IL8 and IL16 levels indicate serum and plasma quality

Kofanova, Olga; Henry, Estelle; Quesada, Rocio Aguilar; Bulla, Alexandre; Linares, Hector Navarro; Lescuyer, Pierre; Shea, Kathi; Stone, Mars; Tybring, Gunnel; Bellora, Camille; Betsou, Fay

How to cite

KOFANOVA, Olga et al. IL8 and IL16 levels indicate serum and plasma quality. In: Clinical Chemistry and Laboratory Medicine, 2018. doi: 10.1515/cclm-2017-1047

This publication URL: https://archive-ouverte.unige.ch/unige:102397
Publication DOI: 10.1515/cclm-2017-1047

Olga Kofanova[a], Estelle Henry, Rocio Aguilar Quesada[a], Alexandre Bulla[a], Hector Navarro Linares, Pierre Lescuyer, Kathi Shea[a], Mars Stone[a], Gunnel Tybring[a], Camille Bellora and Fay Betsou[a,*]

IL8 and IL16 levels indicate serum and plasma quality

https://doi.org/10.1515/cclm-2017-1047
Received November 10, 2017; accepted December 30, 2017

Abstract

Background: Longer pre-centrifugation times alter the quality of serum and plasma samples. Markers for such delays in sample processing and hence for the sample quality, have been identified.

Methods: Twenty cytokines in serum, EDTA plasma and citrate plasma samples were screened for changes in concentration induced by extended blood pre-centrifugation delays at room temperature. The two cytokines that showed the largest changes were further validated for their "diagnostic performance" in identifying serum or plasma samples with extended pre-centrifugation times.

Results: In this study, using R&D Systems ELISA kits, EDTA plasma samples and serum samples with a pre-centrifugation delay longer than 24 h had an IL16 concentration higher than 313 pg/mL, and an IL8 concentration higher than 125 pg/mL, respectively. EDTA plasma samples with a pre-centrifugation delay longer than 48 h had an IL16 concentration higher than 897 pg/mL, citrate plasma samples had an IL8 concentration higher than 21.5 pg/mL and serum samples had an IL8 concentration higher than 528 pg/mL.

Conclusions: These robust and accurate tools, based on simple and commercially available ELISA assays can greatly facilitate qualification of serum and plasma legacy collections with undocumented pre-analytics.

Keywords: interleukin; plasma; preanalytical; quality control; serum.

Introduction

Uncontrolled preanalytical variables can reduce the accuracy and reproducibility of downstream analytical results and hence the specimen's fitness for purpose. For serum and plasma, the most critical preanalytical variables are the type of blood collection tube (presence and type of anticoagulant), the precentrifugation time and temperature, centrifugation conditions, the time of exposure of the isolated serum or plasma to room temperature (RT) (otherwise called "post-centrifugation time") and the long-term storage temperature [1, 2]. Knowledge of these critical variables is essential for determining the fitness for purpose of a specimen for specific downstream applications. For metabolite, cell free DNA, peptide and phosphoprotein based applications and measurements, EDTA plasma is often used, and 3 h of precentrifugation delay at RT is, as a general rule, the limit. For protein analyses in serum or plasma, the critical time depends on the specific protein, however, as a general rule, 24 h at RT is the limit [3, 4].

For legacy serum and plasma collections, where the preanalytical conditions have not been documented [2], it is important to be able to apply retrospective qualification assays, of sufficient sensitivity and specificity, to determine the corresponding preanalytical conditions. High specificity is required in those cases where the downstream analysis is expensive and we want to be certain that a serum or plasma sample is fit for purpose before deciding to apply the analysis. Assays for qualification and quality stratification of different types of specimens were reviewed [5, 6]. However, some gaps exist. While the Lacascore [7] accurately determines the 3 h precentrifugation time limit for EDTA plasma used in metabolomics studies,

[a]International Society for Biological and Environmental Repositories (ISBER) Biospecimen Science Working Group.
***Corresponding author: Fay Betsou,** PhD, HDR, Integrated Biobank of Luxembourg, 6 rue Ernest Barble, 1210 Strassen, Luxembourg, Phone: +003522744641, E-mail: fay.betsou@ibbl.lu
Olga Kofanova, Estelle Henry and Camille Bellora: Integrated Biobank of Luxembourg, Strassen, Luxembourg
Rocio Aguilar Quesada and Hector Navarro Linares: Biobanco del Sistema Sanitario Público de Andalucía (BSSPA), Granada, Spain
Alexandre Bulla and Pierre Lescuyer: Sérothèque Centrale, Département de Médecine Génétique et de Laboratoire, Hôpitaux Universitaires de Genève, Genève, Switzerland
Kathi Shea: Precision for Medicine, Frederick, MD, USA
Mars Stone: Blood Systems Research Institute, San Francisco, CA, USA
Gunnel Tybring: Karolinska Instituten Biobank, Stockholm, Sweden

a major gap, until now, has been the lack of an assay to qualify serum or plasma samples with a precentrifugation delay of more than 24 or 48 h. The precentrifugation time limit for the most common clinical chemistry parameters is 24 h [8]. Hemoconcentration due to movement of water into cells also occurs after 24 h [9].

Cytokines are small proteins, playing a role in leukocyte trafficking and homing, but also in organ development, angiogenesis, tumorigenesis/metastasis and immune responses to microbial infection [10] and the concentration of some cytokines is reputed to be affected by precentrifugation conditions. Among the different cytokines, interleukin 8 (IL-8) is produced by macrophages, lymphocytes and endothelial cells and plays a role in neutrophil and T cell chemotaxis, while interleukin 16 (IL-16) is produced by lymphocytes and eosinophils and is a CD4+ chemoattractant [11].

