Journal of Immunological Methods 465 (2019) 13–19

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

IL8 and EDEM3 gene expression ratio indicates peripheral blood mononuclear cell (PBMC) quality

Olga Kofanova[a,g], Camille Bellora[a], Rocio Aguilar Quesada[b,g], Alexandre Bulla[c,g], Sonia Panadero-Fajardo[b], Marc Keipes[d], Kathi Shea[e,g,1], Mars Stone[f,g], Pierre Lescuyer[c], Fay Betsou[a,g,*]

[a] Integrated Biobank of Luxembourg, 1 rue Louis Rech, 3555 Dudelange, Luxembourg
[b] Biobanco del Sistema Sanitario Público de Andalucía (SSPA Biobank), Avda. del Conocimiento s/n 18016, Granada, Spain
[c] Service de Médecine de Laboratoire, Hôpitaux Universitaires de Genève, 1211 Genève 14, Switzerland
[d] ZithaGesondheetsZentrum, 36 rue Zithe, 2763, Luxembourg
[e] Precision for Medicine, 8425 Progress Drive, Frederick, MD 21701, USA
[f] Blood Systems Research Institute, 270 Masonic Ave., San Francisco, CA 94118, USA
[g] International Society for Biological and Environmental Repositories (ISBER), Biospecimen Science Working Group, Canada

ARTICLE INFO

Keywords:
PBMC
Quality control
Preanalytical

ABSTRACT

Background: Uncontrolled preanalytical variables can reduce the accuracy and reproducibility of downstream analytical results from peripheral blood mononuclear cells (PBMCs).

Methods: PBMCs were isolated from EDTA and citrate-anticoagulated blood samples, obtained from healthy subjects and patients with inflammatory and infectious conditions. PBMC-derived RNA samples were examined for gene expression changes induced by extended blood pre-centrifugation delays at 4 °C and RT. We used Taqman RTqPCR to evaluate the combination of two target genes for their "diagnostic performance" in identifying EDTA and citrate-anticoagulated PBMC samples with extended pre-centrifugation times.

Results: We established the PBMC preanalytical score, a gene expression metric to asses the PBMC quality related to the pre-centrifugation delay at room temperature for different anticoagulants. The PBMC preanalytical score measurement can identify:

(1) EDTA PBMC samples or RNA extracted from these PBMCs with RT precentrifugation times > 48 h with 98% sensitivity and 87% specificity at a cutoff of 57.
(2) citrate PBMC samples or RNA extracted from these PBMCs with RT precentrifugation times of > 48 h with 92% sensitivity and 84% specificity at a cutoff of 348.

Conclusion: The proposed PBMC preanalytical score may enable objective PBMC sample qualification for downstream applications, which may be influenced by blood precentrifugation delays.

1. Introduction

Uncontrolled preanalytical variables can reduce the accuracy and reproducibility of downstream analytical results from peripheral blood mononuclear cells (PBMCs) and hence the specimen's fitness-for-purpose in clinical or experimental immunology. The most critical preanalytical variables for PBMC samples are the type of anticoagulant used for blood collection, precentrifugation time and temperature, centrifugation conditions, time at RT for isolated PBMCs (otherwise called "post-centrifugation time") and the long-term storage temperature (Betsou et al., 2010; Lehmann et al., 2012). Understanding how these critical variables impact specimen quality is essential for determining a specimen's fitness for purpose for specific functional analyses including downstream gene expression.

Abbreviations: PBMC, peripheral blood mononuclear cell; RT, room temperature; RA, rheumatoid arthritis; RIS, RNA Integrity Score; ACD, Acid Citrate Dextrose; CPT, Cell Preparation Tube; PAF, Platelet Activating Factor; RPII, RNA polymerase II gene

* Corresponding author at: Integrated Biobank of Luxembourg, 1 rue Louis Rech, 3555 Dudelange, Luxembourg.
E-mail addresses: olga.kofanova@ibbl.lu (O. Kofanova), camille.bellora@ibbl.lu (C. Bellora), rocio.aguilar.quesada@juntadeandalucia.es (R.A. Quesada), sonia.panadero@juntadeandalucia.es (S. Panadero-Fajardo), marc.keipes@hopitauxschuman.lu (M. Keipes), kathi.shea@brooksbiostorage.com (K. Shea), mstone@bloodsystems.org (M. Stone), Pierre.Lescuyer@hcuge.ch (P. Lescuyer), fay.betsou@ibbl.lu (F. Betsou).
[1] Current affiliation, Brooks Life Science Systems, 2910 Fortune Circle West, Suite E, Indianapolis, IN 46241, USA.

https://doi.org/10.1016/j.jim.2018.11.012
Received 27 March 2018; Received in revised form 19 November 2018; Accepted 19 November 2018
Available online 26 November 2018

Table 1
Characteristics of the different cohorts of samples and baseline ranges of the PBMC score.

