CAT
Critically Appraised Topic

Pre-analytical evaluation of blood calprotectin measurement in Rheumatoid Arthritis

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CLINICAL BOTTOM LINE

There is an additional need for specific biomarkers in diagnosis, prognosis and follow-up of RA. Calprotectin (CLP) has been extensively studied as a mediator of inflammation in the pathogenesis of RA and shown to be related to clinical, laboratory and ultrasound parameters of disease activity. Still, more research is needed to assess its potential as a biomarker for RA. An important problem however is the substantial inter-study variability of CLP in RA, resulting from differences in assays, as well as the use of different sample types. With our study, we wanted to elucidate the effect of different matrices, pre-centrifugation times, storage temperatures and times on blood CLP concentrations. Serum CLP concentrations revealed to be more than twice as high as in EDTA- or citrate plasma. CLP measurement in EDTA showed higher reproducibility compared to serum. Heparin, EDTA and citrate had a stabilizing effect on CLP concentrations up to 6h before centrifugation, whereas significant increases were seen in serum. CLP remained stable refrigerated for up to 7 days in all sample types and up to 72 and 24 hours at room temperature and 30°C respectively.

In conclusion, due to the differences in CLP concentrations between sample types, studies on the clinical value of CLP in RA need to specify the used sample matrix and adjust their reference values or decision cut-offs accordingly. Furthermore, pre-analytical sample handling instructions are more stringent for serum samples and need to be taken into consideration to obtain reproducible CLP concentrations.

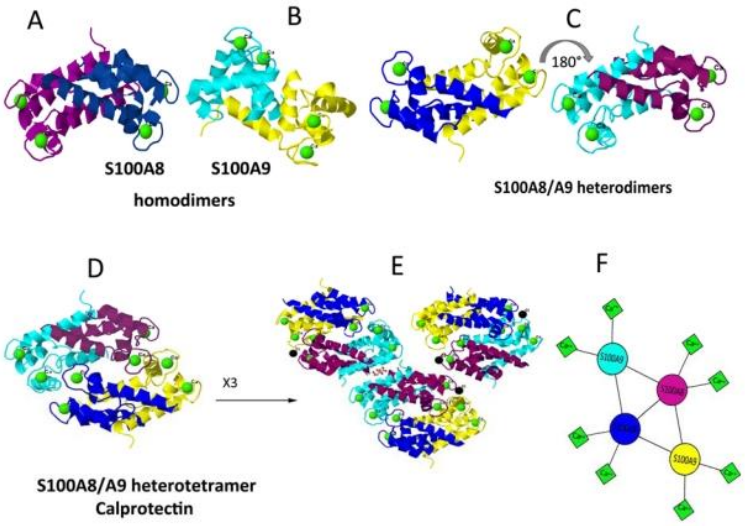
CLINICAL/DIAGNOSTIC SCENARIO

Rheumatoid arthritis (RA) is the most common chronic auto-immune inflammatory joint disease, affecting 0.5-1% of the industrialized world. RA typically presents as a symmetrical polysynovitis of small joints, which if left untreated or undertreated, eventually leads to cartilage destruction and bone erosion. [1,2] Although its cause and pathogenesis are not fully understood, major genetic factors and environmental triggers are described. [1] People with RA show increased mortality and morbidity, affecting daily activities, quality of life, work status and socioeconomic welfare. [3–5] Early disease recognition and start of treatment is of vital importance to prevent or minimize joint damage and related morbidity [6], with the first 12 weeks since symptom onset being regarded as therapeutic window of opportunity.[7,8] In 2010, ACR/EULAR classification criteria were redefined, focusing on early-stage factors associated with later joint destruction (i.e. number of affected joints, elevated C-reactive protein (CRP)/erythrocyte sedimentation rate (ESR), Rheumatoid Factor (RF) and/or Anti-citrullinated protein antibodies (ACPA) positivity and symptom duration). [9] However, diagnosis of early RA remains challenging emphasizing the need for additional specific RA biomarkers in daily practice.[10]

Calprotectin (CLP) is a heterodimeric complex of the S100A8 and S100A9 calcium-binding proteins, also known as myeloid related proteins 8 and 14 (MRP-8/MRP-14) or calgranulin A and B. CLP is classically expressed in neutrophils, monocytes and early stage macrophages. [11,12] In specific circumstances other cell types can express and secrete CLP such as chondrocytes, osteoclasts, fibroblast-like synoviocytes (FLS) and endothelial cells.[13] CLP is mostly secreted through an active, calcium-dependent Protein

