Sample Type and Storage Conditions Affect Calprotectin Measurements in Blood

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Background: Calprotectin, a complex of calcium-binding proteins, is abundant in granulocytes. Increased levels of plasma calprotectin have been found in patients with inflammatory and autoimmune diseases. However, a number of preanalytical factors may affect calprotectin measurement in blood samples.
Methods: Twelve blood samples [4 tubes, 1 each of lithium-heparin (Li-heparin), EDTA, and serum] were drawn from each of 14 healthy individuals. To evaluate the effect of temperature and storage time in the lag time between collection and centrifugation, samples were kept for 2 h at 4 °C, 20 °C, or 37 °C, before centrifugation. Leukocyte, neutrophil, and monocyte counts were measured in EDTA samples on a Sysmex XN-10 hematology analyzer to investigate the relationship between calprotectin concentrations and the granulocyte count.
Results: Calprotectin measurements in EDTA samples were not influenced by temperature or time lag between collection and analysis. Compared to EDTA plasma, significantly higher calprotectin concentrations were found in serum and Li-heparin plasma samples. Furthermore, calprotectin concentrations increased in serum and Li-heparin samples when stored at higher temperatures. There was a linear relationship between the serum calprotectin concentration and neutrophil count in EDTA whole blood.
Conclusions: EDTA is the most suitable anticoagulant for determination of calprotectin in plasma, as this sample matrix does not seem to be affected by temperature or time between sample collection and analysis. Of particular note, neutrophil activation by either clotting or centrifugation should be avoided during the preanalytical process.

IMPACT STATEMENT

Calprotectin is used as a marker of disease activity in patients with many inflammatory conditions and autoimmune diseases. However, influence of different preanalytical variables on calprotectin measurements has not previously been investigated. Results from this study show a temperature-dependent increase in calprotectin concentrations in serum and heparin samples left for some time before centrifugation. Use of EDTA plasma for determination of calprotectin in blood is not affected by temperature or time between collection and analysis. This manuscript advances knowledge in the preanalytical testing phase of calprotectin measurement, an assay that is widely used in clinical settings.

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Calprotectin is a calcium-binding cytosolic protein that constitutes up to 40% of the cytosolic protein or 5% of the total protein content of both neutrophils and monocytes (*1*). The protein belongs to the S100 family, a group of multifunctional proteins with various regulatory roles in different cellular processes (*2*).

Calprotectin is released from neutrophils and monocytes during their activation, and increased systemic levels of the protein have been linked with increased immunological activity and inflammation (*3*). Clinically, calprotectin in blood has been investigated as a marker of disease activity in many inflammatory conditions and autoimmune diseases, such as inflammatory bowel disease, rheumatoid arthritis, and cardiovascular disease (*4*–*6*). However, calprotectin measurements in blood may be affected by preanalytical variables such as type of anticoagulant, and temperature and time of storage. Platelet activation in vitro affects tests in the coagulation laboratory (*7*) and type of anticoagulant; temperature and storage time are known to influence the platelet activation (*8*–*10*).

Activation of leukocytes is influenced by platelets (*11*) and therefore it can be hypothesized that calprotectin levels may be dependent on the same preanalytical factors that affects platelet activation. The aim of the study was to investigate the difference in calprotectin concentration collected in different tubes processed under different conditions. We furthermore investigated the potential relationship between calprotectin levels and the leukocyte count in whole blood.

METHODS

Subjects and samples

Blood samples were collected from 10 female and 4 male nonfasting volunteers 20–61 years of age. From each participant 12 tubes were collected: 4 serum tubes (Catalog No 369032, lot No 5027328), 4 Lithium Heparin (Li-heparin) tubes (Catalog No 367526, lot No 5271003) and 4 EDTA tubes (Catalog No 368861, lot No 5306579), all from BD Vacutainer System. One set of tubes from each volunteer were centrifuged immediately, while the other tubes were stored for 2 h at different temperatures before centrifugation as described in Table 1. After storage, all tubes were centrifuged for 9 minutes at 3600*g* at the same temperature used for storage. The supernatant from all samples was transferred to storage tubes immediately after centrifugation and stored at −80 °C for 3 weeks until calprotectin measurements were performed in a single run with use of the thawed samples. The study was approved by the Regional Ethical Committees for the Region of Southern Denmark, and informed consent was obtained from all participants.