	[a]IBBL	[a]IBBL	[b]HUG	[c]BSRI	[d]P4M	[e]BBSSPA	[f]EWB	IBBL "POP"
Number of donors (M,F)	5 M, 5F	3F	6 M, 10F	5 M, 5F	11	5 M, 5F	3 M, 7F, 1 not specified	7 M, 3F
Age	22–62	25–40	32–62	24–60	Not specified	42–58	23–65	34–57
Health status	healthy	healthy	healthy	healthy	Rheumatoid Arthritis (RA)	HIV positive	4 Acute pancreatitis (AP) 7 Rheumatoid Arthritis (RA)	healthy
Collection tubes	BD K2EDTA, ref. 367,525 BD CPT sodium citrate, ref. 362,761	BD Li Heparin ref. 367,526	BD K2EDTA, ref. 367,525 BD CPT sodium citrate, ref. 362,761	BD K2EDTA, ref. 366,643 BD CPT sodium citrate, ref. 362,761	K2EDTA CPT (exact ref. not specified)	BD K2EDTA, ref. 368,861 BD ACD—B, ref. 367,756	BD K2EDTA, ref. 367,525 BD CPT sodium citrate, ref. 362,780	BD K2EDTA, ref. 367,525
Pre-centrifugation conditions tested	30 min, 3 h, 23–24 h, 48 h at 4 °C 30 min, 3 h, 23–24 h, 48 h at RT	3 h, 24 h, 48 h at RT	30 min, 3 h, 23–24 h, 48 h at RT	30 min, 3 h, 23–24 h, 48 h at RT	30 min at RT	3 h, 48 h at RT	3 h, 48 h at RT	2–4 h at RT
Centrifugation program	2000 *g* RT 20 min brake 5	2000 g RT 20 min brake 5	1800 g RT 20 min brake 5	2000 g RT 20 min brake 5	Not specified	1800 g RT 20 min brake 5	2000 g RT 20 min	2000 g RT 20 min brake 5
Range of PBMC score at pre-centrifugation conditions ≤ 3 h RT	EDTA, 0–6 CPT, 0–1	Heparin, 6–18	EDTA, 0–27 CPT, 0–155	EDTA, 2–42 CPT, 59–560	EDTA, 279–1078 CPT, 421–2060	EDTA, 0–17 ACD—B, 3–47	EDTA RA, 3–14 EDTA AP, 18–47 CPT RA, 5–63 CPT AP, 128–233	EDTA, 0–2.5

[a] IBBL, Integrated Biobank of Luxembourg.
[b] HUG, Hôpitaux Universitaires de Genève.
[c] *BSR*I, Blood Systems Research Institute.
[d] P4M, Precision for Medicine.
[e] BBSSPA, SSPA Biobank.
[f] EWB, EastWestBio.

PBMC-based gene expression profiling is frequently used for discovery of clinically relevant biomarkers in different clinical areas (Staratschek-Jox et al., 2009). The precentrifugation time is a critical preanalytical variable in the scope of viable or non-viable PBMC gene expression analyses. Active metabolism of the blood cells at RT leads to oxygen depletion and many clinically important gene expression pathways are affected by the resulting hypoxia (Liu et al., 2012). Both the anticoagulant type and processing delays as short as 2 hours can influence blood cell transcriptomic profiles (Baechler et al., 2004; Barnes et al., 2010; Debey et al., 2004; Hebels et al., 2013).

The time between venipuncture and cryopreservation has been shown to be the most critical factor affecting PBMC recovery and function. It has been shown that a 24 hour delay in blood processing leads to more than ten-fold increase of granulocyte contamination of the PBMCs and impairs T cell function (McKenna et al., 2009). Such delays lead to significantly lower recovery, viability and ELISPOT reactive T cells (Bull et al., 2007; Weinberg et al., 1998), and lower ability of NK cells to degranulate and secrete cytokines (Naranbhai et al., 2011).

Thus, for legacy PBMC collections, where the preanalytical conditions may not have been documented (Lehmann et al., 2012), it is important to be able to apply retrospective qualification assays of sufficient sensitivity and specificity to determine how unknown preanalytical conditions may have affected specimen quality which impacts downstream applications. High specificity is required particularly in those cases where the downstream analysis is expensive and confirmation is needed of a PBMC sample's fitness for purpose before proceeding with analysis. Assays for qualification and quality stratification of different specimen types were reviewed (Betsou et al., 2013; Betsou et al., 2016). For viable PBMC suspensions, assays for concentration, sterility, viability, purity (absence of granulocyte contamination), genomic stability, and biological activity (response to antigen stimulation) are well established. These assays are useful to assess the feasibility of downstream gene expression or functional analyses but are insufficient to guarantee the accuracy of such downstream analyses. Similarly, once RNA has been extracted from PBMCs, assays for RNA concentration, purity and integrity are well established. These assays are useful to assess the feasibility of downstream gene expression analyses, but again, are insufficient to guarantee their accuracy. For this, an assay that can “diagnose” prolonged precentrifugation times of either viable or non-viable PBMC samples is needed.