Kinase C (PKC) pathway [14], next to the passive leakage from necrotic cells and release in neutrophil extracellular traps (NET).[13,15] S100A8 and S100A9 proteins can be found as homodimers and heterotetramers, but the heterodimer is the most stable form and accounts for most of its biological functions [16] (Figure 1). In the presence of excess Ca^{2+}, heterodimers associate to form heterotetramers which are more resistant to proteolysis. [17]

Figure 1: Tertiary and quaternary structures of S100A8 and S100A9 proteins. Adapted from Vogl et al. [18]



Recently, CLP has been extensively studied as an important mediator of inflammation in RA. [19] CLP is part of the innate immune response, acting as an endogenous ligand of Toll-like receptor 4 (TLR4) and receptor of advanced glycation end-products (RAGE).[13] Following receptor activation, S100A8/A9 expression is upregulated, creating a positive feedback loop with autocrine and paracrine stimulation of effector cells (e.g. endothelial cells, osteoclasts, FLSs). [20] Furthermore, expression of proinflammatory cytokines, matrix metalloproteinases and adhesion molecules is upregulated facilitating leukocyte migration and infiltration in the synovial cavity. [21,22] CLP is released at the local site of inflammation and plasma levels have been suggested to be a biomarker that reflects local disease activity in inflammatory diseases [23] , in contrast to conventional acute-phase proteins such as CRP, which are mainly produced by hepatocytes after non-specific, systemic inflammatory activity. [24]

A recent meta-analysis of 16 studies with 849 patients and 266 controls revealed that CLP levels in serum and synovial fluid were significantly higher in RA patients compared to controls. In addition, CLP was significantly correlated to disease activity measured by CRP and Disease Activity 28-joint (DAS28) score. [25] Similar findings were shown by a recent systematic review and additionally, it was objectified that the high serum/plasma CLP levels in RA patients fell with effective treatment and that high baseline levels of CLP were predictive of future erosion. [23] Several studies show that serum CLP is significantly correlated with ultrasound (US) synovitis [26,27] and that CLP is a better predictor of US synovitis than CRP. [28,29] Moreover, CLP seems to better reflect inflammation in RA patients with moderate to high disease activity and a normal CRP level [30,31], which is seen in approximately 35-40% of RA patients. [32] Similarly, in patients treated with IL-6 inhibitors, CLP showed superiority to CRP in discriminating disease status. [33,34] This can be explained by the direct blocking effect of IL-6 inhibitors on CRP, which precludes its use to assess treatment response.[34] Studies showed that CLP more accurately discriminates disease activity than CRP and ESR even in patients with low inflammatory activity[35] and that CLP could discriminate subclinical disease activity from US-defined remission in patients that were in clinical remission. [36] Furthermore, baseline CLP levels were higher in TNF-α treatment responders and a decrease predicted treatment response. [35,37] Similar findings were

observed in other studies, with higher baseline CLP in Disease Modifying Anti-Rheumatic Drug (DMARD) responders and a decrease in CLP after start of treatment. [27,38,39] It has also been shown that higher baseline CLP values were predictive of relapse during treatment with biological DMARD treatment in patients with low disease activity, whereas no difference in CRP or ESR between relapsers and non-relapsers was observed. Moreover, CLP levels significantly increased during relapse. [40] Furthermore, a recent study observed higher CLP levels at the moment of treatment tapering or stopping in RA patients that experienced relapse within 12 months. [41] These findings suggest that CLP might be a promising biomarker in treatment monitoring and follow-up of drug-free remission. However, further investigations are required to evaluate the value of CLP on prognosis and therapeutic decisions in RA.

Despite being a promising biomarker, a substantial inter-study variability of CLP in RA has been described, resulting from differences in assays, as well as the use of different sample types and other pre-analytical factors. [24,42–44] The current lack of harmonization hampers adequate comparison of studies. In most of the previous studies, serum was used for CLP measurement. However, only limited evidence is available on how preanalytical factors, such as storage conditions and sample type, affect CLP levels [42,44] and there is no clear consensus on the matrix that should be used. In this study we want to investigate the impact of different storage conditions and sample types on CLP blood concentrations.

QUESTION

1) To investigate the effect of pre-analytical variables (sample type, pre-centrifugation time, storage time/temperature) on blood calprotectin measurement.

