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Increasing sensitivity of a particle-enhanced turbidimetric immunoassay for quantitative analysis of calprotectin

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Biotechnology, Molecular biology

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Abstract

Calprotectin is a protein that is released by activated leukocytes and monocytes. Increased levels of the protein are found in patients with inflammatory and autoimmune conditions, including juvenile idiopathic arthritis (JIA), a childhood group of diseases causing inflammation of the joints.

In this thesis, the Gentian Calprotectin Immunoassay (GCAL®) was adjusted to allow accurate measurements of calprotectin concentrations under 0.5 mg/L in human blood samples. The adjusted highly sensitive assay (hsGCAL) was tested on serum samples from children with various forms of JIA for a selection of laboratory and clinical parameters. The effects on sensitivity of using different parts of the avian antibody immunoglobulin Y (IgY) in the assay was also assessed.

The results show that the hsGCAL assay (with adjusted calibrator set and 8 uL sample volume) meets the criteria for linearity in the range 0.05 – 10.4 mg/L (on Abbott Architect c4000) with a security zone up to at least 60 mg/L (highest concentration tested). The results also show improved/lower CV (%) compared to the reference assay (GCAL®). The results indicate that the adjustments performed and tested in this thesis have the potential to increase the sensitivity of the GCAL® assay.

Also, a stronger turbidimetric signal was observed when only using the immunoparticles coated with the monomeric fraction of anti-calprotectin IgY only, compared to fractions containing combinations of different fractions of IgY. This indicates that the GCAL® assay may benefit from using monomeric antibodies instead of total IgY.

An increased sensitivity in the lower calprotectin concentration range could be beneficial when used to diagnose diseases where there is little difference in the calprotectin concentration between a healthy and a diseased person. However, no statistical differences between GCAL® and hsGCAL were found in serum samples of JIA patients, indicating that for this patient group there is no need for the added sensitivity provided by the hsGCAL assay.

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1. Introduction

Calprotectin is a protein that is released by activated leukocytes and monocytes during inflammation. The protein can be detected in different body fluids such as blood, saliva, urine, feces and synovial fluid. There is increasing evidence that calprotectin is a better marker for inflammation, as elevated calprotectin levels are associated with bacterial infection, sepsis, but also a range of other diseases such inflammatory bowel diseases, rheumatic diseases and certain cancers (Pruenster *et al.*, 2016).

The Norwegian company Gentian AS has developed a particle-enhanced turbidimetric immunoassay (PETIA) for quantitative analysis of total calprotectin in plasma. The Gentian Calprotectin Immunoassay (GCAL®) can be used for the measurement of calprotectin in plasma in the diagnosis of inflammation. The assay can be applied on a wide range of automated turbidimetric clinical chemistry analyzers.

However, with calprotectin being a relatively new marker, there are a couple of challenges when using the calprotectin immunoassay in clinical settings. To start with, blood calprotectin has different concentrations depending on the matrix: serum gives the highest calprotectin values, EDTA plasma the lowest values and Lithium Heparin plasma in between (Pedersen *et al.*, 2018). In addition, calprotectin has not yet an international standard, meaning that there is no internationally approved reference material with a determined calprotectin value which can be used to calibrate a new diagnostic test against. Therefore, each commercial and in-house test must define its own calibration and cutoff values, and there are variations between them.

For Gentian's turbidimetric calprotectin test (for blood plasma), the obtained values have been compared with the BÜHLMANN ELISA calprotectin test (**Figure 1)**. For unknown reasons, the values of BÜHLMANN ELISA calprotectin test are a factor 3 higher than the turbidimetric Gentian plasma calprotectin test, although the two methods correlated well and are highly commutable (Nilsen, Sunde and Larsson, 2015).

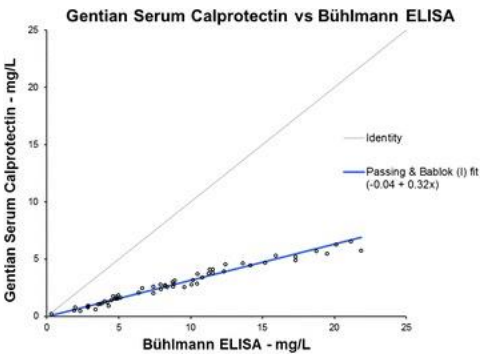


Figure 1. Method comparison. Gentian Serum Calprotectin (GCAL®) vs Buhlmann ELISA (Nilsen, Sunde and Larsson, 2015)

The lower quantification limit (LoQ) for GCAL® is ~0.30 mg/L (LoQ is instrument dependent) for Lithium Heparin plasma. However, the LoQ comes with high error and high Coefficient of Variation (CV), which is a measurement of relative variability. With this high potential for error in the lower concentrations, the potential 1/3 lower measurements for GCAL® compared to other calprotectin tests, and low calprotectin values in EDTA plasma, it may be useful to increase the sensitivity of the current test This will allow measurement of low calprotectin concentrations in blood. **Such a sensitive assay can be useful in situations where it is important that the calprotectin values in the lower concentration areas are precisely/accurately measured.**

1.1. Objectives and research questions

The goal of this study was to improve sensitivity of the Gentian Calprotectin Immunoassay (GCAL®), to allow accurate measurements of calprotectin concentrations under 0.5 mg/L in human blood.

