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Short communication

Effect of storage duration on cytokine stability in human serum and plasma

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ABSTRACT

Quantification of analytes such as cytokines in serum samples is intrinsic to translational research in immune diseases. Optimising pre-analytical conditions is critical for ensuring study quality, including evaluation of cytokine stability. We aimed to evaluate the effect on cytokine stability of storage duration prior to freezing of serum, and compare to plasma samples obtained from patients with systemic lupus erythematosus (SLE). Protein stability was analysed by simultaneously quantifying 18 analytes using a custom multi-analyte profile in SLE patient serum and plasma samples that had been prospectively stored at 4 °C for pre-determined periods between 0 and 30 days, prior to freezing. Six analytes were excluded from analysis, because most tested samples were above or below the limit of detection. Amongst the 12 analysed proteins, 11 did not show significant signal degradation. Significant signal degradation was observed from the fourth day of storage for a single analyte, CCL19. Proteins levels were more stable in unseparated serum compared to plasma for most analytes, with the exception of IL-37 which appears slightly more stable in plasma. Based on this, a maximum 3 days of storage at 4 °C for unseparated serum samples is recommended for biobanked samples intended for cytokine analysis in studies of human immune disease.

1. Introduction

Systemic lupus erythematosus (SLE) is an idiopathic systemic autoimmune disease which can potentially affect any organ [1]. Measurements that enable physicians to stratify patients and make optimal therapeutical decisions are lacking. Assessing the activity of the disease, and aligning this with analysis of key pathological pathways, is a hallmark of translational biomarker studies. Most such studies to date have focused on serum analysis of candidate cytokines, such as B cell-activating factor (BAFF), a proliferation-inducing ligand (APRIL), interferon-α (IFN-α), macrophage migration inhibitory factor (MIF), interleukin (IL)-6, IL-10, IL-17, IL-37 and IL-38 [2–5].

Biological fluid sample quality is a crucial prerequisite for translational biomarker research. Pre-analytical conditions, from patient preparation to serum collection, handling and storage, are potentially critical for serum cytokine measurement, as these analytes could undergo degradation leading to signal decay or *ex vitro* cell release leading to increased measure concentrations [6–9]. Thus, identification of optimal pre-analytical procedures could lead to improved accuracy and reproducibility of cytokine measurements in the clinical setting.

Only a few studies have focused on the potential effects of storage condition on serum cytokine stability between centrifugation and freezing steps, most of those using separated serum samples [9–13]. One group has studied cytokine stability in unseparated serum, stored for up to six days at 4 °C, reporting some effect on TNF-α, IL-8 and epidermal growth factor (EGF) [10]. In an effort to optimize standard operating procedures (SOP) for serum sample collection, processing and storage, we designed a study to explore the effect of different durations of serum storage prior to freezing on measured cytokine concentration. 18 proteins were simultaneously quantified using a custom Multi-Analyte Profile (MAP) (multiplex immunoassay) in serum samples from 10 SLE patients, as well as in paired serum and plasma samples from 3 SLE patients. Samples were subjected to a range of pre-determined storage periods between centrifugation and freezing, to evaluate the

Abbreviations: ACR, American College of Rheumatology; APRIL, a proliferation-inducing ligand; BAFF, B cell-activating factor of the tumor necrosis factor (TNF) family; IFN, interferon; IL, interleukin; IQR, inter-quartiles range; MAP, multi-analyte profile; MIF, macrophage migration inhibitory factor; ra, receptor antagonist; SD, standard deviation; SEM, standard error of mean; SLE, systemic lupus erythematosus; SLEDAI-2k, SLE disease activity index 2000; SLICC, systemic Lupus International Collaborating Clinics; SOP, standard operating procedure