SEARCH TERMS

1) MeSH Database (PubMed): MeSH term: "Leukocyte L1 Antigen Complex" AND "Rheumatoid Arthritis"
2) Pubmed (Medline, Embase, Cochrane): (calprotectin AND rheumatoid arthritis) OR (serum calprotectin AND rheumatoid arthritis)) OR (plasma calprotectin AND rheumatoid arthritis))) OR (("Leukocyte L1 Antigen Complex"[Mesh]) AND "Arthritis, Rheumatoid"[Mesh])))) OR (calprotectin AND rheumatic disease))) OR (calprotectin and edta))) OR (calprotectin AND citrate)) OR (calprotectin AND heparin)) OR ((serum calprotectin) AND assay[Title/Abstract])) OR ((serum calprotectin) AND matrix[Title/Abstract])) OR ((serum calprotectin) AND ELISA[Title/Abstract])) OR ((serum calprotectin) AND reference values[Title/Abstract])

RELEVANT EVIDENCE/REFERENCES

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Materials and methods

Samples

We recruited 10 healthy controls, defined as having no fever or pain of any kind, no systemic rheumatic disease in the medical history or current anti-inflammatory or corticoid treatment. CRP measurement (Cobas c501, Roche Diagnostics, Germany) was performed to confirm non-inflammatory status of the controls.
Imprecision analysis was performed on three patient pools of different CLP concentrations (940, 330 and 70 µg/ml) and a low (<200 µg/mL) and high (939 µg/mL) commercially available kit control.

Evaluation of preanalytical aspects

-Sample types and sample processing
For each participant, one blood collection was performed with Sarstedt S-MONOVETTE® tubes, following the standard hospital procedure. Five different sample types were collected:
1) Serum (clot activator): 7.5 ml (ref 01.1601.001) and 1.2 ml (ref. 06.1663.001)
2) Serum with gel (clot activator + separator gel): 7.5 ml (ref 01.1602.001) and 4.9 ml (ref. 04.1935.001)
3) Lithium-Heparin (25 IU/ml): 4.9 ml (ref. 04.1940.001)
4) K3-EDTA (1.6 mg/ml) : 2.7 ml (ref: 05.1167.001) and 1.2 ml (ref. 06.1664.001)
5) 9NC Trisodium Citrate (0.106 mol/l): 3ml (ref: 05.1165.001) and 1.4 ml (ref. 06.1668.001).

After collection, serum samples were allowed to coagulate for at least 30 minutes (and maximum 2 hours; t=0) before centrifugation at 3500 rpm for 8 minutes. Two sample tubes of every sample type were stored at room temperature until centrifugation at t=6h and t=24h after sample collection. After centrifugation at t=0, serum/plasma was pooled per sample type and divided in aliquots for measurement at different storage time points and conditions.

-Storage conditions
CLP was measured at three different storage conditions (room temperature (18-25 °C), in refrigerator (2-8°C) and incubator (30°C)) at three different storage time points (t=24h, t=72h and t= 7 days). Additionally, CLP concentrations were analyzed after 5 freeze-thaw cycles and after 7 days and 3 months of storage at -20°C. Immediately following every CLP analysis, the aliquot was stored at -20°C.

Ethics and trial registration

The study was approved by the local Ethics Committee OLV Hospital Aalst with Belgian registration number 126201942003.

Calprotectin assay

Blood CLP was measured in serum and plasma with the EliA™ Calprotectin 2 test, a sandwich-based fluoro-enzyme-immunoassay (FEIA), on a Phadia™ 200 instrument (Phadia A.B., Thermo Fisher Scientific, Sweden) using a for-research-use-only protocol. Samples were diluted 1:50 with EliA Sample diluent prior to the assay. In summary, this assay uses polystyrene wells, coated with monoclonal anti-calprotectin antibodies that will bind to CLP in the sample. After sample incubation and a first washing step, a β-galactosidase-conjugated, monoclonal mouse anti-calprotectin antibody is added, creating a sandwich complex. Following conjugate incubation and a second washing step, substrate (4-Methylumbelliferyl-β-D-galactoside) is added to generate a fluorophore. After stopping the reaction by the addition of a stop solution, the fluorescence of the eluate is measured using a fluorometer. The CLP value is calculated, by comparing the fluorescence signal to that of a 5-point logistic calibration curve. The technical validation of each analytical run is based on the duplicate analysis of a curve

control to verify the validity of the calibration curve on the one hand and the analytical performance of an EliA Calprotectin positive and negative iQC on the other.