In this study, we first screened serum, EDTA plasma and citrate plasma samples with cytokine assays on the Mesoscale MSD platform and identified the two most promising cytokine markers for use in the diagnosis of extended precentrifugation times. We then validated these markers, using seven independent cohorts, in a collaboration between members of the ISBER Biospecimen Science Working Group.

Materials and methods

Samples used

Serum and plasma samples from healthy individuals from the Integrated Biobank of Luxembourg (IBBL), the Biobank of the Geneva University Hospitals, the San Francisco Blood Systems Research Institute (BSRI) and Karolinska Institute Biobank were used. Serum and plasma samples from rheumatoid arthritis (RA) patients were provided by Precision for Medicine (Frederick, MD, USA). Plasma samples from HIV patients were provided by the BSSPA Biobank (Andalucía), and finally serum and plasma samples from acute pancreatitis and RA patients were procured from EastWestBio (Ukraine). HIV positive diagnosis was established on the basis of LIAISON® XL murex HIC Ab/Ag positive assays. RA 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 data of the samples used.

Five IBBL EDTA plasma and CPT plasma samples, and ten IBBL serum samples, were exposed to RT for 30 min, 24 h and 48 h, after plasma or serum separation ("post-centrifugation" time) and after one freeze-thaw (FT) cycle.

All donors gave their informed consent and the protocol has received approval from the Ethics Committee (CNER approval ##201107/02, CNER approval ##20093/04).

Table 1: Characteristics of the different cohorts of samples.

	IBBL	HUG	BSRI	P4M	BSSPA	KIB	EWB
Number of donors (M, F)	5 M, 5 F	6 M, 10 F	5 M, 5 F	11	5 M, 5 F	8 M, 8 F	3 M, 7 F, 1 not specified
Age	22–62	32–62	24–60	Not specified	42–58	24–62	23–65
Health status	Healthy	Healthy	Healthy	RA	HIV positive	Healthy	4 Acute pancreatitis 7 RA
Collection tubes	BD CAT serum, ref. 367896	BD CAT serum, ref. 367896	BD SST serum, ref. 367988	CAT	BD K2EDTA, ref. 368861	BD K2EDTA, ref. 368861	BD CAT serum, ref. 367896
	BD K2EDTA, ref. 367525	BD K2EDTA, ref. 367525	BD K2EDTA, ref. 366643	K2EDTA	BD ACD-B, ref. 367756		BD K2EDTA, ref. 367525
	BD CPT sodium citrate, ref. 362761	BD CPT sodium citrate, ref. 362761	BD CPT sodium citrate, ref. 362761	CPT (exact ref. not specified)			BD CPT sodium citrate, ref. 362780
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	30 min, 3 h, 23–24 h, 48 h at RT	30 min at RT	3 h, 48 h at RT	1 h, 24 h, 36 h at RT	3 h, 48 h at RT
	30 min, 3 h, 23–24 h, 48 h at RT						
Centrifugation program	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 10 min brake 9	2000 *g* RT 20 min

IBBL, Integrated Biobank of Luxembourg; HUG, Hôpitaux Universitaires de Genève; BSRI, Blood Systems Research Institute; P4M, Precision for Medicine; BSSPA, Biobanco del Sistema Sanitario Público de Andalucía; KIB, Karolinska Instituten Biobank; EWB, EastWestBio: RA, rheumatoid arthritis; M, male; F, female.

Meso scale discovery (MSD) analyse

Meso scale discovery (MSD) assays are based on MULTI-ARRAY® technology with electrochemiluminescence detection. We used the Human Pro-Inflammatory Panel I V-plex Plus kit with the Pro-Inflammatory Control Pack, and the Human Cytokine Panel I V-plex Plus kit with the Cytokine Control Pack, following the manufacturer's instructions, on the MSD QuickPlex SQ120 instrument and with the MSD Discovery Workbench 4.0 software. Three internal controls (control 1, 2, 3) were used, according to the manufacturer's instructions. Serum, EDTA plasma and CPT plasma samples from three IBBL healthy donors, with precentrifugation times of 30 min and 53 h at either 4 °C or RT were diluted two-fold and results were read on a QuickPlex SQ120 reader. The following 20 analytes were measured in duplicate: granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-12, IL-15, IL-16, IL-17, IL-1α, IL-5, IL-7, tumor necrosis factor (TNF)-β, vascular endothelial growth factor (VEGF), interferon (IFN)-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8 and TNF-α.

IL8 measurements

IL8 was measured in all serum and citrate plasma samples of different precentrifugation times at RT, with the R&D Systems kit "Human IL-8/CXCL8 Quantikine ELISA kit" D8000C and control material "Quantikine ImmunoAssay Control Group 8" QC-23 from R&D Systems. Calibration curve was prepared following the protocol with dilution series from 31.25 to 2000 pg/mL with a four parameter logistic curve fit. Samples were diluted 1:1 and measured in duplicate.

IL16 measurements

IL16 was measured in all EDTA plasma samples of different precentrifugation times at RT, with the R&D Systems kit "Human IL-16 Quantikine ELISA kit" D1600 from R&D Systems and control material "Quantikine ImmunoAssay Control Group 4" QC-21 from R&D Systems. A calibration curve was prepared following the protocol with dilution series from 31.25 to 2000 pg/mL with a log/log curve fit. Samples were diluted 1:1 and measured in duplicate.

Statistical analyses

Summary statistics were performed in Excel version 2013. Student's t and ANOVA on ranks analyses were performed with SigmaPlot version 12.5. Receiver-operating characteristics (ROC) curve analyses, including calculations of the confidence intervals of the diagnostic sensitivities and specificities, were performed with the AnalyseIT software version 2.30. The cut-off values that were selected were those corresponding to the combinations of higher specificity and sensitivity.