Based on previous literature, we selected two unstable PBMC gene targets; IL-8 whose gene expression increases (Baechler et al., 2004; Rainen et al., 2002) and EDEM3 whose gene expression decreases (Benita et al., 2009) with precentrifugation delay. The performance of their combination was evaluated for the “diagnosis” of the precentrifugation conditions of the original blood samples, and a PBMC preanalytical score was established.

2. Materials and methods

2.1. Samples

PBMC samples from healthy individuals from the Integrated Biobank of Luxembourg, the Biobank of the Geneva University Hospitals and the San Francisco Blood Systems Research Institute were used. Ten IBBL EDTA PBMC samples from an independent collection of healthy donors (“POP”) and three IBBL heparin PBMC samples were also used. PBMC samples from rheumatoid arthritis (RA) patients were provided by Precision for Medicine (Frederick, MD). PBMC samples from HIV patients were provided by the SSPA Biobank (Andalucía), and finally PBMC samples from acute pancreatitis (AP) and rheumatoid arthritis (RA) patients were procured from EastWestBio (Ukraine). Citrate-anticoagulated blood was collected in either ACD or CPT blood collection tubes. HIV positive diagnosis was established on the basis of LIAISON® XL murex HIC Ab/Ag positive assays. Rheumatoid arthritis diagnosis was established on the basis of functional impairment of joints. Acute pancreatitis diagnosis was established on the basis of ultrasound sonography and elevated levels of α-amylase. Table 1 summarizes the demographic and clinical data of the donors and the preanalytical conditions of the blood specimens used. PBMCs were isolated with Ficoll (Eurobio, ref. CMCMSL01 or GE Healthcare, ref. 17-1440-03), and aliquots of $5x10^6$ cells in RNA Cell protect Reagent (Qiagen, ref. 76526) were prepared and frozen at -80 °C until analysis. All donors gave their informed consent and the protocol has received approval from the ethics committee (CNER approval ##201107/02, CNER approval ##20093/04).

2.2. RNA processing

RNA was extracted from $5x10^6$ frozen PBMCs with the miRNeasy Mini kit (Qiagen, ref. 217004), including DNase digestion (Qiagen ref. 79254), according to manufacturer's instructions. RNA concentration was measured by spectrophotometry with a Synergy™ Mx instrument (Biotec). RNA integrity was measured with a Qiaxcel Advanced instrument and Qiaxcel RNA QC kit (Qiagen ref. 929102) providing RNA Integrity Score (RIS) measurements.

2.3. RTqPCR

A quantity of 100 ng of RNA was reverse transcribed at a concentration of 10ng/µl, using the High Capacity cDNA reverse transcription kit (Life Tech ref. 4368814), with random primers, Multiscribe Reverse Transcriptase and RNase inhibitor (Life Technologies, ref. N8080119). The RT reaction was carried out for 10 min at 25 °C, followed by 120 min at 37 °C and 5 min at 85 °C on a BioRad thermocycler.

The following primers and probes, from Life Technologies, were used: TaqMan Gene Expression Assay Hs99999034_m1 (IL8), Hs00228632_m1 (EDEM3), Hs00172187_m1 (POLR2A). A volume of 5 µl of cDNA was used for each qPCR reaction, in a total reaction volume of 50 µl. The qPCR reactions were performed with TaqMan Universal PCR Master Mix (Life Technologies ref. 4304437). The following thermocycling program was applied: 2 min at 50 °C, followed by 10 min at 95 °C and 40 cycles of 15 sec at 95 °C and 1 min at 60°C. The Human Universal Reference Total RNA (Clontech ref. 636538) was used as calibrator and internal positive control sample. Each run included an RT negative control, a PCR negative control, and a positive control (calibrator sample). Each qPCR reaction was performed in triplicate on a 7500 Fast Real-Time PCR instrument. The amplification efficiency was calculated for each of the three targets, with six serial twofold dilutions of the positive control cDNA sample.

Data analysis was performed with the 7500 Fast System SDS software version 1.1.4 secure. Outliers were identified and removed when a Ct value deviated more than 0.5 Ct from the mean of the triplicate. qPCR reactions with $Ct > 35$ were considered to be below the detection limit. The POLR2A target was used for normalisation.