Methodology and data analysis
The CLP concentrations are presented in µg/mL as median +/- 95% confidence interval (CI).

A verification of the imprecision of the EliA calprotectin 2 assay was performed on three patient pools and two commercially available kit controls during ten different runs. A F-test for comparison of standard deviations was used to verify if the imprecision of the EliA assay on the Phadia 200 differed significantly from the results obtained on Phadia 250. [42] For each storage time and storage condition, the CLP concentration was compared to the baseline measurement (t=0) using a Wilcoxon test (non-parametric test, paired samples).

Mean differences compared to baseline were calculated. A mean difference of +/- 2*total imprecision (2*CVa) was considered clinically relevant. A Mann-Whitney test (non-parametric test, independent samples) was used to compare CLP concentrations at t=0 in the different sample types and to compare reference CLP concentrations in serum and EDTA with previous findings.[42] The Grubbs test was used for outlier detection.

All data analysis was performed in MEDCALC® (version 17.1, Ostend, Belgium). A p-value < 0.05 was considered statistically significant.

Results

Demographics
The male/female ratio of the healthy controls included in the study was 40%. Age ranged from 25-62 years. CRP values of all healthy controls were <5 mg/L.

Imprecision
Imprecision results are shown in Table 1. CV% ranged from 4.6% to 10.8% and were comparable to previously determined CV%'s on Phadia 250. [42] The highest CV% did not significantly differ from the previously determined CV% (10.8 vs. 9.2, F-Test: p=0.326).

Table 1: Imprecision on Phadia 200

	Patient pools			Thermo Fisher iQC	
Calprotectin	**high**	**medium**	**low**	**iQC pos**	**iQC neg**
Mean (µg/mL)	0.936	0.329	0.069	0.960	0.112
SD (µg/mL)	0.050	0.017	0.007	0.044	0.011
CV (%)	5.4	5.3	10.8	4.6	10.2

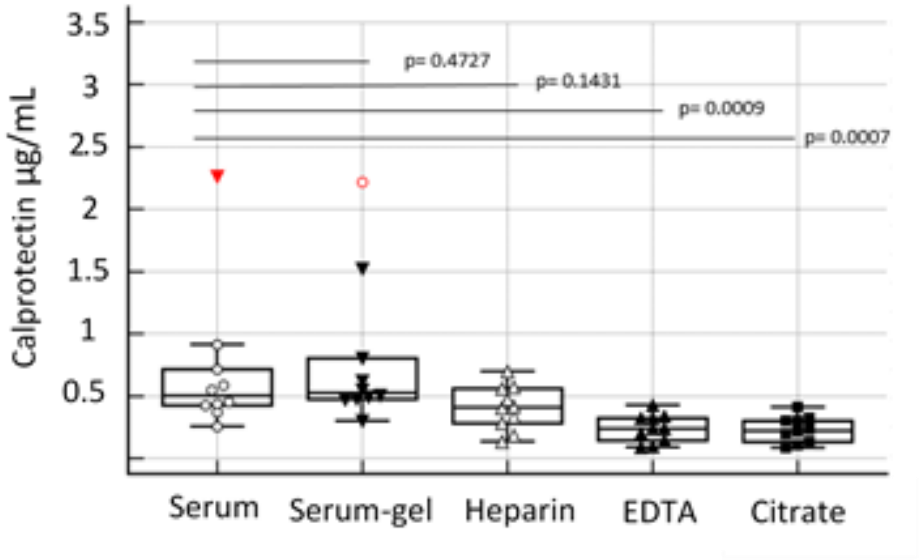
Evaluation of preanalytical conditions

-Comparison of calprotectin concentrations in different matrices in healthy controls (Figure 2).

At baseline, median CLP values in serum and serum-gel samples were more than twice the values in EDTA and citrate, i.e. 0.501 (0.395-0.818) µg/mL and 0.525 µg/mL (0.470-1.180) vs. 0.237 µg/mL (0.117-0.327) (p = 0.0009) and 0.220 (0.118-0.310) µg/mL (p = 0.0007), respectively. Median CLP value in heparin was 0.409 µg/mL (0.229-0.562) and did not significantly differ from serum (p = 0.1431) but

was significantly higher than in EDTA (p = 0.0413) and citrate (p = 0.0284). Outlier values were observed in serum(-gel) but not in plasma (Grubbs' test; resp. 2.260 µg/mL and 2.215 µg/mL; p<0.05).