Results

MSD analyses

The Human Pro-Inflammatory Panel I V-plex Plus kit and the Human Cytokine Panel I V-plex Plus kit were used to screen for 20 pro-inflammatory factors/cytokines with serum and plasma samples from three donors, before selecting the proteins with the most promising performance in terms of high fold-change and low inter-individual variability, for validation with samples from more donors.

In the Human Pro-Inflammatory Panel I V-plex Plus kit and the Human Cytokine Panel I V-plex Plus kit, the majority (77%) of the measurements were above the lower limit of quantification of the kits. All the results are shown in Supplemental Data 1. Almost all changes corresponded to increased cytokine concentrations and occurred only at RT. The only changes that were observed when blood stood at 4 °C before centrifugation were a decrease in the concentration of IL8 in serum, and a small increase in the concentration of IL16 in EDTA and citrate plasma, after >53 h delay. The cytokines that showed the highest fold changes and a statistically significant increase ($p < 0.1$) at >53 h RT delay relative to the 30 min RT time were IL-8 ($p < 0.05$ in serum, $p < 0.01$ in citrate plasma), TNF-α ($p < 0.01$ in EDTA plasma and $p < 0.1$ in citrate plasma), IL-5 ($p < 0.01$ in serum), IL-10 ($p < 0.1$ in EDTA plasma), IL-7 ($p < 0.1$ in serum), IL-16 ($p < 0.1$ in EDTA plasma) and VEGF ($p < 0.05$ in citrate plasma and $p < 0.1$ in serum). Among all the cytokines and conditions tested, the ones which showed detectable levels in all three donors, the most striking differences, in terms of fold-change, between 30 min and >53 h precentrifugation delays, and CV% of change <70% were the following:

- IL8 in serum for RT precentrifugation conditions, with an average fold increase of 296× (STDEV 139×)
- IL8 in citrate plasma for RT precentrifugation conditions, with an average fold increase of 14× (STDEV 4×)
- IL16 in EDTA plasma for RT precentrifugation conditions, with an average fold increase of 16× (STDEV 11×) (Table 2).

IL6 showed the highest fold increase in serum, but with extremely high inter-individual variability (CV% = 165%). GM-CSF showed the highest fold increase in citrate plasma, but in one donor only, while it was undetectable in the other two donors. IL1β showed the highest fold increase in EDTA plasma, but in one donor only, while it was undetectable in the other two donors (Supplemental Data 1).

IL8 in serum

In the diagnosis of 24 h RT precentrifugation delay of non-anticoagulated blood, ROC plot analysis was applied to each population of samples and the optimal cut-off

Table 2: Concentrations of IL8 (pg/mL) and IL16 (pg/mL) in the MSD screening (three biological replicates).

	4 °C		RT	
	30 min	53 h	30 min	53 h
SERUM IL-8				
Donor 1	16.4	7.1	19.6	2838.4
Donor 2	57.7	4.4	9.3	3839.2
Donor 3	13.6	6.1	12.5	4177.9
CPT PLASMA IL-8				
Donor 1	5.5	6.5	6.4	71.2
Donor 2	4.2	4.7	4.5	83.1
Donor 3	4.2	6	4.6	61.5
EDTA PLASMA IL-16				
Donor 1	155.5	183.7	124.3	1241.2
Donor 2	100.2	178.1	118.7	3304
Donor 3	195.1	284.8	179.5	1716.1

values are shown in Table 3. When the measurements from all the populations were analyzed together in a single ROC plot (92 true negative samples, with delay ≤3 h and 69 true positive samples, with delay ≥24 h), the area under the curve (AUC) was 0.98 (95%CI, 0.96–0.99) (Figure 1A and B). The optimal cut off value above which a serum sample was "positive", meaning that it has been isolated from blood with precentrifugation delay ≥24 h at RT, was 125 pg/mL.

Table 3: Diagnostic performance (precentrifugation >24 h RT or >48 h RT) of IL8 in serum from different populations.

	Cut-off, pg/mL	Sensitivity	Specificity
Precentrifugation >24 h RT			
IBBL	44.2	100% (83%–100%)	100% (83%–100%)
HUG	125.3	100% (89%–100%)	100% (89%–100%)
BSRI	36.7	100% (81%–100%)	100% (83%–100%)
BSSPA	NA[a]		
KIB	NA[a]		
EWB	NA[a]		
Precentrifugation >48 h RT			
IBBL	44.2	100% (69%–100%)	100% (83%–100%)
HUG	125.3	100% (79%–100%)	100% (89%–100%)
BSRI	38.6	100% (59%–100%)	100% (83%–100%)
BSSPA	NA[a]		
KIB	NA[a]		
EWB	NA[a]		

95% confidence intervals of the sensitivity and specificity are indicated in parentheses. [a]NA, not available samples. IBBL, Integrated Biobank of Luxembourg; HUG, Hôpitaux Universitaires de Genève; BSRI, Blood Systems Research Institute; P4M, Precision for Medicine; BSSPA, Biobanco del Sistema Sanitario Público de Andalucía; KIB, Karolinska Instituten Biobank; EWB, EastWestBio.

In the diagnosis of 48 h RT precentrifugation delay of non-anticoagulated blood, ROC plot analysis was applied to each population of samples and the optimal cut-off values are shown in Table 3. When the measurements from all the populations were analyzed together in a single ROC plot (92 true negative samples, with delay ≤3 h and 33 true positive samples, with delay ≥48 h), the AUC was 0.99 (95%CI, 0.98–1.00) (Figure 1A and C). The optimal cut-off value above which a serum sample was "positive", meaning that it has been isolated from blood with precentrifugation delay ≥48 h at RT, was 528 pg/mL.