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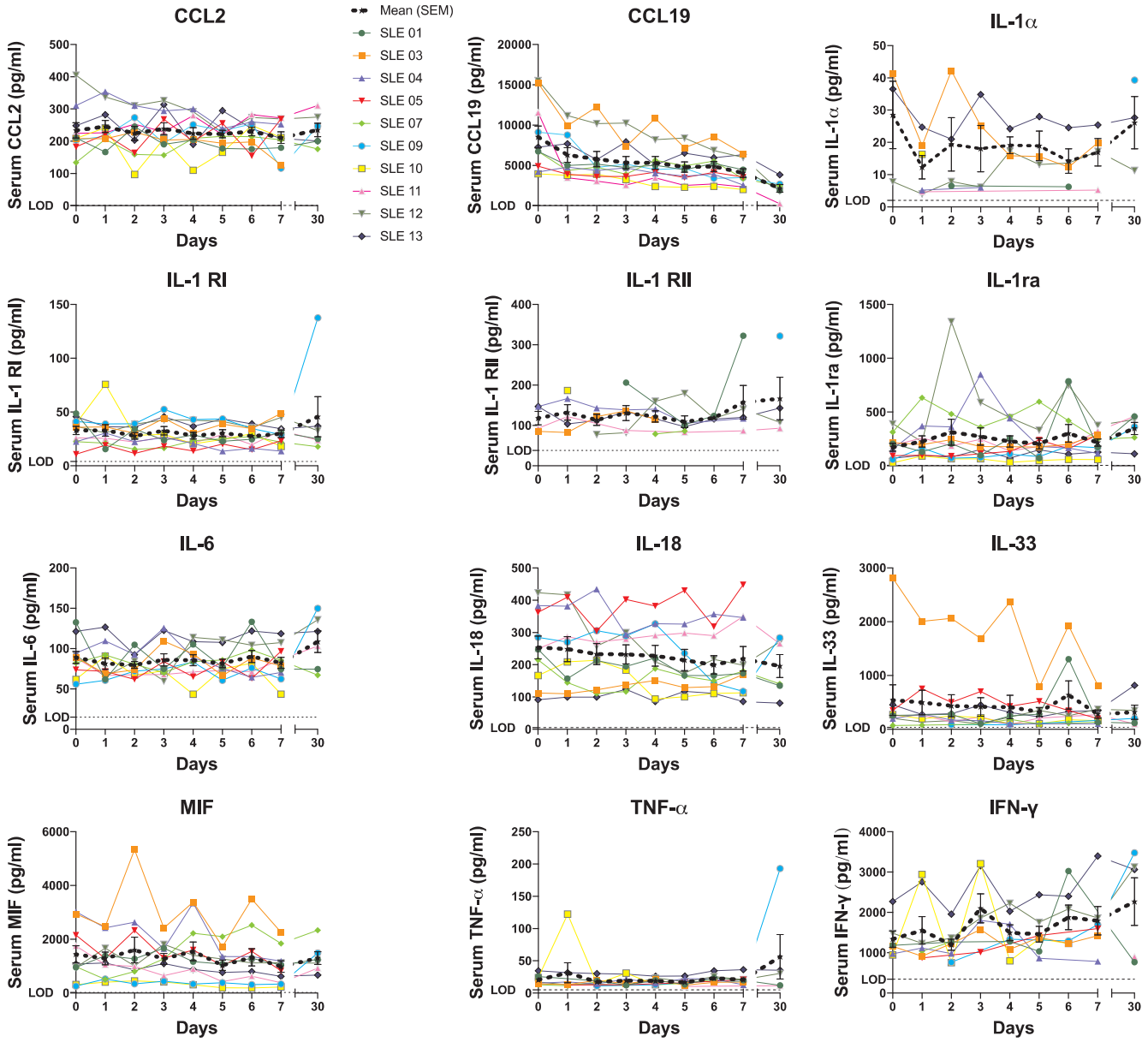

Fig. 1. Single analyte time series for tested proteins in individual SLE patients. Serum concentrations of CCL2, CCL19, IL-1α, IL-1 RI, IL-1 RII, IL-1ra, IL-6, IL-18, IL-33, MIF, TNF-α, and IFN-γ over time (n = 10). Serum cytokine concentrations are expressed in pg/ml. Boldfaced dotted line indicates the mean with SEM. LOD: limit of detection.

effect of storage duration at 4 °C on cytokine stability in unseparated serum and plasma.