Calprotectin assay

Analysis of serum/plasma calprotectin was performed as previously described (*6*) by use of the automated ELIA Calprotectin for ImmunoCap250 (Thermo Fisher Scientific). The established reference interval for serum calprotectin (2.5–97.5%) is 1040–4262 μg/L. Establishment of the reference interval for the serum calprotectin assay was conducted with use of serum samples obtained from a cohort of 120 healthy adult Danish blood donors (62 females and 58 males, 19–66 years of age) at Odense University Hospital. Participants were excluded if any of the following was present: pregnancy, autoimmune diseases, AIDS/HIV, infectious diseases, cancer, allergy, high (diastolic BP > 90 mmHg) or low (systolic BP < 110 mmHg) blood pressure, known cardiovascular disease (including thromboses), diabetes requiring treatment, severe

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Table 1. Calprotectin measurements at different storage conditions (time and temperature) prior to centrifugation.

	Serum Median (range) (µg/L)	Li-heparin Median (range) (µg/L)	EDTA Median (range) (µg/L)
Effect of storage temperature[a]			
4 °C	1370 (400–4270)	1370 (420–4590)	590 (<50–3230)
20 °C	4850 (181–11020)	3180 (1080–5010)	480 (<50–1690)
37 °C	5250 (2320–13080)	2710 (600–4660)	600 (<50–1660)
P values[b]	<0.001	<0.001	0.15
Effect of storage time			
Basic values[c]	4820 (2010–17640)	2020 (1150–8550)	530 (<50–1620)
Stored for 2 h (at 20 °C)	4850 (1810–11020)	3180 (1080–5010)	480 (<50–1700)
P values[d]	0.74	0.26	0.95

[a] Stored for 2 h.
[b] Between increasing temperature and 4 °C.
[c] Immediate centrifugation, freezing, and analysis.
[d] 2 h values compared to basic values.

skin diseases treated with steroids, ulcer, current use of narcotics or medicine, recent operation/tattoo/piercing, or treatment with hormones. All samples were aliquoted immediately after sampling and stored at −80 °C until analysis (*6*).

Blood count

Enumeration of leukocytes, neutrophils, and monocytes was determined in the EDTA whole blood samples immediately after sampling (tube: EDTA) with a Sysmex XN-10 hematology analyzer.

Statistical analysis

Variables are presented as medians and ranges. Because data tested with the D'Agostino-Pearson omnibus test did not follow a normal distribution, nonparametric tests were used. The Mann–Whitney *U*-test was used to evaluate differences between 2 samples, while Friedman's test was used for more than 2 samples.

Correlation between variables was evaluated with Spearman's rank correlation and interpreted according to the guidelines by Colton (*12*). Data were analyzed by use of GraphPad Prism and differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of storage temperature

The results of the calprotectin measurements for different storage conditions are shown in Table 1. Calprotectin concentrations in EDTA samples stored for 2 h before analysis at 4 °C, 20 °C, or 37 °C did not differ ($P = 0.15$). After storage, calprotectin concentrations in serum and Li-heparin samples increased with increasing temperature, and the differences between all storage temperatures were significant for both sample tubes ($P < 0.001$).

Effect of storage time

Calprotectin concentrations did not differ between samples centrifuged immediately and samples stored for 2 h at 20 °C in EDTA, Li-heparin, or serum samples ($P = 0.95$, $P = 0.26$, and $P = 0.74$, respectively).

Table 2. Calprotectin measurements in samples with different types of anticoagulant stored for 2 h.

	Serum Median (range) (µg/L)	Li-heparin Median (range) (µg/L)	*P* value[a]	EDTA Median (range) (µg/L)	*P* value[b]
4 °C	1370 (400–4270)	1370 (420–4590)	0.98	590 (<50–3230)	<0.001
20 °C	4850 (2320–13080)	3180 (600–4660)	0.09	480 (<50–1660)	<0.001
37 °C	5250 (1810–11020)	2710 (1080–5010)	<0.001	600 (<50–1690)	<0.001

[a] Li-heparin compared to serum.
[b] EDTA compared to serum.

Effect of anticoagulant

The results of the calprotectin measurements for different types of anticoagulant are shown in Table 2. Pairwise comparison of samples stored for 2 h revealed no differences between serum and Li-heparin when stored at 4 °C and 20 °C. At 37 °C serum calprotectin concentrations were significantly higher than in Li-heparin samples ($P < 0.001$). Calprotectin levels in EDTA tubes were significantly lower than calprotectin concentrations in serum at all temperatures. For samples stored at 4 °C, the median concentrations in EDTA samples were approximately 2 times lower than in serum ($P < 0.001$). At 20 °C the EDTA plasma concentrations were 8.6-fold lower than in serum ($P < 0.001$), and in samples stored at 37 °C EDTA plasma concentrations were 10.3-fold lower than in serum ($P < 0.001$).