In the acute pancreatitis and RA samples from Ukraine, the average IL8 concentration at baseline (<3 h precentrifugation time) was 20.5 pg/mL (STDEV 12.1 pg/mL). In the RA samples from the USA, the average

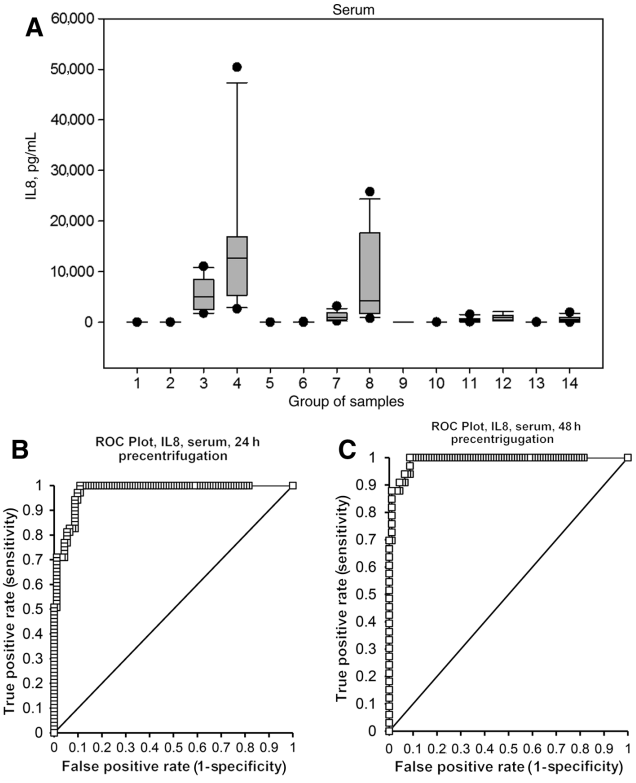

Figure 1: Summary of IL8 measurements in serum samples from five independent collections.
(A) Box plots with medians, 10th, 25th, 75th, 90th percentiles and outliers, corresponding to different precentrifugation times at RT. (1), IBBL 30 min, (2) IBBL 3 h, (3) IBBL 24 h, (4) IBBL 48 h, (5) HUG 30 min, (6) HUG 3 h, (7) HUG 24 h, (8) HUG 48 h, (9) BSRI 30 min, (10) BSRI 3 h, (11) BSRI 24 h, (12) BSRI 48 h, (13) EWB 3 h, (14) P4M 30 min. (B) Corresponding ROC curve analysis and global performance in the diagnosis of precentrifugation conditions equivalent to 24 h at RT (control group, 30 min combined with 3 h). (C) Corresponding ROC curve analysis and global performance in the diagnosis of precentrifugation conditions equivalent to 48 h at RT (control group, 30 min combined with 3 h).

IL8 concentration at baseline (<3 h precentrifugation time) was 649.3 pg/mL (STDEV 562.5 pg/mL).

The average concentration of IL8 in 10 serum samples from healthy donors was 14.6 pg/mL (STDEV 5.5 pg/mL) after 30 min exposure of isolated serum to RT, 22.6 pg/mL (STDEV 22 pg/mL) after 24 h exposure of the serum to RT and 26.5 pg/mL (STDEV 32.8 pg/mL) after 48 h RT exposure. ANOVA on ranks showed no statistically significant differences ($p = 0.722$), therefore post-centrifugation delays at RT do not have a significant impact on the IL8 concentration in serum and do not interfere in the diagnostic performance of the IL8 assay for the precentrifugation conditions.

IL8 in citrate plasma

No change was observed for IL8 in citrate plasma after 24 h of precentrifugation delay. In the diagnosis of 48 h RT precentrifugation delay of citrate blood, ROC plot analysis was applied to each population of samples and the optimal cut-off values are shown in Table 4. When the measurements from all the populations were analyzed together in a single ROC plot (138 true negative samples, with delay ≤3 h and 56 true positive samples, with delay ≥48 h), the AUC was 0.94 (95%CI, 0.91–0.97) (Figure 2) and the optimal cut-off value above which a plasma sample was "positive", meaning that it has been isolated from blood with precentrifugation delay ≥48 h at RT, was 21.5 pg/mL.

In the acute pancreatitis and RA samples from Ukraine, the average IL8 concentration at baseline (<3 h precentrifugation time) was 18.3 pg/mL (STDEV 14.8 pg/mL). In the RA samples from the USA, the average IL8 concentration at baseline (<3 h precentrifugation time) was 8.9 pg/mL (STDEV 6.7 pg/mL).

The average concentration of IL8 in five citrate plasma samples from healthy donors was 11.2 pg/mL (STDEV 0.9 pg/mL) after 30 min exposure of isolated plasma to RT, 25.9 pg/mL (STDEV 31.9 pg/mL) after 24 h exposure of the plasma to RT and 33.1 pg/mL (STDEV 47.4 pg/mL) after 48 h RT exposure. ANOVA on ranks showed no statistically significant differences ($p = 0.756$), therefore post-centrifugation delays at RT do not have a significant impact on the IL8 concentration in citrate plasma and do not interfere in the diagnostic performance of the IL8 assay.

The analytical imprecision of the IL8 assay, calculated with the three internal quality control (QC) samples, across 19 different runs, corresponded to a CV of 10%–12%.

Table 4: Diagnostic performance (precentrifugation >48 h RT) of IL8 in citrate plasma from different populations.

	Cut-off, pg/mL	Sensitivity	Specificity
Precentrifugation >48 h RT			
IBBL	18.1	100% (69%–100%)	100% (88%–100%)
HUG	15	94% (70%–100%)	83% (70%–93%)
BSRI	13.2	90% (56%–100%)	97% (83%–100%)
BSSPA	6.8	100% (69%–100%)	100% (69%–100%)
KIB	NA[a]		
EWB	42.5	100% (69%–100%)	100% (69%–100%)

95% confidence intervals of the sensitivity and specificity are indicated in parentheses. [a]NA, not available samples. IBBL, Integrated Biobank of Luxembourg; HUG, Hôpitaux Universitaires de Genève; BSRI, Blood Systems Research Institute; P4M, Precision for Medicine; BSSPA, Biobanco del Sistema Sanitario Público de Andalucía; KIB, Karolinska Instituten Biobank; EWB, EastWestBio.