2. Materials and methods

2.1. Patients and clinical assessments

Adult SLE patients fulfilling the 1997 American College of Rheumatology (ACR) or 2012 Systemic Lupus International Collaborating Clinics (SLICC) criteria for SLE classification, followed as part of the Australian Lupus Registry and Biobank [14], were eligible. Fourteen SLE patients were prospectively included between May and June 2017. Ten SLE patients were selected based on their disease activity score, using the SLE disease activity index 2000 (SLEDAI-2k), as previously described [2], to include subsets of patients according to disease activity, as follows: 3, 4 and 3 patients with SLEDAI-2k < 4, SLEDAI-2k = 4 and SLEDAI-2k > 4, respectively. In this cohort, median (IQR) age and disease duration were 47.2 (36.5, 56.9) and 12.4 (4.1, 26.1) years, respectively. All patients were female, half were of Asian ethnicity, and the median (IQR) SLEDAI-2k score was 4 (0, 6). All experimental protocols were approved by the Human Research Ethics Committee, Monash Health and written informed consent was obtained from all subjects. All methods were carried out in accordance with the relevant guidelines.

2.2. Collection of human serum sample

Following whole blood collection by venepuncture into BD Vacutainer® Plastic SST™ II Advance tubes with Gold Hemogard Closure, samples were stored at room temperature for at least 30 min to clot the sample. After centrifugation at 1300*g* for 15 min at 4 °C, each serum sample was stored in its respective blood collection tube (defined as unseparated) at 4 °C for pre-determined periods of time before separating and aliquoting serum into sterile microtubes and storing at -80 °C until further use. Thus, serum was frozen after 4 h, 1, 2, 3, 4, 5, 6, and 7 days, and 1 month storage at 4 °C.