Figure 2: Calprotectin baseline measurement in different matrices in healthy controls



The reference values for CLP in serum and EDTA-plasma as reported in a previous study were verified using healthy controls. [42]

Median serum CLP in healthy controls significantly differed from previous data (0.846 µg/mL [0.655 – 1.188] vs. 0.456 µg/mL [0.376 – 0698], p = 0.0058), whereas in EDTA, no difference between previous and our current data was observed (0.176 µg/mL [0.140 – 0.249] vs. 0.237 µg/mL [0.117 – 0.318], p= 0.5093). This observation was also reflected in the comparison of the 95th percentiles of both data sets, where serum showed a noticeable difference (i.e. 1.402 µg/mL vs. 0.832 µg/mL) in contrast to EDTA (0.408 µg/mL vs. 0.385 µg/mL).

-Stability of calprotectin under different storage conditions at different time points in healthy controls.

Results are shown in Table 2. Significant p-values and mean differences outside the analytical acceptance limits (2 * CVa, i.e. 20%) are highlighted. For all sample types, no significant change in median CLP values was observed after 5 freeze-thaw cycles and after 7 days and 3 months of storage at -20°C. The highest observed mean difference was 7.8%.
No significant change in median CLP values was observed at 2-8°C for up to 7 days of storage for all sample types with the exception of serum samples. However, the increases in serum at 72h and 7 days (8.5% and 8.2% respectively) were within analytical acceptance limits and found not to be clinically relevant. At room temperature, CLP values were stable in all sample types up to 72h. At 7 days of storage a decrease of 40.8% was observed in EDTA whereas the other sample types showed increasing CLP values, ranging from 14.9 to 25.6%.
Regarding the storage conditions at 30°C, all sample types revealed, clinically relevant increases in CLP values after 72h. For EDTA samples, a clinically relevant decrease of 28.4% was already obtained after 24h of storage at 30°C. After 7 days of storage at 30°C, CLP values in EDTA had almost declined to zero (decrease of 95.6%) whereas the other sample types revealed increases ranging from 40.5% to 43.7%.

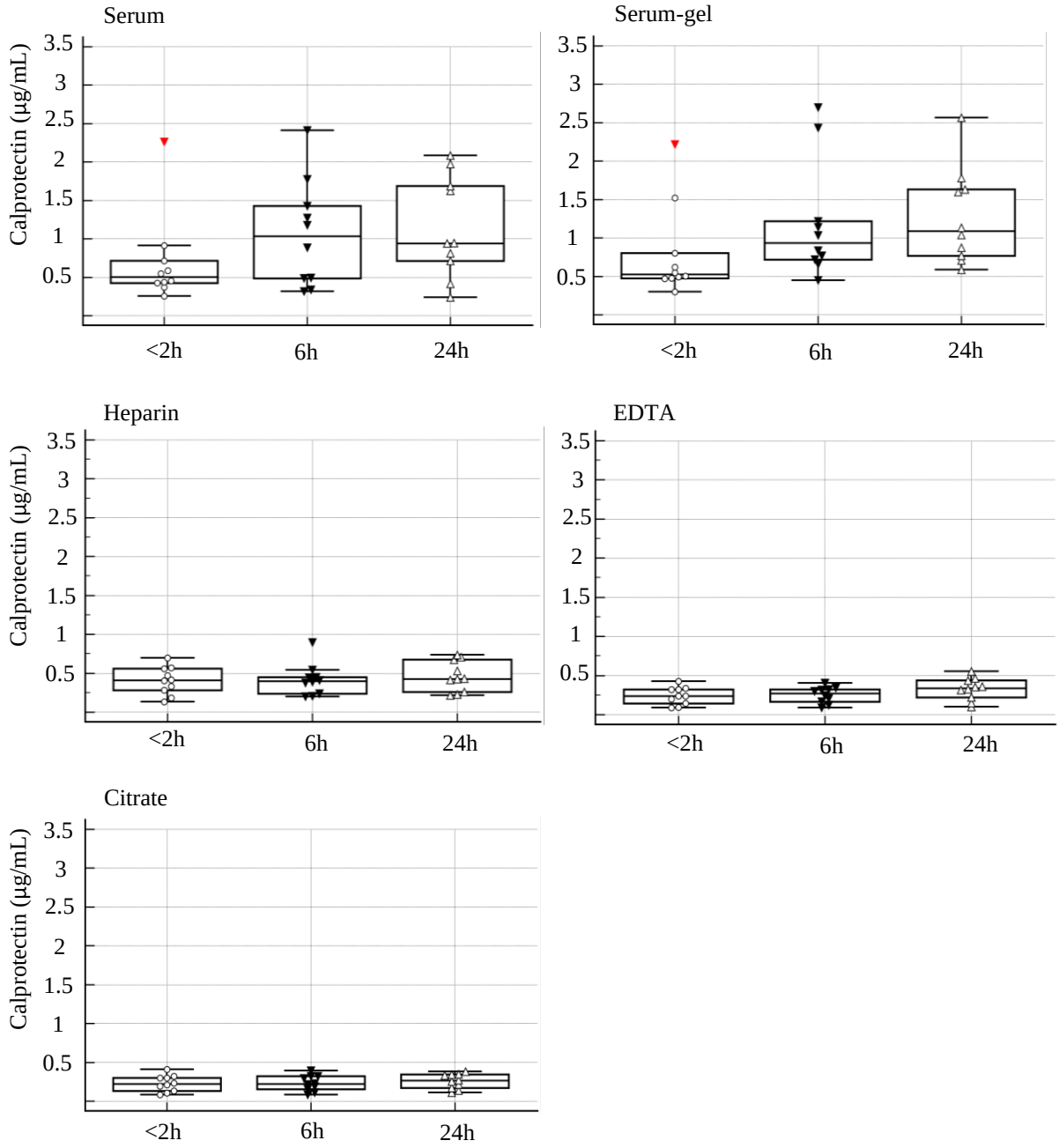
Table 2: % increase/decrease at different time points and storage conditions compared to baseline calprotectin measurement (t = 0h) of healthy controls. Significant p-values and mean differences outside the analytical acceptance limits (2 * CVa, i.e. 20%) are highlighted.