2.4. Statistical analyses

Summary statistics were performed in Excel version 2013. ROC curve analyses, including calculations of the diagnostic sensitivities and specificities (and their confidence intervals), of the ratio fold-change IL8 to fold-change EDEM3, for the diagnosis of precentrifugation delays at RT, were performed with the AnalyseIT software version 2.30. In the evaluation of the diagnostic performance for the 24 hour delay, samples with ≥24 hour were considered positive, while samples with 3 hour delays were considered negative (control group). In the evaluation of the diagnostic performance for the 48 hour delay, samples with ≥48 hour were considered positive, while samples with 3 hour delays were considered negative (control group).The cut-off values that were

selected were those corresponding to the combinations of higher specificity and sensitivity.

3. Results

We isolated PBMCs, extracted RNA and analysed the samples from seven different collections: four collections from healthy donors (IBBL, HUG, BSRI, IBBL "POP") and three collections from donors with infectious or inflammatory conditions (P4M, BBSSPA, EWB). We analysed samples from donors with Rheumatoid Arthritis (RA) and Acute Pancreatitis (AP) because IL8 production is known to be increased in these conditions. Therefore, such samples were the most relevant to assess the robustness and field of application of our method. Each donor provided multiple blood tubes, and each tube underwent different preanalytical processing (Table 1).

3.1. RNA integrity

The RNA integrity values were measured by Qiaxcel, as RNA Integrity Score (RIS) values. They were in the range of 7.2 - 8.2 at +4°C and 6.8 - 8.1 at RT, for the EDTA-anticoagulated samples, and in the range of 7.1 - 8.5 at +4°C and 6.4 - 8.5 at RT for CPT-anticoagulated samples, with lower RIS observed at longer pre-centrifugation times.

3.2. PBMC preanalytical score

The RTqPCR method was analytically validated. The amplification efficiency for each of the three targets was 79.4 % for IL8, 79.1 % for EDEM3 and 86.6 % for POLR2A. The stability of the normalisation target POLR2A across the experimental conditions was verified, and corresponded to an average fold-change of 1.09 (standard deviation 0.16) in EDTA-anticoagulated samples, and a fold-change of 0.95 (standard deviation 0.16) in citrate-anticoagulated samples.

The formula used for the calculation of the PBMC preanalytical score was the following:

PBMC preanalytical score = $2^{(-\Delta\Delta Ct_{IL8})} / 2^{(-\Delta\Delta Ct_{EDEM3})}$, where $\Delta\Delta Ct_{IL8} = \Delta Ct_{IL8\_sample} - \Delta Ct_{IL8\_calibrator}$ and $\Delta\Delta Ct_{EDEM3} = \Delta Ct_{EDEM3\_sample} - \Delta Ct_{EDEM3\_calibrator}$. The baseline (corresponding to < 3 hour RT precentrifugation conditions) reference ranges of the PBMC preanalytical score in the different populations of samples are shown in Table 1. The analytical reproducibility of the assay was calculated on raw Ct values, obtained with an internal control sample. Standard deviations obtained were 0.07 Ct for the IL8 target, 0.07 Ct for the EDEM3 target and 0.1 Ct for the POLR2A target (n = 36 assays).

The "diagnostic performance" of the assay refers to the ability of the PBMC preanalytical score to differentiate RNA samples, extracted from PBMCs with long (≥24 hr or ≥48 hr) precentrifugation times, from RNA samples, extracted from PBMCs with short (≤3 hr) pre-centrifugation times. A sample with long precentrifugation time is considered as a "positive" sample.

3.3. Diagnostic performance of the PBMC preanalytical score in PBMC samples from EDTA-anticoagulated blood

The diagnostic performance was evaluated in each of the three independent cohorts of samples from healthy donors separately and then in the whole healthy population. No significant change in gene expression was observed at 4°C (data not shown). When EDTA blood samples were stored at RT for 24 hours before centrifugation, the PBMC preanalytical score showed a sensitivity of 99% (CI 92–100%, $p < 0.05$) and a specificity of 98% (CI 89–100%, $p < 0.05$) in the diagnosis of the ≥24 hour delay against the ≤3 hour delay, at a cut-off value of 28, in the whole healthy donor population (Table 2A, Figure 1A). When EDTA blood samples were stored at RT for 48 hours before centrifugation, the PBMC preanalytical score showed sensitivity of 97% (CI 85–100%, $p < 0.05$) and specificity 98% (CI 89–100%, $p < 0.05$) in the diagnosis of the ≥48 hour delay against the ≤3 hour delay, at a cut-off value of 42.5, in the group of the healthy samples (Table 2B, Figure 1B).

Table 2
Diagnostic performance of the PBMC preanalytical score in the detection of precentrifugation delays of ≥24 h in EDTA samples from healthy donors (Table 2A) or ≥ 48 h (Table 2B) in EDTA samples from healthy and diseased donors. The number of valid measurements is indicated in parentheses (n).