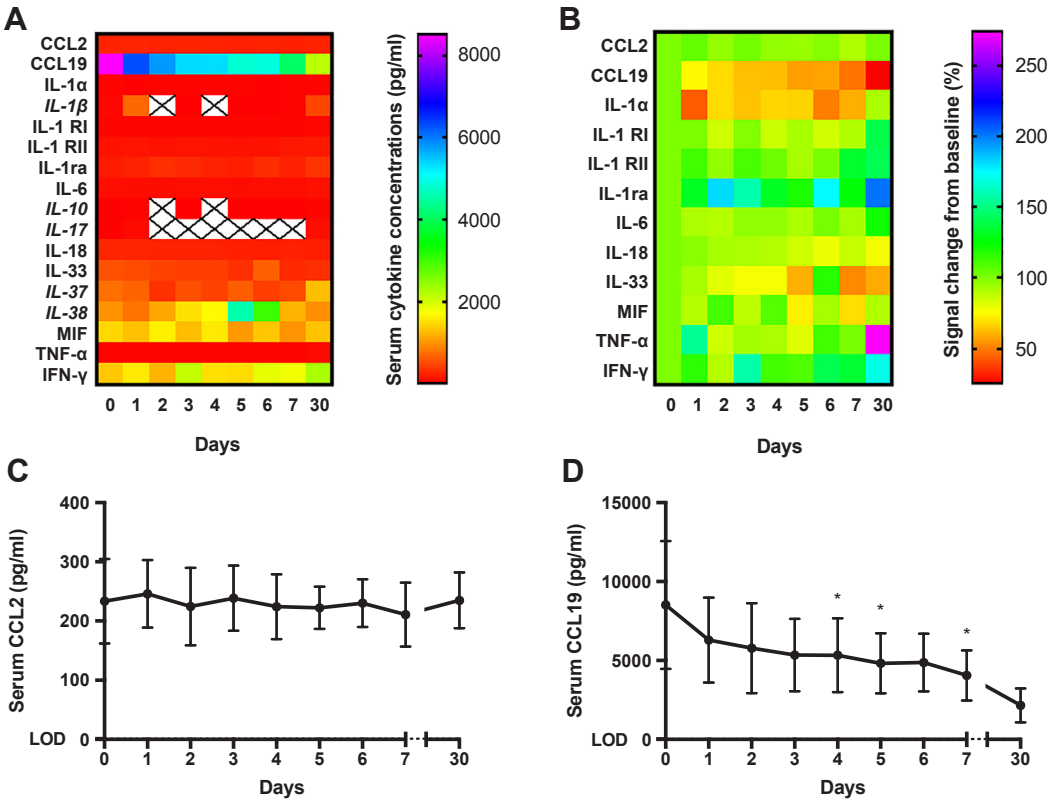

Fig. 2. MAP time series for tested serum proteins in SLE patients. (A) Heat map of serum cytokine concentrations of multi-analyte time series in 10 SLE patients. Mean serum cytokine concentrations are shown. A rainbow-colour scale has been applied, with red and purple as the lowest and highest values, respectively. Cytokines are shown in italic where most values were below the LOD and analysis not performed. (B) Heat map of percentage of signal change from baseline in analysed cytokines (n = 12). Each mean cytokine concentration has been normalized to 100% at the first time point (day 0). A rainbow-colour scale has been applied, with red and purple as the lowest and highest values, respectively. Serum (C) CCL2 and (D) CCL19 concentrations over time in the SLE cohort; n = 10 for all samples except day 30 (n = 6). All serum proteins are expressed in pg/ml. In panels C-D, horizontal bars indicate the mean with SD. LOD: limit of detection. *$P < 0.05$ (with Bonferroni *post-hoc* correction). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Collection of human plasma sample

Following collection of whole blood by venepuncture into BD Vacutainer® Plastic K2EDTA tubes, tubes were gently inverted 180° and back 8 to 10 times, and were stored at 4 °C for at least 30 min. After centrifugation at 1300*g* for 15 min at 4 °C, plasma was collected and aliquoted into sterile microtubes. Plasma aliquots were stored at 4 °C for 4 h, 1, 2, 3, 4, 5, 6, and 7 days, and 1 month before storing at −80 °C.

2.4. Serum and plasma protein quantification

A panel of 18 analytes was selected encompassing a broad range of cytokines, soluble receptors and a hormone, as follows: IL-1α, IL-1β, IL-1 receptor antagonist (ra), IL-1 receptor type (R)1, IL-1 R2, IL-6, IL-10, IL-17, IL-18, IL-33, IL-37, IL-38, MIF, C-C motif chemokine 2 (CCL)2, CCL19, TNF-α, IFN-γ, and leptin. Proteins were quantified using a customised MAP Quantibody 18-plex (Raybiotech, Norcross, GA, USA) performed by Crux Biolab (Scoresby, Victoria, Australia). A 1:2 dilution was applied to serum and plasma samples, which were tested in quadruplicate, as per manufacturer instructions. Concentrations are reported in pg/ml for all proteins. The limit of detection (LOD) was defined by the RayBiotech Q-Analyzer software which added three standard deviations to the mean optical density of four blank sample replicates.

2.5. Statistical analysis

Statistical analyses were performed using R (v.3.2.1), Stata (v.14.2) and GraphPad Prism (V 7.0c) software. Continuous data were described as mean [standard deviation (SD)] or median ([inter-quartile range (IQR)]). Categorical data were described as frequency (%). For each analyte, Shapiro-Wilk normality test were performed on pooled measurements, accompanied by histogram to determine normality. To compare repeated measures, for Gaussian distributed values, paired *t* tests were used, while for non-Gaussian distributed values, Wilcoxon matched-pairs signed rank tests were used, with *post-hoc* Bonferroni correction. To compare matching plasma and serum samples in the group of three patients, we assumed a normal distribution and used a paired *t* test. A *P* value < 0.05 was considered statistically significant.