	calprotectin (µg/mL)		**Fridge 2-8 °C**			**Room temp. 18-25 °C**			**Incubator 30°C**			**Centrifugation**		**Freeze-Thaw**	**Freezer -20°C**
		t = 0h	24h	72h	7d	24h	72h	7d	24h	72h	7d	6h	24h	5 cycli	7d
serum	**median (µg/mL)**	**0.456**	**0.457**	**0.484**	**0.510**	**0.517**	**0.568**	**0.598**	**0.492**	**0.576**	**0.607**	**0.885**	**0.942**	**0.446**	**0.474**
	***p* (Wilcoxon)**		0.0781	0.0234	0.0273	0.0195	0.0039	0.0039	0.0039	0.0039	0.0039	0.0195	0.0195	0.0742	0.7344
	mean difference (%)		6.2%	8.5%	8.2%	7.3%	15.4%	25.6%	10.8%	20.4%	43.7%	74.2%	87.0%	7.8%	-0.3%
serum gel	**median (µg/mL)**	**0.505**	**0.518**	**0.533**	**0.549**	**0.516**	**0.593**	**0.595**	**0.521**	**0.600**	**0.770**	**0.837**	**1.040**	**0.536**	**0.517**
	***p* (Wilcoxon)**		0.1641	0.3008	0.5703	0.0547	0.0547	0.0039	0.0273	0.0039	0.0039	0.0039	0.0039	0.2500	0.8203
	mean difference (%)		2.6%	5.4%	1.2%	5.1%	8.2%	14.9%	6.4%	18.7%	41.6%	58.4%	83.6%	3.8%	-0.3%
heparin	**median (µg/mL)**	**0.409**	**0.397**	**0.415**	**0.400**	**0.418**	**0.451**	**0.454**	**0.467**	**0.468**	**0.588**	**0.400**	**0.427**	**0.422**	**0.453**
	***p* (Wilcoxon)**		0.3750	0.0645	0.2500	0.0371	0.0020	0.0020	0.0098	0.0020	0.0020	0.9219	0.4316	0.7695	0.2754
	mean difference (%)		4.1%	7.6%	6.5%	7.1%	14.3%	19.6%	10.4%	22.5%	42.6%	7.7%	25.4%	2.0%	5.1%
EDTA	**median (µg/mL)**	**0.237**	**0.237**	**0.230**	**0.224**	**0.216**	**0.199**	**0.146**	**0.173**	**0.32**	**0.076**	**0.271**	**0.339**	**0.243**	**0.235**
	***p* (Wilcoxon)**		0.840	0.7695	0.2324	0.0645	0.0020	0.0020	0.0020	0.0020	0.0020	0.0742	0.0273	0.3008	0.6963
	mean difference (%)		3.4%	0.5%	-3.6%	-5.2%	-14.8%	-40.8%	-28.9%	-82.2%	-95.6%	7.5%	53.3%	1.2%	0.4%
citrate	**median (µg/mL)**	**0.220**	**0.221**	**0.227**	**0.236**	**0.246**	**0.255**	**0.260**	**0.239**	**0.304**	**0.339**	**0.220**	**0.264**	**0.220**	**0.205**
	***p* (Wilcoxon)**		0.2754	0.0195	0.0840	0.840	0.0020	0.0020	0.0020	0.0020	0.0020	0.7695	0.0096	0.5703	0.4316
	mean difference (%)		3.5%	7.5%	6.1%	7.5%	16.9%	22.6%	10.8%	29.2%	40.5%	2.1%	17.8%	4.3%	-2.1%