	Cut-off	AUC	Sensitivity (95% CI)	Specificity (95% CI)
A				
IBBL (*n* = 39)	57	1.00	1.00 (0.82–1.00)	1.00 (0.83–1.00)
HUG (*n* = 46)	76	1.00	1.00 (0.88–1.00)	1.00 (0.79–1.00)
BSRI (*n* = 29)	27	0.99	1.00 (0.82–1.00)	0.90 (0.56–1.00)
Global/healthy (*n* = 114)	28	1.00	0.99 (0.92–1.00)	0.98 (0.89–1.00)
B				
IBBL (n = 29)	57	1.00	1.00 (0.66–1.00)	1.00 (0.83–1.00)
HUG (*n* = 32)	89	1.00	1.00 (0.79–1.00)	1.00 (0.79–1.00)
BSRI (*n* = 19)	27	0.99	1.00 (0.66–1.00)	0.90 (0.56–1.00)
Global/healthy (*n* = 80)	42.5	1.00	0.97 (0.85–1.00)	0.98 (0.89–1.00)
Global/healthy and disease (*n* = 130)	56.6	0.94	0.98 (0.90–1.00)	0.87 (0.77–0.94)

In the HIV positive population of EDTA samples (n = 20 samples), the PBMC preanalytical score had a sensitivity of 100% (CI 69–100%, $p < 0.05$) and a specificity of 100% (CI 69–100%, $p < 0.05$) at the cut-off value 67.6 for the detection of RNA samples extracted from cells with 48 hr pre-centrifugation delay at RT.

In the inflammatory population of EDTA samples (n = 30), the diagnostic cut-off values were much higher. For the detection of pre-centrifugation times of ≥48 hrs, the PBMC preanalytical score threshold of 1258 gave 100% sensitivity (CI 69–100%, $p < 0.05$) and 100% specificity (CI 83–100%, $p < 0.05$).

When all cohorts of samples, both those collected from healthy donors and from inflammatory and infected patients, were analysed together, the PBMC preanalytical score, at a threshold of 57, could detect EDTA-anticoagulated samples with pre-centrifugation times ≥48 hrs with a sensitivity of 98% (CI 90–100%, $p < 0.05$) and a specificity of 87% (CI 77–93.5%) (Figure 2A). The AUC values are shown in Table 2.

3.4. Diagnostic performance of the PBMC preanalytical score in PBMC samples from citrate-anticoagulated blood

The diagnostic performance was evaluated in each of the three independent populations of samples from healthy donors separately and then in the whole healthy population. No significant change in gene expression was observed at 4°C (data not shown). When CPT blood samples were stored at RT for 24 hours before centrifugation, the PBMC preanalytical score showed a sensitivity of 80% (CI 68–89%, $p < 0.05$) and a specificity of 84% (CI 70–93%, $p < 0.05$) in the diagnosis of the ≥24 hour delay against the ≤3 hour delay, at a cut-off value of 167, for samples from healthy subjects (Table 3A, Figure 1C). When CPT blood samples stood at RT for 48 hours before centrifugation, the PBMC preanalytical score showed a sensitivity of 90% (CI 73–98%, $p < 0.05$) and a specificity of 91% (CI 78–98%, $p < 0.05$) in the diagnosis of the ≥48 hour delay compared with the ≤3 hour delay at a cut-off value of 277 in the group of the whole healthy donor population (Table 3B, Figure 1D).

In the HIV positive population of ACD samples (n = 20 samples), the diagnostic cut-off values were much higher. The PBMC preanalytical score had a sensitivity of 100% (CI 69–100%, $p < 0.05$) and a specificity of 100% (CI 69–100%, $p < 0.05$) at the cut-off value 1186.

In the inflammatory population of CPT samples (n = 29), the optimum diagnostic cut-off values were also much higher. For the detection of precentrifugation times of ≥48 hours, the PBMC preanalytical score threshold of 1948 led to 78% sensitivity (CI 40–97%, $p < 0.05$)

