ALAS2L, ALAS2LC, SLC4A1,* and *CA1* in RNA samples extracted via a manual method from DBSs generated from finger-prick capillary blood samples after rhEPO injections.

The expression levels were compared with the %RET (gray line). Data were normalized to the corresponding levels of the reference genes. The dashed lines indicate subcutaneous injections of rhEPO (~50 IU/kg).

Figure 2. Correlations between paired DBS samples extracted using manual and automated methods (n = 40).

Figure 3. Relative expression levels of *ALAS2L, ALAS2LC, SLC4A1,* and *CA1* in RNA samples extracted via an automated method from DBSs generated from venous blood samples after rhEPO injections.

Data were normalized to the corresponding levels of the reference genes. The dashed lines indicate subcutaneous injections of rhEPO (~50 IU/kg).

Table 1. Inter-individual variation of RNA samples generated via manual or automated extraction methods from DBSs collected via a finger-prick or the TAP™ device. Subject 1: female; subject 2: male.

Supporting figures

Supporting Figure 1. Relative expression levels of *ALAS2L, ALAS2LC, SLC4A1,* and *CA1* in DBSs generated from finger-prick capillary blood samples in the second volunteer after rhEPO injections.

The expression levels were compared with the %RET (gray line). Data were normalized to the corresponding levels of the reference genes. The dashed lines indicate subcutaneous injections of rhEPO (~50 IU/kg).

Supporting Figure 2. Relative expression levels of *ALAS2L, ALAS2LC, SLC4A1,* and *CA1* in DBSs generated from venous blood samples in a second volunteer after rhEPO injections.

Data were normalized to the corresponding levels of the reference genes. The dashed lines indicate subcutaneous injections of rhEPO (~50 IU/kg).

FIGURE 1

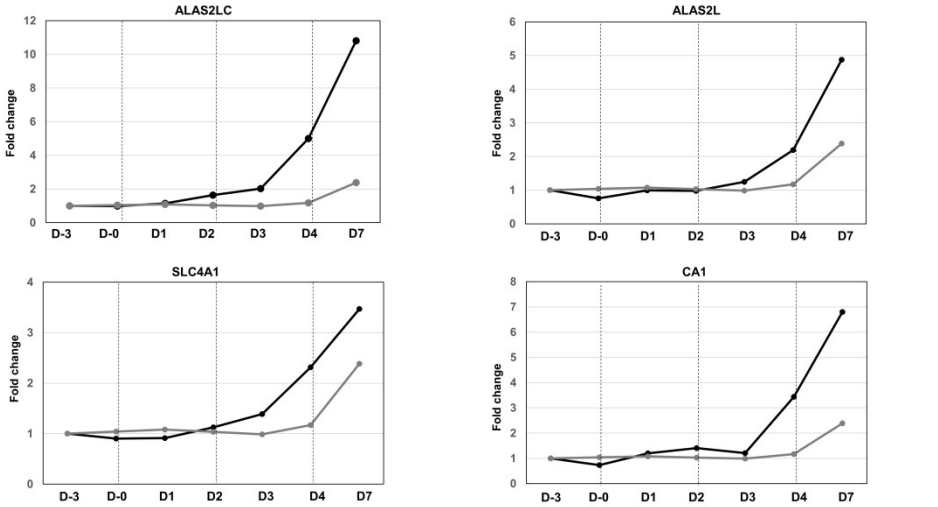

Figure 1. Relative expression levels of ALAS2L, ALAS2LC, SLC4A1, and CA1 in RNA samples extracted via a manual method from DBSs generated from finger-prick capillary blood samples after rhEPO injections. The expression levels were compared with the %RET (gray line). Data were normalized to the corresponding levels of the reference genes. The dashed lines indicate subcutaneous injections of rhEPO (~50 IU/kg).