-Effect of time to centrifugation on calprotectin concentrations in healthy controls (Figure 3).

In serum and serum-gel tubes, relevant increases in CLP concentrations were observed already at the 6h-centrifugation time point (74.2% and 58.4% respectively). In heparin, EDTA and citrate, CLP concentrations remained stable at the 6h-centrifugation time point (increases of 7.7%, 7.5% and 2.1% respectively). At the 24h-centrifugation time point, CLP concentrations remained stable in citrate only (increase of 17.8%).

Figure 3: Effect of time to centrifugation on calprotectin concentration in healthy controls.



Discussion

Blood CLP is suggested as potential biomarker for the evaluation of inflammation in RA[13]. In an arthritis-induced murine model, mice lacking functional CLP did not show cartilage destruction in contrast to controls, emphasizing the role of CLP in the pathogenesis of RA. [19] Since CRP is normal in up to 50% of RA patients, and treatment with IL-6 inhibitors rapidly blocks CRP levels, the addition of supplementary biomarkers to assess treatment response or the occurrence of infection in RA is warranted. Recent literature reveals the correlation of CLP with clinical, laboratory and ultrasound parameters of disease activity in RA,[23,25-29,33,35,37,38,40–43,45–53] even in patients with normal CRP levels.[30,31,34,36]

Despite being a promising biomarker, a substantial inter-study variability of serum CLP levels has been described. [24,42] Besides analytical variables, important pre-analytical factors contribute to this lack of harmonization between CLP studies. [42] Regarding sample types, in previous studies examining pre-analytical aspects, only serum, EDTA and heparin blood samples were analyzed. [42,44] In our study, we also included citrate blood samples. As confirmed by our current study results, baseline CLP concentrations (time to centrifugation <2h) measured in serum are at least two times higher compared to EDTA/citrate-plasma, [42–44] and heparin CLP concentrations are somewhere in between serum and EDTA/citrate CLP concentrations. [44,53,54] Different hypotheses corroborate the observed differences in baseline CLP concentrations:

1) in vitro CLP release by neutrophils

Plasma CLP levels are suggested to more accurately reflect in vivo CLP concentrations since the higher concentrations observed in serum are likely due to the in vitro release of CLP by neutrophils after sampling. [43,44] Both EDTA and citrate chelate calcium, thereby blocking the active secretion of CLP through the calcium-dependent PKC pathway.[14]

2) *in vitro CLP release through coagulation and platelet activation*

Coagulation and platelet activation in serum tubes leads to activation of neutrophils and formation of NETs, thereby inducing more CLP release [15], contributing to the higher levels of CLP in serum compared to plasma.

3) in vitro change of CLP conformation

In the presence of Ca^{2+}, CLP dimers and oligomers are generated, causing significant alterations in the protein structure and thus availability of antigenic epitopes for binding in sandwich ELISA assay. [43] Since Ca^{2+} concentration in resting cells is approximately 100 nM compared to 2 mM extracellular, the heterodimer is regarded as the major cytosolic form, whereas the Ca^{2+}-bound heterotetramer is the most important extracellular form. [55] Thus, one can hypothesize that in serum and heparin tubes, where Ca^{2+} is freely circulating, mainly tetramers are found. These tetramers might produce a double fluorescence signal in the FEIA assay, when compared to heterodimers that are expected to be the main form in EDTA and citrate plasma. This theory however needs verification, preferably by a Western blotting experiment which can objectively discriminate between the different CLP structures.