IL16 in EDTA plasma

In the diagnosis of 24 h RT precentrifugation delay of EDTA blood, ROC plot analysis was applied to each population of samples and the optimal cut-off values are shown in Table 5. When the measurements from all the populations were analyzed together in a single ROC plot (118 true negative samples, with a delay ≤3 h and 120 true positive samples, with delay ≥23–24 h), the AUC was 0.98 (95%CI, 0.97–0.99) (Figure 3A and B). The optimal cut-off value above which a plasma sample was "positive", meaning that it has been isolated from blood with precentrifugation delay ≥24 h at RT, was 313 pg/mL.

In the diagnosis of 48 h RT precentrifugation delay of EDTA blood, ROC plot analysis was applied to each population of samples and the optimal cut-off values are shown in Table 5. When the measurements from all the populations were analyzed together in a single ROC plot (118 true negative samples, with delay ≤3 h and 56 true positive samples, with delay ≥48 h), the AUC was 1.00 (95%CI, 1.00–1.00) (Figure 3A and C). The optimal cut-off value above which a plasma sample was "positive", meaning that it has been isolated from blood with precentrifugation delay ≥48 h at RT, was 897 pg/mL.

In the acute pancreatitis and RA samples from Ukraine, the average IL16 concentration at baseline (<3 h precentrifugation time) was 167.3 pg/mL (STDEV 45.9 pg/mL). In the RA samples from the USA, the average IL16 concentration at baseline (<3 h precentrifugation time) was 426.1 pg/mL (STDEV 216.3 pg/mL).

The average concentration of IL16 in 5 EDTA plasma samples from healthy donors was 123.1 pg/mL (STDEV

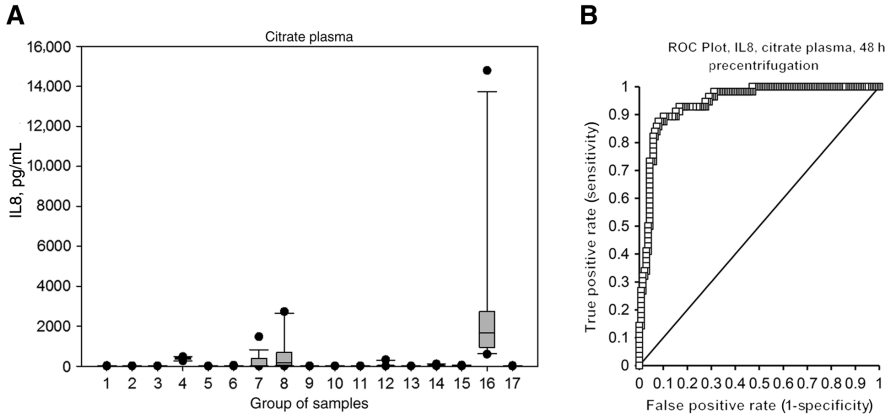

Figure 2: Summary of IL8 measurements in citrate plasma samples from six independent collections.
(A) Box plots with medians, 10th, 25th, 75th, 90th percentiles and outliers, corresponding to different precentrifugation times at RT. (1) IBBL 30 min, (2) IBBL 3 h, (3) IBBL 24 h, (4) IBBL 48 h, (5) HUG 30 min, (6) HUG 3 h, (7) HUG 24 h, (8) HUG 48 h, (9) BSRI 30 min, (10) BSRI 3 h, (11) BSRI 24 h, (12) BSRI 48 h, (13) BSSPA 3 h, (14) BSSPA 48 h, (15) EWB 3 h, (16) EWB 48 h, (17) P4M 30 min. (B) Corresponding ROC curve analysis and global performance in the diagnosis of precentrifugation conditions equivalent to 48 h at RT (control group, 30 min combined with 3 h and 24 h).

Table 5: Diagnostic performance (precentrifugation >24 h RT or >48 h RT) of IL16 in EDTA plasma from different populations.

	Cut-off, pg/mL	Sensitivity	Specificity
Precentrifugation >24 h RT			
IBBL	185.5	100% (83%–100%)	100% (83%–100%)
HUG	197.1	100% (89%–100%)	100% (89%–100%)
BSRI	222.2	100% (83%–100%)	100% (83%–100%)
BSSPA	NA[a]		
KIB	171.1	93% (77%–99%)	100% (79%–100%)
EWB	NA[a]		
Precentrifugation >48 h RT			
IBBL	185.5	100% (69%–100%)	100% (83%–100%)
HUG	197.1	100% (79%–100%)	100% (89%–100%)
BSRI	222.2	100% (69%–100%)	100% (83%–100%)
BSSPA	212.6	100% (69%–100%)	100% (69%–100%)
KIB[b]	171.1	100% (74%–100%)	100% (79%–100%)
EWB	246	100% (69%–100%)	100% (69%–100%)

95% confidence intervals of the sensitivity and specificity are indicated in parentheses. [a]NA, not available samples; [b]36 h instead of 48 h. IBBL, Integrated Biobank of Luxembourg; HUG, Hôpitaux Universitaires de Genève; BSRI, Blood Systems Research Institute; P4M, Precision for Medicine; BSSPA, Biobanco del Sistema Sanitario Público de Andalucía; KIB, Karolinska Instituten Biobank; EWB, EastWestBio.