3. Results and discussion

Amongst the 18 analysed proteins, most values for IL-1β, IL-10, IL-17, IL-37 and IL-38 were below the LOD, while leptin was significantly above the upper level of detection in all samples; these analytes were excluded from further analysis. Of the remaining 12 proteins, IL-1α, IL-1RII, IL-33, IFN-γ and TNF-α were below the limit of detection in some tested samples, which were excluded from analysis (Fig. 1A-L). However, to ensure that this did not mask potentially significant loss of signal, we also performed analysis on extrapolated values and found no significant differences (data not shown). Amongst the 12 analysed serum proteins, 11 did not show significant change in signal over up to 30 days (Figs. 1 and 2A and B), exemplified by the CCL2 time series in Fig. 2C. Statistically significant signal degradation was observed from the fourth day of storage for one analyte, CCL19 (Fig. 2D). It is worth noting that paired *t* test was performed on CCL19 based on positive normality test results, which explains why day 4 has drastically lower *P* values than day 3 (0.04 versus 0.12), while both having comparable

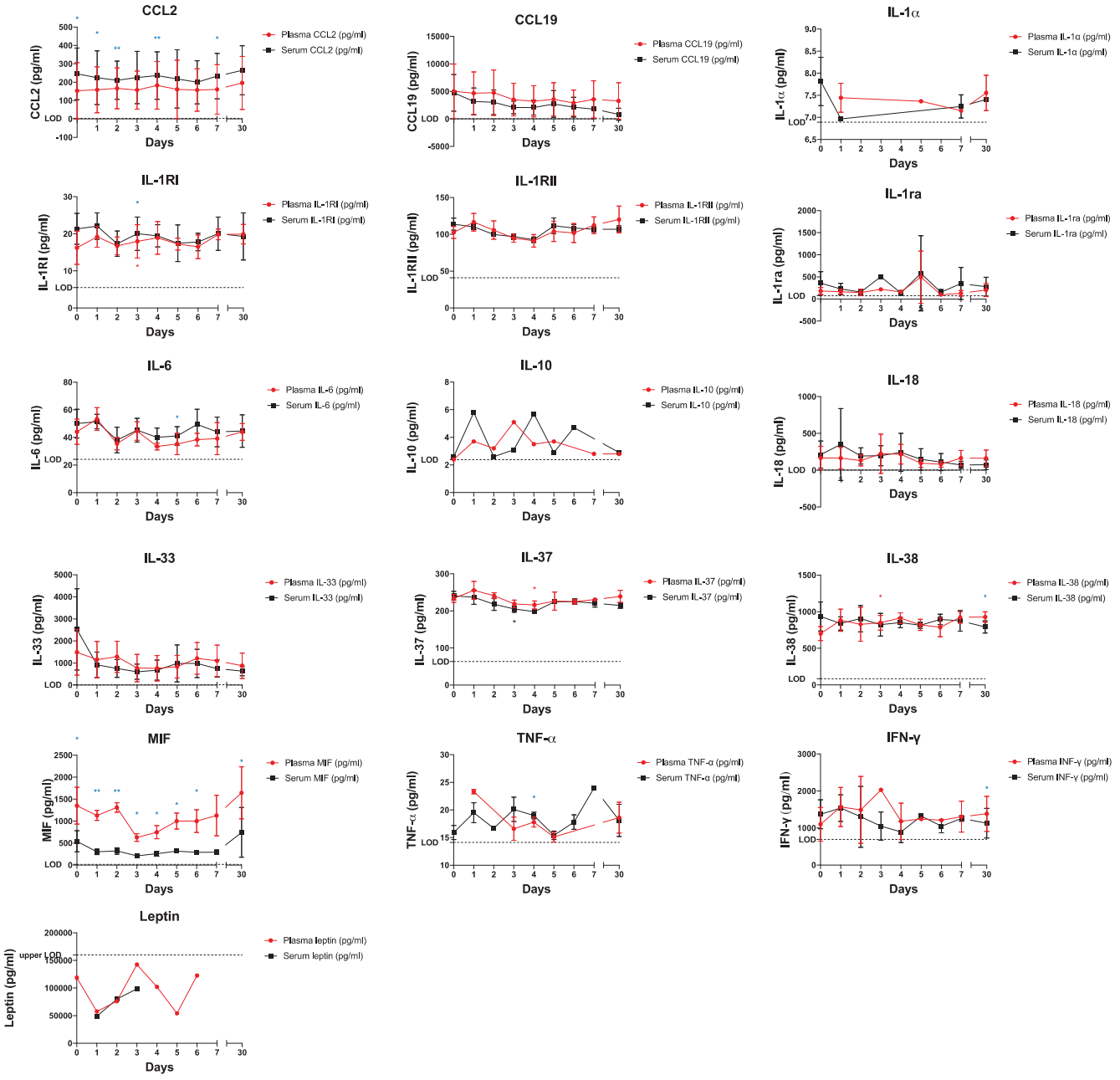

Fig. 3. Single analyte time series for tested proteins in serum and plasma from SLE patients. Serum and plasma concentrations of CCL2, CCL19, IL-1α, IL-1 RI, IL-1 RII, IL-1ra, IL-6, IL-10, IL-18, IL-33, IL-37, IL-38, MIF, TNF-α, and IFN-γ over time are shown (n = 3). All values within detection ranges are shown. Blue * is shown for paired statistical analysis examining the difference in protein levels between serum and plasma samples, performed using paired plasma and serum samples at each time point. Black * is shown for paired statistical analysis comparing protein levels at each time point to baseline (day 0) in serum. Red * is shown for paired statistical analysis comparing protein levels at each time point to baseline (day 0) in plasma. Serum and plasma cytokine concentrations are expressed in pg/ml. LOD: limit of detection. $^{**}P < 0.01$; $^{*}P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