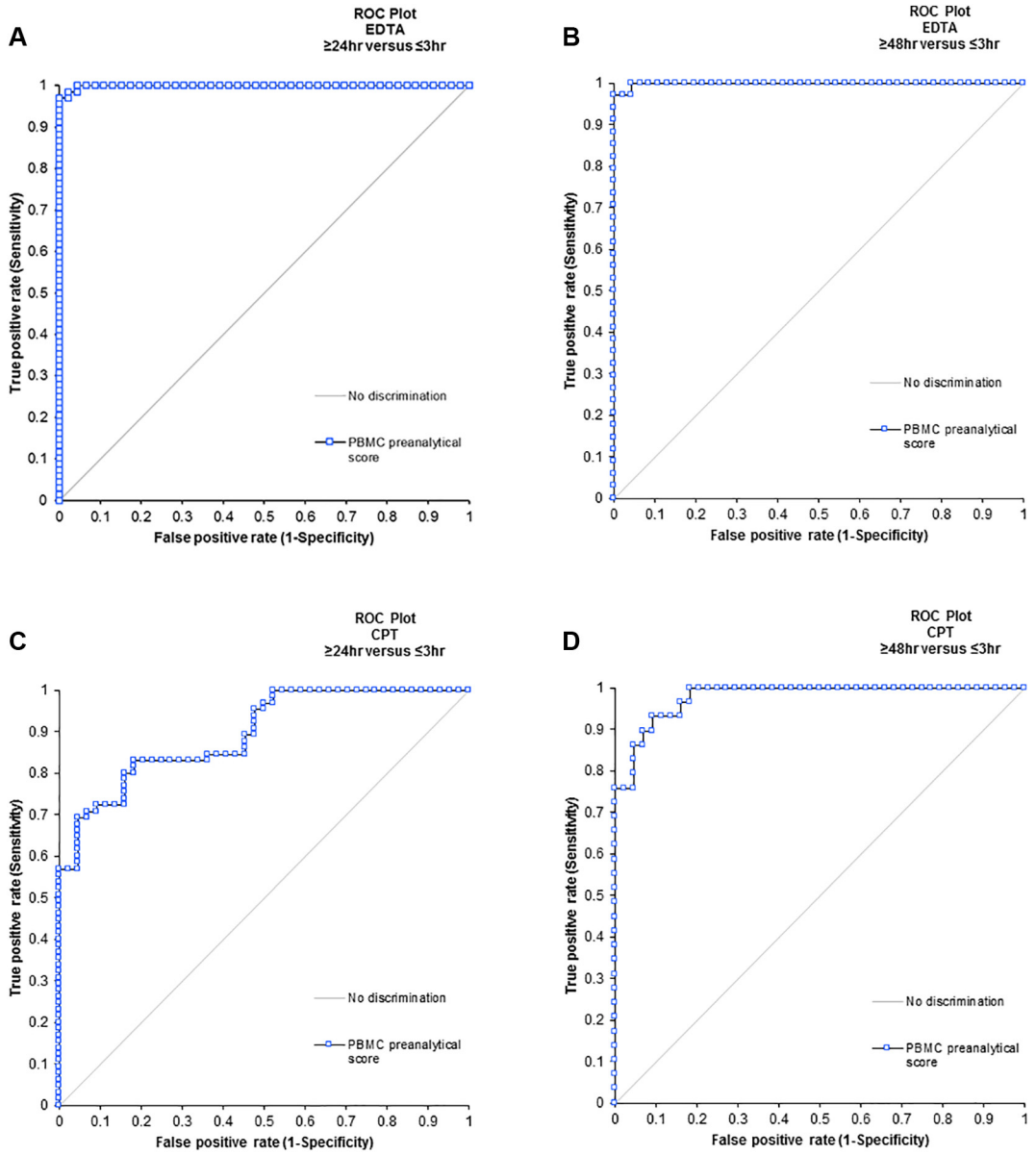

Fig. 1. ROC analysis based on the PBMC preanalytical score, in healthy donors.
Analysis of EDTA-anticoagulated PBMC samples, for 24 h (A) and 48 h (B) delayed processing, and of citrate-anticoagulated PBMC samples, for 24 h (C) and 48 h (D) delayed processing.

and 95% specificity (CI 75–100%, $p < 0.05$).

When all populations of samples, both those collected from healthy donors and from inflammatory and infected patients, were analysed together, the PBMC preanalytical score, at a threshold of 348, could detect citrate-anticoagulated samples of pre-centrifugation times ≥48 hours with sensitivity of 92% (CI 80–98%, $p < 0.05$) and specificity of 84% (CI 73–91%) (Fig. 2B). The AUC values are shown in Table 3.

3.5. PBMC preanalytical score in PBMC samples from heparin-anticoagulated blood

The field of application of the PBMC preanalytical score was found to extend also to heparin anticoagulated samples. The range of the score in the samples collected from three healthy donors was 6 – 18 at 3 hours, 341 – 615 at 24 hours and 2854 – 3462 at 48 hours of pre-centrifugation delay at room temperature.

4. Discussion

The focus of our study is PBMC isolated from anticoagulated whole blood, collected without RNA stabilisers. This study does not concern RNA from blood collected in tubes, such as PAXgene RNA (PreAnalytiX) or Tempus (Applied Biosystems). These tubes contain solutions that inhibit RNA degradation and metabolic activities by lysing blood cells and stabilizing nucleic acids. Recently, a blood collection tube has been commercialised which stabilises gene expression levels whilst maintaining intact cells (Cell-Free RNA BCT by Streck Inc) upon three-day precentrifugation delays at RT (Das et al., 2014) and could be considered for future studies.