Correlation between calprotectin and cell count

The results of the cell counts for the 14 volunteers are shown in Table 3.

There was a moderately strong linear relationship between serum calprotectin concentrations at 20 °C and the leukocyte count (Spearman's $r = 0.58$, $P = 0.032$), as well as the neutrophil count (Spearman's $r = 0.70$, $P < 0.001$; Fig. 1), but not between serum calprotectin and monocyte count (Spearman's $r = -0.12$, $P = 0.7$). The correlation between the total leukocyte count and calprotectin levels in Li-heparin and EDTA samples did not achieve statistical significance (Spearman's $r = 0.47$, $P = 0.09$) and Spearman's $r = 0.29$, $P = 0.33$ respectively).

DISCUSSION

The key findings in this study were 1) a significant effect of storage temperature of uncentrifuged samples on the calprotectin concentration in serum and Li-heparin samples, 2) a significantly lower concentration of calprotectin in EDTA samples compared to serum at all temperatures, 3) a stable calprotectin concentration in EDTA samples regardless of storage temperature or time, and 4) a moderately strong linear relationship between serum calprotectin and the leukocyte and neutrophil count.

A previous study compared calprotectin levels in matching EDTA-plasma and serum samples, and revealed no difference in fresh nonfrozen samples

Table 3. The results of cell count for the 14 volunteers.

	Leukocyte count (Ref.: 3.5–8.8 × 10^9/L) Median (ranges)	Neutrophil count (Ref.: 1.50–7.50 × 10^9/L) Median (ranges)	Monocyte count (Ref.: 0.20–0.80 × 10^9/L) Median (ranges)
14 volunteers	6.11 (4.34–11.91)	3.47 (1.70–7.79)	0.50 (0.27–2.09)

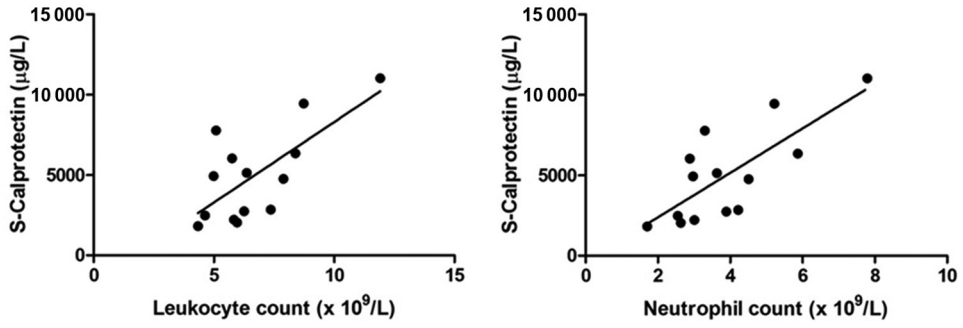

Fig. 1. Correlation between serum calprotectin in samples clotted at room temperature versus total leucocyte and neutrophile count in matched EDTA samples.

(*6*). This suggests that the difference between matrices happens during sample processing after sampling. Also, this indicates that the level of calprotectin is stable in EDTA regardless of post-sampling processing. We therefore believe the concentration in EDTA samples reflects the patient level the most accurately (at sampling).

During aggregation and activation platelets release a wide variety of proteins (e.g., proinflammatory cytokines) into serum. Exposure to proinflammatory cytokines causes activation of leukocytes and subsequent release of factors involved in an inflammatory response from the cells (*11*). Our finding of increased calprotectin concentrations due to neutrophil activation is also consistent with earlier reports (*13*). On the basis of this, we hypothesize that in-vitro leukocyte activation, and thereby calprotectin levels, may depend on anticoagulant, storage time, and storage temperature.

The temperature-dependent increase in serum calprotectin concentrations and the positive correlation between serum calprotectin and the leukocyte and neutrophil counts support the hypothesis that granulocytes are activated during serum clotting and release of granulocyte-activating substances leads to release of calprotectin.

Li-heparin samples also displayed a temperature-dependent increase in calprotectin concentrations suggesting that blood clotting is not the only factor necessary for release of calprotectin in vitro. Heparin has been widely investigated as an antiinflammatory agent, (*14*) and low molecular weight heparin has been shown to cause release of cytokines from human leukocytes (*15*). It is possible that heparin from the sample tubes stimulates release of calprotectin from granulocytes.