25.4 pg/mL) after 30 min exposure of isolated plasma to RT, 128.7 pg/mL (STDEV 26.6 pg/mL) after 24 h exposure of the plasma to RT and 126.3 pg/mL (STDEV 24.3 pg/mL) after 48 h RT exposure. ANOVA on ranks showed no statistically significant differences ($p = 0.941$), therefore post-centrifugation delays at RT do not have a significant impact on the IL16 concentration in EDTA plasma and do not interfere in the diagnostic performance of the IL16 assay.

The analytical imprecision of the IL16 assay, calculated with the three internal QC samples, across 14 different runs, corresponded to a CV of 18%–21%.

Discussion

Electrochemiluminescent detection offers sensitivity and an extended dynamic range. Background signals are minimal because the stimulation mechanism (electricity) is decoupled from the signal (light), while multiple excitation cycles of each label amplify the signal. The high sensitivity (in the low pg/mL) and high dynamic range (3 logs for all tested cytokines) of the platform allowed us to identify the two most promising cytokine markers (IL-8 and IL-16) in one run, among 20 cytokines tested.

Acute pancreatitis and RA are associated with high IL8 levels [12, 13] and in particular IL16 has been proposed as a predictive biomarker in RA [14]. Therefore, it was important to assess the field of application of the new serum and plasma qualification methods in the scope of these clinical conditions. Similarly, we wanted to assess the feasibility in the context of a viral disease, such as HIV1 infection, which increases the IL16 levels [15]. None of the above conditions limits the use of the IL8 and IL16-based QC assays of serum and plasma, with the exception of RA serum samples in which the baseline concentration of IL8 was significantly higher than in plasma. This is probably linked to the fact that the IL8 levels in serum are already 10 times higher

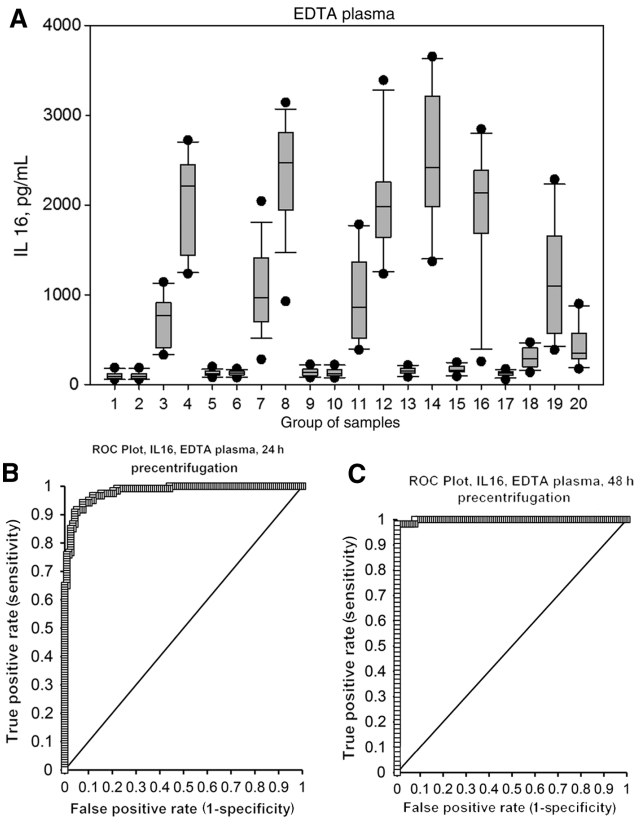

Figure 3: Summary of IL16 measurements in EDTA plasma samples from seven independent collections.
(A) Box plots with medians, 10th, 25th, 75th, 90th percentiles and outliers, corresponding to different precentrifugation times at RT. (1) IBBL 30 min, (2) IBBL 3 h, (3) IBBL 24 h, (4) IBBL 48 h, (5) HUG 30 min, (6) HUG 3 h, (7) HUG 24 h, (8) HUG 48 h, (9) BSRI 30 min, (10) BSRI 3 h, (11) BSRI 24 h, (12) BSRI 48 h, (13) BSSPA 3 h, (14) BSSPA 48 h, (15) EWB 3 h, (16) EWB 48 h, (17) KIB 1 h, (18) KIB 24 h, (19) KIB 36 h, (20) P4M 30 min. (B) Corresponding ROC curve analysis and global performance in the diagnosis of precentrifugation conditions equivalent to 24 h at RT (control group, 30 min combined with 1 h and 3 h). (C) Corresponding ROC curve analysis and global performance in the diagnosis of precentrifugation conditions equivalent to 48 h at RT (control group, 30 min combined with 1 h and 3 h).

than in plasma in healthy individuals [16]. We have no explanation as to why the IL8 levels were much higher in serum samples from RA patients from Ukraine than those in serum samples from RA patients from the USA. One hypothesis may be linked to the levels of alcohol consumption in certain populations in Eastern Europe and the "extremely high" serum IL8 levels (>100 pg/mL) associated with this consumption [17].

Furthermore, elevated IL-16 levels have been reported in overweight adolescents [18] and in patients suffering from trauma [19]. However, the reported levels are similar to the levels we found in the RA patient samples and therefore we believe that the field of application of the assays covers serum and plasma samples from both healthy and diseased donors. One possible exception is the application of the IL8 assay in serum from certain autoimmune disease patients, where the sensitivity of the assay may be lower. Another exclusion from the scope of application of the IL8 assay is the plasma of patients with septic shock in which IL8 levels are 50–500 times higher than in samples from healthy individuals [20].

Concerning the robustness of the diagnostic performance of the three qualification assays that have been validated in this work (IL8 for serum, IL8 for citrate plasma and IL16 for EDTA plasma), exposure of isolated serum or plasma to RT conditions does not influence the levels of IL8 and IL16. Stability in serum, and EDTA plasma has been shown for 10 FT cycles and also in isolated serum or plasma, exposed 20 days to RT, 4 °C or –70 °C [21]. We have further confirmed that there is no significant impact of exposure to RT on IL8 and IL16 levels in isolated serum or plasma. Therefore, the assay can be performed independently of post-centrifugation exposure to RT. If post-centrifugation delays at RT need to be assessed, sCD40L measurement is the appropriate qualification assay [22].