Four mRNA biomarkers (USP32, LMNA, FOSB, TNRFSF10C) have previously been suggested for monitoring the preanalytical quality of mRNA in blood samples (Zhang et al., 2014), however no formula for their use has been proposed and no ROC curve analysis has been published.

Interleukin-8 (IL-8) is a potent neutrophil chemoattractant and

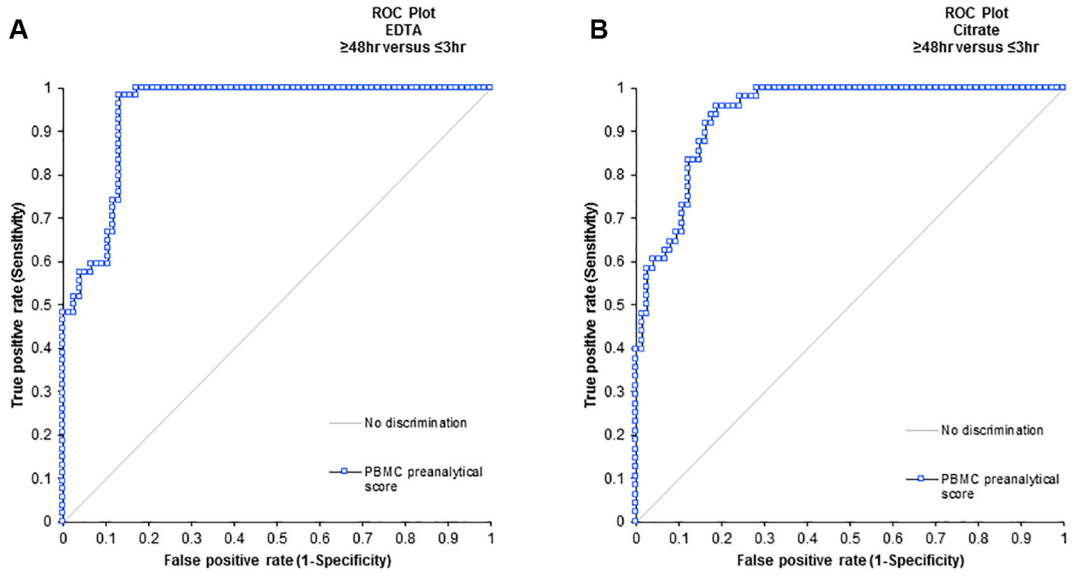

Fig. 2. ROC analysis based on the PBMC preanalytical score, in both healthy and diseased donors.
Analysis of EDTA-anticoagulated PBMC samples, for 48 h delayed processing (A), and of citrate-anticoagulated PBMC samples, for 48 h delayed processing (B).

Table 3
Diagnostic performance of the PBMC preanalytical score in the detection of precentrifugation delays of ≥24 h in citrate samples from healthy donors (Table 3A) or ≥ 48 h (Table 3B) in citrate samples from healthy and diseased donors. The number of valid measurements is indicated in parentheses (n).

	Cut-off	AUC	Sensitivity (95% CI)	Specificity (95% CI)
A				
IBBL (n = 38)	2.8	1.00	1.00 (0.82–1.00)	1.00 (0.83–1.00)
HUG (n = 41)	139	0.98	0.96 (0.81–1.00)	0.93 (0.66–1.00)
BSRI (n = 30)	560	0.96	0.80 (0.56–0.94)	0.90 (0.56–1.00)
Global/healthy (n = 109)	167	0.89	0.80 (0.68–0.89)	0.84 (0.70–0.93)
B				
IBBL (n = 28)	119	1.00	1.00 (0.63–1.00)	1.00 (0.83–1.00)
HUG (n = 25)	401	1.00	1.00 (0.72–1.00)	1.00 (0.77–1.00)
BSRI (n = 20)	664	1.00	1.00 (0.69–1.00)	1.00 (0.69–1.00)
Global/healthy (n = 73)	277	0.98	0.90 (0.73–0.98)	0.91 (0.78–0.98)
Global/healthy and disease (n = 122)	348	0.94	0.92 (0.80–0.98)	0.84 (0.73–0.91)

activator, produced by different cell types during inflammation. It has recently been confirmed in a microarray experiment comparing different times of exposure of EDTA blood to RT that IL-8 is the gene with the maximum fold-change (Franken et al., 2016). The mechanism of increased IL-8 gene expression in PBMCs upon prolonged blood storage may be linked to the platelet activating factor (PAF) (Denault et al., 1997), which is increased during storage of whole blood (Silliman et al., 1994). We included samples from RA patients because it is known that monocytes from RA patients express higher levels of IL8 mRNA than cells from healthy donors (Schulze-Koops et al., 1997).