Because the volunteers included here were not selected on the basis of health status, we cannot be sure of absence of diseases contributing to increased calprotectin levels in some of the blood samples. However, this does not conflict with the aim of the study, which is an investigation of preanalytical factors influencing calprotectin measurements independent of calprotectin concentrations pretesting. Thus, our findings contribute information to the characteristics of the preanalytical phase in laboratory testing of calprotectin, which is important information because this analysis is widely used in various clinical settings.

The study has some limitations that should be noted. We only examined the effect of increasing temperatures over a 2-h period, which does not allow general conclusions to be drawn regarding temperature and time effects on calprotectin measurements. Also, we did not compare the levels of calprotectin before and after freezing, so we cannot eliminate that the freezing procedure had an impact

on measurements. As mentioned earlier, a previous comparison of calprotectin levels in fresh, nonfrozen serum and EDTA samples did not show any difference (*6*), suggesting that freezing of serum/plasma may affect the calprotectin levels differently in the 3 samples types. If serum calprotectin is investigated in biobanked samples, further study of a possible freeze–thaw effect in other sample types is warranted.

In conclusion, the temperature-dependent increase in calprotectin concentrations in serum and heparin tubes left for some time before centrifugation indicate these collection conditions are suboptimal for clinical purposes. We therefore recommend use of EDTA plasma for determination of calprotectin in blood, as this sample matrix does not appear to be affected by temperature or time between collection and analysis when investigated over a 2-h period. Also, neutrophil activation due to clotting or centrifugation should be avoided during the preanalytical process.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

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REFERENCES

1. Ehrchen JM, Sunderkotter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. J Leukoc Biol 2009;86(3): 557–66.
2. Sedaghat F, Notopoulos A. S100 protein family and its application in clinical practice. Hippokratia 2008;12: 198–204.
3. Naess-Andresen CF, Egelandsdal B, Fagerhol MK. Calcium binding and concomitant changes in the structure and heat stability of calprotectin (L1 protein). J Clin Pathol 1995;48:278–284.
4. Østgård RD, Deleuran BW, Dam MY, Hansen IT, Jurik AG, Glerup H. Faecal calprotectin detects subclinical bowel inflammation and may predict treatment response in spondylarthritis. Scand J Rheumatol 2017;26:1–8.
5. Bae SC, Lee YH. Calprotectin levels in rheumatoid arthritis and their correlation with disease activity: a meta-analysis. Postgrad Med 2017;129:531–37.
6. Pedersen L, Nybo M, Poulsen MK, Henriksen JE, Dahl J, Rasmussen LM. Plasma calprotectin and its association with cardiovascular disease manifestations, obesity and the metabolic syndrome in type 2 diabetes mellitus patients. BMC Cardiovasc Disord 2014;14:196.
7. Magnette A, Chatelain M, Chatelain B, Ten Cate H, Mullier F. Pre-analytical issues in the haemostasis laboratory: guidance for the clinical laboratories. Thromb J 2016; 14:49.
8. Semple JW, Freedman J. Platelets and innate immunity. Cell Mol Life Sci 2010;67:499–511.
9. Blok SL, Engels GE, van Oeveren W. In vitro hemocompatability testing: the importance of fresh blood. Biointerphases. 2016;11:029802
10. Ho CH, Chan IH. The influence of time of storage, temperature of storage, platelet number in platelet-rich plasma, packed cell, mean platelet volume, hemoglobin concentration, age, and sex on platelet aggregation test. Ann Hematol 1995;71:129–33.
11. Bazzoni G, Dejana E, Del Maschio. Platelet-neutrophil interactions: possible relevance in the pathogenesis and inflammation. Haematologica. 1991;76:491–99.
12. Colton T. Statistics in Medicine. 1st ed. Boston: Little, Brown; 1974.
13. Cotoi OS, Dunér P, Ko N, Hedblad B, Nilsson J, Björkbacka H, Schiopu A. Plasma S100A8/A9 correlates with blood neutrophil counts, traditional risk factors, and cardiovascular disease in middle-aged healthy individuals. Arterioscler Thromb Vasc Biol 2014; 34:202–10.
14. Koller M, Kutscha-Lissberg F, Brom J, Weidinger G, Muhr G. Influence of low molecular weight heparin (certoparin) and unfractionated heparin on the release of cytokines from human leukocytes. Inflammation 2001;25:331–7.
15. Young E. The anti-inflammatory effects of heparin and related compounds. Thromb Res 2008;122:743–52.