IL8 and IL16 levels are stable across different age groups [23], therefore the field of application is not limited to any specific age group.

IL8 and IL16 levels in heparin, EDTA and citrate plasma were measured and found to be the same with the three anticoagulants [16], indicating that these quality markers might be applicable also to heparine plasma, but further studies would be needed to establish this.

IL16, but not IL8, has been found to increase significantly after 2 h precentrifugation time at RT in EDTA plasma, and the presence of protease inhibitors (PIs) did not significantly affect cytokine levels [24]. Therefore, the qualification methods presented here should also be robust to the use of PIs in blood collection tubes (e.g. BD P100 tubes).

The BSRI cohort samples had lower initial (baseline) concentration of IL8 in serum and less marked increase of IL8 in citrate plasma. Leukapheresis samples have been reported to have lower levels of IL8 [25]. The BSRI plasma was not cleared and so, we have no explanation why these samples had lower levels of IL8.

IL8 has recently been suggested, among other cytokines, for the diagnosis of serum precentrifugation times longer than 24 h, with a sensitivity of 81% and a specificity of 78% at the cut-off of 23 pg/mL [26]. The optimal diagnostic threshold that we found was higher (125 pg/mL). This difference can be explained, first by the fact that in the study performed by Lee et al. the

samples were kept before centrifugation, not in the blood collection tube itself, but in 1.5 mL Eppendorf tubes, which might have affected the kinetics of IL8 production. Second and more importantly, the measurement kit used by Lee et al. was the Milliplex Map Human Cytokine/Chemokine Magnetic Bead Panel kit-Immunology Milliplex Assay (Millipore), which is different from the kit we used (R&D Systems). These differences underline the critical importance of using common reference (QC) materials for cytokine measurements, as it was recommended by Aziz et al. [21] and/or participating in international ring trials [27]. Interestingly, the MSD and R&D kits used in our study, showed similar concentration results for IL16 in EDTA plasma, but the R&D kit showed 3–5 times higher concentration results for IL8 in serum and in citrate plasma than the MSD kit, using the same samples from three IBBL donors. This highlights the fact that different ELISA kits are not comparable. The conclusions of our study and the established cut-off values are valid with the R&D kits.

The possible explanation for the increase of IL8 and IL16 in blood samples before isolation of serum/plasma is that monocytes, lymphocytes and granulocytes may release *de novo* synthesized or already synthesized interleukins in a nonspecific way, post-collection. Data published by Reinsberg et al. [28] indicate that *de novo* IL8 synthesis in blood cells takes place upon storage of whole blood at RT. It seems that different mechanisms are at work. IL16 increases tremendously in EDTA plasma, slightly in citrate plasma and not at all in serum. IL16 is produced by eosinophils, monocytes and lymphocytes, but the mechanism of release or secretion of IL16 is still unknown. Our results suggest that release of IL16 by granulocytes is abundant in EDTA but not in citrate, and absent in serum where leucocytes may be trapped in the blood clot. IL8, which is produced, not only by granulocytes, but also by platelets [29], increases tremendously in serum, probably due to platelet activation and formation of platelet-monocyte aggregates [30], significantly in citrate plasma and less in EDTA plasma.

Conclusions

- IL8 concentration measurement by ELISA can be used to identify serum samples with RT precentrifugation times of more than 24 h with sensitivity 97% and specificity 90% at the cutoff 125 pg/mL.
- IL8 concentration measurement by ELISA can also be used to identify serum samples with RT precentrifugation times of more than 48 h with sensitivity 94% and specificity 94% at the cut-off 528 pg/mL.
- IL8 concentration measurement by ELISA can be used to identify citrate plasma samples with RT precentrifugation times of more than 48 h with sensitivity 86% and specificity 93% at the cut-off 21.5 pg/mL.
- IL16 concentration measurement by ELISA can be used to identify EDTA plasma samples with RT precentrifugation times of more than 24 h with sensitivity 91% and specificity 96% at the cut-off 313 pg/mL.
- IL16 concentration measurement can also be used to identify EDTA plasma samples with RT precentrifugation times of more than 48 h with sensitivity 98% and specificity 100% at the cut-off 897 pg/mL.

The above assays are easily applicable and immediately accessible to all laboratories and should facilitate qualification of legacy collections or quality stratifications of serum and plasma samples with undocumented preanalytics. This initial qualification can be applied before expensive proteomic analyses are undertaken.

Acknowledgments: We are grateful to EastWestBio for procurement of samples, to Arnaud Verlinde and Celine Weckerle for support with the Mesoscale technology and to Dr Mark Keipes for collection of healthy donor samples. We are grateful to Clara Martínez-Montes, Benilde Ayala-Moscoso and Sonia Panadero-Fajardo for support with collection of HIV samples. We are also thankful to all volunteer blood donors.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: IBBL.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. Betsou F, Lehmann S, Ashton G, Barnes M, Benson EE, Coppola D, et al. Standard preanalytical coding for biospecimens: defining the sample PREanalytical code. Cancer Epidemiol Biomarkers Prev 2010;19:1004–11.
2. Lehmann S, Guadagni F, Moore H, Ashton G, Barnes M, Benson E, et al. Standard preanalytical coding for biospecimens: review and implementation of the Sample PREanalytical Code (SPREC). Biopreserv Biobank 2012;10:366–74.