338x190mm (300 x 300 DPI)

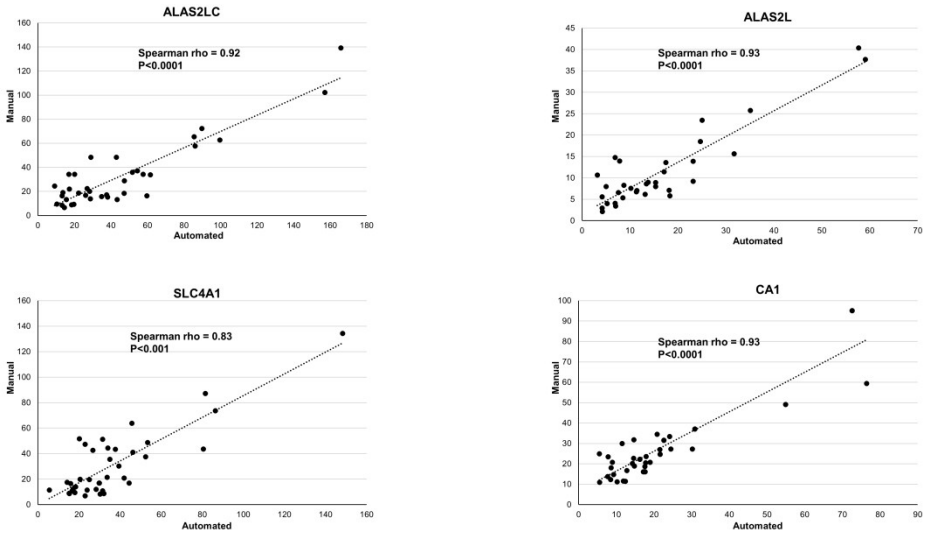

Figure 2. Correlations between paired DBS samples extracted using manual and automated methods (n = 40).

338x190mm (300 x 300 DPI)

FIGURE 3

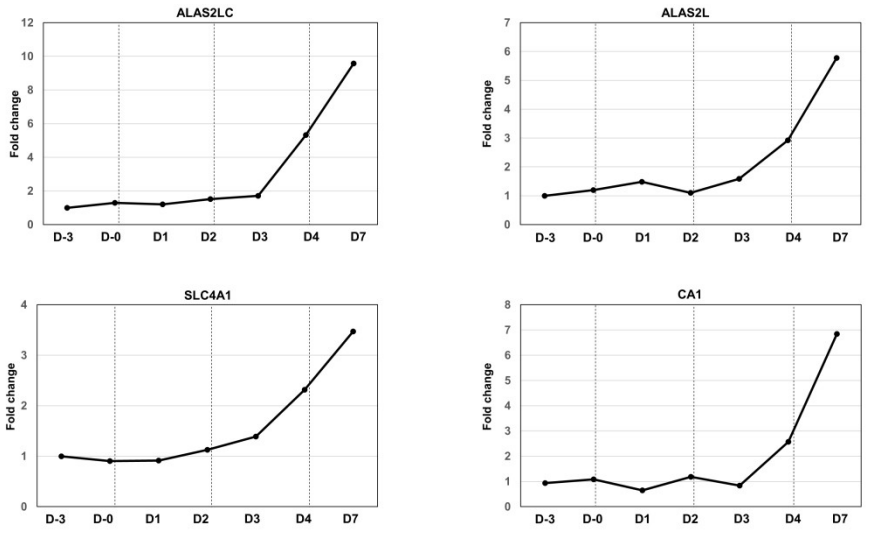

Figure 3. Relative expression levels of ALAS2L, ALAS2LC, SLC4A1, and CA1 in RNA samples extracted via an automated method from DBSs generated from venous blood samples after rhEPO injections.
Data were normalized to the corresponding levels of the reference genes. The dashed lines indicate subcutaneous injections of rhEPO (~50 IU/kg).

338x190mm (300 x 300 DPI)

Table 1

	Manual extraction (CV%)			
	Subject 1		Subject 2	
	Finger prick	TAP device	Finger prick	TAP device
ALAS2LC	12.4	13.1	14.4	14
ALAS2L	17.1	15.2	13.1	13.4
SLC4A1	16.1	15.8	14.4	11.3
CA1	15.4	14.6	7.4	4.6
	Automated extraction (CV%)			
	Subject 1		Subject 2	
	Finger prick	TAP device	Finger prick	TAP device
ALAS2LC	11.1	12.9	13.2	13.5
ALAS2L	15.3	15.4	13.1	12.4
SLC4A1	15.9	12.7	10.2	10.1
CA1	14.9	13.2	5.5	7.8

Table 1. Inter-individual variation of RNA samples generated via manual or automated extraction methods from DBSs collected via a finger-prick or the TAP™ device in non treated subjects. Subject 1: female; subject 2: male.

338x190mm (300 x 300 DPI)