Endoplasmic reticulum degradation-enhancing α-mannosidase-like protein 3 (EDEM3) is an α1,2-mannosidase involved in quality control of misfolded proteins (Hirao et al., 2006).

The RNA polymerase II gene (POLR2A) was selected for normalisation, since this gene has been shown to be the most stable and robust to different cellular types and cellular activation conditions (Radonić et al., 2004).

Concerning the precentrifugation temperature, short-term storage of the blood at RT is best practice for viable PBMC recovery. Indeed, higher viability and lower granulocyte contamination are obtained from blood stored at RT (McKenna et al., 2009). Refrigerated temperature, on the other hand, has the advantage of slowing down cell metabolic activity and is more fit-for-purpose for gene expression studies. However, in the majority of clinical trials collecting viable PBMCs for both functional and gene expression assays, and where functional assays are considered to be the most important endpoint, the precentrifugation temperature is RT.

Overall, the PBMC preanalytical score was much higher in citrate-anticoagulated than EDTA-anticoagulated samples. This may be due to citrate altering IL-8 transcription (Ashbrook et al., 2015). We have no explanation for the much lower PBMC preanalytical score at 24 hours in the IBBL CPT samples relative to the HUG and BSRI samples.

In inflammatory samples, the assay showed excellent sensitivity, but lower specificity with a risk of discarding samples of good quality. However, detection of good quality samples, with short precentrifugation delays, was 100% reliable. Therefore, also in collections of samples in inflammatory disease areas, the assay is useful when molecular immunologists want to confirm the preanalytical quality of PBMC samples before engaging in expensive analyses or when they need to be certain of the absence of preanalytical bias.

We could not reliably calculate a diagnostic threshold for the detection of 24-hour and 48-hour precentrifugation delay at RT in heparin-anticoagulated PBMC samples, due to the small number of donors examined. However, the assay is also applicable to heparin PBMC samples since the PBMC preananalytical score showed an increase of roughly 50-fold at 24 hours and 300-fold at 48 hours relative to the 3-hour precentrifugation delay.

Derbey-Pascher *et al* studied the instability of PBMC transcriptome to long-term cryopreservation of PBMCs in liquid nitrogen and found 190 genes to be affected by freezing time of up to 60 months (Debey-Pascher et al., 2011). Neither the IL-8 nor the EDEM3 were among these genes. Furthermore, IL8 expression levels were not altered by 6 year storage of stabilized PBMCs at -80°C (Duale et al., 2014). Therefore, the PBMC preanalytical score assay is expected to be robust to long-term cryopreservation of either stabilized or unstabilized PBMCs.

More extensive validation needs to be conducted to establish the robustness of this assay to more extended precentrifugation delays, as a decrease of IL-8 gene expression level after a certain time point might be possible, and therefore might influence the sensitivity of the PBMC preanalytical score. Further validation would also lead to more robust calculation of baseline reference ranges and diagnostic threshold(s) in heparin samples.

In conclusion, blood precentrifugation conditions are critical parameters to avoid bias in PBMC-based gene expression or functional

studies. It is now possible to assess the precentrifugation conditions, based on the proposed PBMC preanalytical gene expression score.

- Blood collected with EDTA anticoagulant:
  - ○ PBMC preanalytical score higher than 28 indicates PBMC samples, or RNA extracted from these PBMCs, with RT precentrifugation times of > 24 h (99% sensitivity, 98% specificity) (applicable in healthy donor populations).
  - ○ PBMC preanalytical score higher than 57 indicates PBMC samples, or RNA extracted from these PBMCs, with RT precentrifugation times of > 48 h (98% sensitivity, 87% specificity) (applicable in healthy or diseased populations). The same threshold seems to be applicable to samples collected with heparin anticoagulant.
- Blood collected with citrate anticoagulant:
  - ○ PBMC preanalytical score higher than 167 indicates PBMC samples, or RNA extracted from these PBMCs, with RT precentrifugation times of > 24 h (80% sensitivity, 84% specificity) (applicable in healthy donor populations).
  - ○ PBMC preanalytical score higher than 348 indicates PBMC samples, or RNA extracted from these PBMCs, with RT precentrifugation times of > 48 h (92% sensitivity, 84% specificity) (applicable in healthy or diseased populations).

This score will enable objective PBMC sample qualification for downstream assays that are sensitive to blood precentrifugation delays, such as gene expression analyses and antigen-specific response assays.

Acknowledgements

We are grateful to EastWestBio for procurement of samples. We are also grateful to all volunteer blood donors.

Olga Kofanova and Fay Betsou designed the study and wrote the article, Camille Bellora performed the experiments, Rocio Aguilar Quesada, Alexandre Bulla, Sonia Panadero-Fajardo, Kathi Shea, Marc Keipes, Mars Stone and Pierre Lescuyer collected, processed and provided samples.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit secors.

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