3. Hsieh SY, Chen RK, Pan YH, Lee HL. Systematical evaluation of the effects of sample collection procedures on low-molecular-weight serum/plasma proteome profiling. Proteomics 2006;6:3189–98.
4. Di Girolamo F, Alessandroni J, Somma P, Guadagni F. Pre-analytical operating procedures for serum low molecular weight protein profiling. J Proteomics 2010;73:667–77.
5. Betsou F, Gunter E, Clements J, DeSouza Y, Goddard KA, Guadagni F, et al. Identification of evidence-based biospecimen quality-control tools: a report of the International Society for Biological and Environmental Repositories (ISBER) Biospecimen Science Working Group. J Mol Diagn 2013;15:3–16.
6. Betsou F, Bulla A, Cho SY, Clements J, Chuaqui R, Coppola D, et al. Assays for qualification and quality stratification of clinical biospecimens used in research: a technical report from the ISBER Biospecimen Science Working Group. Biopreserv Biobank 2016;14:398–409.
7. Trezzi J-P, Bulla A, Bellora C, Rose M, Lescuyer P, Keihntopf M, et al. LacaScore: a novel plasma sample quality control tool based on ascorbic acid and lactic aceid levels. Metabolomics 2016;12:96.
8. Heins M, Heil W, Withold W. Storage of serum or whole blood samples? Effects of time and temperature on 22 serum analytes. Clin Chem Lab Med 1995;33:231–8.
9. Boyanton BL, Blick KE. Stability studies of twenty-four analytes in human plasma and serum. Clin Chem 2002;48:2242–7.
10. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol 2014;32:659–702.
11. Cruikshank WW, Kornfeld H, Center DM. Interleukin-16. J Leukocyte Biol 2000;67:757–66.
12. Malmstrøm ML, Hansen MB, Andersen AM, Ersbøll AK, Nielsen OH, Jørgensen LN, et al. Cytokines and organ failure in acute pancreatitis: inflammatory response in acute pancreatitis. Pancreas 2012;41:271–7.
13. Sharma J, Bhar S, Devi CS. A review on interleukins: the key manipulators in rheumatoid arthritis. Mod Rheumatol 2017;27:723–46.
14. Murota A, Suzuki K, Kassai Y, Miyazaki T, Morita R, Kondo Y, et al. Serum proteomic analysis identifies interleukin 16 as a biomarker for clinical response during early treatment of rheumatoid arthritis. Cytokine 2016;78:87–93.
15. Bader A, Brockmeyer N, Schnaitmann E, Mertins L, Otteken A, Kurth R, et al. Interleukin-16 serum levels during the course of HIV-1 infection. Aids 2001;15:528–9.
16. Biancotto A, Feng X, Langweiler M, Young NS, McCoy JP. Effect of anticoagulants on multiplexed measurement of cytokine/chemokines in healthy subjects. Cytokine 2012;60:438–46.
17. Gonzalez-Quintela A, Campos J, Gude F, Perez L-F, Tomé S. Serum concentrations of interleukin-8 in relation to different levels of alcohol consumption. Cytokine 2007;38:54–60.
18. Lichtenauer M, Franz M, Fritzenwanger M, Figulla H-R, Gerdes N, Jung C. Elevated plasma levels of interleukin-12p40 and interleukin-16 in overweight adolescents. Biomed Res Int 2015;2015:940910.
19. Shimonkevitz R, Northrop J, Harris L, Craun M, Bar-Or D. Interleukin-16 expression in the peripheral blood and CD8 T lymphocytes after traumatic injury. J Trauma Acute Care 2005;58:252–8.
20. Endo S, Inada K, Ceska M, Takakuwa T, Yamada Y, Nakae H, et al. Plasma interleukin 8 and polymorphonuclear leukocyte elastase concentrations in patients with septic shock. J Inflamm 1995;45:136–42.
21. Aziz N, Nishanian P, Mitsuyasu R, Detels R, Fahey JL. Variables that affect assays for plasma cytokines and soluble activation markers. Clin Diagn Lab Immuno 1999;6:89–95.
22. Lengelle J, Panopoulos E, Betsou F. Soluble CD40 ligand as a biomarker for storage-related preanalytic variations of human serum. Cytokine 2008;44:275–82.
23. Kleiner G, Marcuzzi A, Zanin V, Monasta L, Zauli G. Cytokine levels in the serum of healthy subjects. Mediat Inflamm 2013;2013:434010.
24. Ayache S, Panelli M, Marincola FM, Stroncek DF. Effects of storage time and exogenous protease inhibitors on plasma protein levels. Am J Clin Pathol 2006;126:174–84.
25. Wadhwa M, Seghatchian M, Dilger P, Contreras M, Thorpe R. Cytokine accumulation in stored red cell concentrates: effect of buffy-coat removal and leucoreduction. Transfus Sci 2000;23:7–16.
26. Lee J-E, Kim J-W, Han B-G, Shin S-Y. Impact of whole-blood processing conditions on plasma and serum concentrations of cytokines. Biopreserv Biobank 2016;14:51–5.
27. Gaignaux A, Ashton G, Coppola D, De Souza Y, De Wilde A, Eliason J, et al. A biospecimen proficiency testing program for biobank accreditation: four years of experience. Biopreserv Biobank 2016;14:429–39.
28. Reinsberg J, Dembinski J, Dorn C, Behrendt D, Bartmann P, van der Ven H. Determination of total interleukin-8 in whole blood after cell lysis. Clin Chem 2000;46:1387–94.
29. Boehlen F, Clemetson KJ. Platelet chemokines and their receptors: what is their relevance to platelet storage and transfusion practice? Transfusion Med 2001;11:403–17.
30. Takahashi D, Fujihara M, Azuma H, Wakamoto S, Sato S, Kato T, et al. Adhesive interaction between peripheral blood mononuclear cells and activated platelets in the presence of anti-human leukocyte antigen Class I alloantibody causes production of IL-1β and IL-8. Vox Sang 2012;102:250–7.

Supplemental Material: The online version of this article offers supplementary material (https://doi.org/10.1515/cclm-2017-1047).