Due to the differences in CLP concentrations between sample types, studies on the clinical value of CLP in RA need to specify the used sample matrix and adjust their reference values or decision cut offs accordingly.

Furthermore, to our knowledge, we are the first to report the effect of delayed centrifugation and 3 different storage conditions [room temperature (18-25 °C), refrigerator (2-8°C) and incubator (30°C)] on CLP stability in different sample matrices. For all sample types, we found that CLP was stable (i.e.

mean difference <20%) after 5 free-thaw cycles, up to 3 months of storage at -20°C and up to 7 days when refrigerated. At room temperature and at 30°C, stability was observed up to 72h and 24h respectively.
Instability of CLP manifested as an increase in serum, heparin and citrate and as a decrease in EDTA. Two hypotheses to motivate these changes in time:

1) enhanced in vitro CLP release by neutrophils due to a delay in separation from cellular matrix

In contrast to serum, plasma can be separated from cells immediately after sampling, thereby limiting potential in vitro release of CLP from neutrophils. Up to date, the effect of a delayed centrifugation has not yet been extensively studied. Pedersen et al. didn't reveal any difference in CLP concentrations in serum, heparin and EDTA concentrations when centrifuged immediately vs. after two hours of storage at room temperature. [44] Dale et al. described a stabilizing effect of EDTA on CLP concentrations up to three days before centrifugation in contrast to significant increases of CLP levels in citrated blood and serum tubes. [56] In our current study, we've compared CLP concentrations when centrifuged within two hours versus 6 and 24 hours. In plasma tubes, we didn't reveal any difference in CLP concentrations between 2h and 6h, but a significant increase after 24h of storage before centrifugation. In serum tubes however, we already observed significantly increased CLP concentrations (mean increase 74.2%) after 6h of storage before centrifugation, most likely due to an enhanced in vitro CLP release from neutrophils. In addition, the usage of serum tubes without gel separator might lead to higher CLP, due to incomplete separation of neutrophils and monocytes from serum, [43] but this was not observed in our data.

CLSI GP44 'Guidelines on handling and processing blood specimens' allows a pre-centrifugation time of 24-48h for the most common lab tests. This guideline does not account for serum CLP and a more strict pre-centrifugation time (< 2h after sampling) has to be taken into consideration to obtain reliable CLP serum concentrations. If plasma samples are used, these pre-analytical considerations are less strict (centrifugation < 6h after sampling), which make plasma tubes more suitable for CLP analysis when samples are taken at e.g. the general practices.

2) conformational CLP changes and increased sensibility to plasma proteases

In serum, heparin and citrate samples, we've obtained a moderate increase (max. 8.2% refrigerated to 43.7% at 30°C) in CLP concentrations over time. The underlying cause of this observation is not clear yet, but might be related to shifting in CLP configurations (dimer/tetramer) over time. Remarkably, after 7 days of storage at 30°C, CLP concentrations declined to almost zero in EDTA. It has been shown that the Ca^{2+}-dependent formation of heterotetramers provides protection to breakdown by proteases [55] and enhances thermal stability.[57] Since EDTA strongly chelates calcium, the plasma is expected only to contain the more "vulnerable" heterodimers, that are more easily destructed by protease activity, at a higher rate at 30°C compared to room temperature. Remarkably, this theory does not hold true for citrate since the citrate matrix seems to have a stabilizing effect on CLP heterodimers over time.

Interestingly, we also revealed a higher reproducibility of CLP reference values in EDTA than in serum, when comparing data from healthy donors to a previous study. [42] Furthermore, outlier values were observed in serum tubes but not in plasma. These findings suggest that future usage of plasma matrices might provide more reproducible CLP concentrations between studies.

Conclusion

Due to the differences in CLP concentrations between sample types, studies on the clinical value of CLP in RA need to specify the used sample matrix and adjust their reference values or decision cut-offs accordingly. Furthermore, pre-analytical sample handling instructions are more stringent for serum samples and need to be taken into consideration to obtain reproducible CLP concentrations.

To Do/Actions

1) Expanding our data set with blood sampling of 5 RA patients.
2) Confirming the CLP conformational hypothesis with Western Blotting analysis.
3) Performing a meta-analysis on current CLP literature with focus on pre-analytical variables.