means and SEMs (Fig. 2D).

Of note, serum cytokine concentrations were increased in day 30 sample for one patient (SLE09) for 11/12 (91.7%) of tested analytes, while this pattern was not observed in other patients (Fig. 1). Exclusion of this potential outlier at day 30 for all tested proteins did not lead to a change in significance for the reported *P* values after *post-hoc* Bonferroni correction (data not shown). This may be the result of a faulty separation tube, allowing protein transfer from the cell fraction into the serum. Release of cytokines from cells and platelet degranulation within the clot after centrifugation as a consequence of the coagulation process and complement activation may trigger post-sampling cytokine release [7,10,15]. However, the gel present in the tube, which acts as a barrier between the clot and serum, should be impermeable to cytokines, according to the manufacturer (unpublished data).

Previous studies have suggested that detectability of TNF-α decreases in concentration after 30 days of storage of separated serum/plasma at 4 °C, but not at seven days [10,12]. In our study, no significant change in serum TNF-α concentration was observed, even after 30 days. The reason for this difference is not clear; but could be due to differences in collection and processing protocols or a difference between separated and unseparated serum. However, a previous study has shown a similar effect, with decreased TNF-α levels at six days, while increases in measured IL-8 and EGF concentration were observed in unseparated, but not in separated serum samples [10]. These data

suggest that shorter periods of pre-freeze storage are preferable, but that serum cytokine concentrations tolerate several days of storage at 4 °C, prior to freezing.

We have examined the concentrations of each of the 18 analytes in serum compared to plasma, at each time point, in a group of three SLE patients. All values were below the LOD for IL-1β and IL-17, most values were below the LOD for IL-1α, IL-10 and TNF-α, while nearly all values were above the upper level of detection for leptin. We found a statistically significant difference in levels of seven proteins between paired serum and plasma samples, namely CCL2 (days 0, 1, 2, 4, and 7), IL-1 RI (day 3), IL-6 (day 5), IFN-γ (day 30), MIF (days 0 to 6, and day 30), TNF-α (day 4), and IL-38 (day 30) (Fig. 3 & Supplementary Fig. 1). It is worth noting that concentrations of analytes were not all higher in serum compared to plasma, with some analytes such as MIF where plasma concentrations were significantly higher than in serum. To ensure that the exclusion of values outside of the detection ranges did not mask potentially significant difference between matched serum and plasma, we also performed analysis using extrapolated values. This led to a statistically significant difference in levels of three other proteins between serum and plasma, namely IL-1α (days 0 and 1), IL-1β (day 0) and IL-17 (days 0 and 6), as well as statistically significant difference in levels of IFN-γ at other time points (days 0 and 5).

We have also examined the change in protein signal over 30 days in serum compared to plasma samples in these three SLE patients. Amongst the 18 analytes, IL-1α, IL-1β, IL-10, IL-17 and leptin were outside of the detection ranges in most serum and plasma samples, and IL-1ra, IFN-γ, and TNF-α were outside of the detection ranges in most plasma samples, precluding meaningful statistical analysis. In serum matrix, we only observed a significant decrease in signal for IL-37 at day 3 ($P = 0.02$). In contrast, in plasma matrix we found a significant decrease in signal for IL-37 at day 4 ($P = 0.03$), and a significant increase in signal for IL-1 RI and IL-38 at day 3 ($P = 0.01$ for both) (Fig. 3). Analysis performed in all samples, including those with values outside of the detection range, did not affect these analysis outcomes. This data suggests a more stable protein levels in unseparated serum compared to plasma for most analysed analytes, with the exception of IL-37 which appears slightly more stable in plasma compared to serum.

We also examined whether the difference in serum/plasma matrix could affect the individual ranking of each of these three SLE patients according to each protein concentration. For this purpose, because of the small sample size, we analysed all samples including those with extrapolated values. Patients were ranked in the same order at all time points only for CCL19. In contrast, patients were ranked differently in serum compared to plasma, at at least 2 time points for all other proteins (Supplementary Fig. 2). Altogether, relative values for most proteins differed according to the matrix studied, suggesting that SLE biomarker studies using plasma or serum should be compared with caution. Of note, we also observed that patient rankings changed over time in the same sample matrix (Supplementary Fig. 2), emphasizing the importance of analysing samples with homogeneous storage duration period.

Some caveats apply to the interpretation of our findings. Firstly, the number of patients was small. However, the sample size was large enough to demonstrate statistically significant signal degradation overtime for CCL19 and also to demonstrate the lack of such degradation for the other 11 analytes. Secondly, only six patient samples were tested at day 30. In addition, five analytes were not reliably detected in a majority of samples, precluding statistical analysis. Serum decay analysis for those analytes would require more patients to be included in future prospective study. Finally, it cannot be assumed that all cytokines behave similarly to those measured here.

In conclusion, only one of twelve analysed proteins showed significant signal degradation in unseparated serum over 0 to 30 days of storage at 4 °C prior to freezing, and all analytes were stable for up to 3 days under these conditions. Protein also appeared more stable in unseparated serum compared to plasma for most studied analytes. This suggests that storage of unseparated serum for up to 3 days prior to freezing is unlikely to result in significant degradation of cytokine concentrations, although this cannot be assured for all analytes. This results in considerable increased feasibility of serum biobanking for cytokine analysis, especially in a busy clinical setting.

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Conflict of interest

EFM has been a consultant to GSK and Eli Lilly. The other authors have no conflict of interest to declare.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.06.009.

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