It was hypothesized that such a sensitive assay might be useful in 1) diseases where calprotectin levels are elevated but where the difference between control and patient is small, 2) testing in EDTA plasma which generally has lower calprotectin levels compared to Lithium Heparin plasma and serum and 3) when testing children as it is uncertain if calprotectin baseline levels in the pediatric population differs from adults.

Leading up to this study was the increasing evidence that calprotectin is a relevant biomarker in diagnosis, prediction of flares and evaluating treatment effect in Juvenile Idiopathic Arthritis (JIA), a pediatric disease closely related to rheumatic arthritis (RA) in adults (Kopeć-Mędrek, Widuchowska and Kucharz, 2016). A recent study by Nordal *et al.*, (2018) showed that testing calprotectin in EDTA had the strongest associations with assessments of disease activity in RA, and the question arises if this could be true for JIA as well.

Values for systemic onset JIA are very high, but for other forms of JIA, they are moderate and in healthy children (controls) they should be even lower (around 0.3 mg/L in many studies, but because of the abovementioned variation, potentially much lower in EDTA with the GCAL® assay).

Research questions addressed in this thesis are:

- What is the effect of varying assay parameters, such as sample volume and calibration range, on the sensitivity of the GCAL® assay?
- Do different fractions of total avian immunoglobulin (IgY) show higher affinity for calprotectin? Does this result in higher sensitivity?
- Does increased sensitivity of the GCAL® assay lead to a better predictive value in JIA (clinical significance)?

1.2. Background information

1.2.1. Calprotectin

S100 proteins were discovered in the 60s and were partly soluble in 100% saturated ammonium sulphate, which explains the name S100-proteins. In 1980, calprotectin was first isolated from granulocytes by Fagerhol and his colleagues and provisionally named L1 protein.

The term calprotectin is often used for the hetero-complex of S100A8/A9, reflecting its protective role in epithelial defense and its antimicrobial activity (Kopeć-Mędrek, Widuchowska and Kucharz, 2016; Pruenster et al., 2016). S100A8/A9 and other S100 proteins are only found in vertebrates (Vogl, Gharibyan and Morozova-Roche, 2012). Calprotectin is also known as myeloid related proteins 8 and 14 (MRP8/14) because they are mainly expressed in myeloid lineage cells, and as Calgranulin A/B due to their ability to bind Ca^{2+}. It is also, but in a lesser degree, referred to in the literature as L1 protein, 27E10 antigen, cystic fibrosis antigen, myeloid-histiocyte antigen and CP-10 (this refers to the light chain only).

Calprotectin (S100A8/A9) is a heterodimeric protein of 24 kDa, consisting of α subunit S100A8 (10.8 kDa, 93 aa) and β subunit S100A9 (13,2 kDa, 114 aa). S100A9 also has a truncated isoform S100A9* (12,7 kDa, 110 aa) though differences in biological function are not yet discovered.

Both S100A8 and S100A9 are members of the S100 sub-family of EF-hand calcium-binding proteins, which includes 25 known members in humans. The EF-hand motif is composed of two alpha helices “E” and “F” joined by a loop of 12-amino acid residues. These charged amino acid residues gives the protein a high affinity for binding Ca^{2+} (Pruenster *et al.*, 2016).

Each S100A8 and S100A9 monomer has two EF-hand regions that can bind one Ca^{2+} ion each. Binding of Ca^{2+} causes a conformational change in the C-terminus, exposing a hydrophobic cleft that enhances protein-protein interaction and improves affinity for transition metal ions. Without bound Ca^{2+}, the protein interaction site is buried. (Fritz *et al.*, 2010; Pruenster *et al.*, 2016). Because extracellular Ca2+ levels are high, the tetrameric form is expected to be the most abundant and relevant extracellular form (Nakashige *et al.*, 2016).

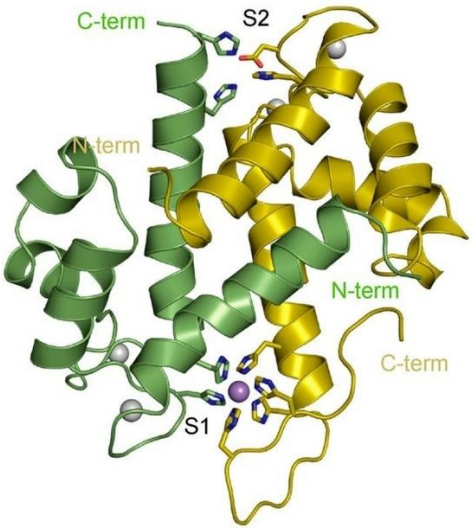


Figure 2. X-ray crystal structure of Mn-bound calprotectin. The calprotectin heterodimer composed of S100A8 (green) and S100A9 (yellow). The four white spheres represent Ca^{2+} ions, and the purple sphere a Mn^{2+} ion. The two transition metal binding sites are labeled S1 (Mn/Zn-specific) and S2 (Zn-specific) (Damo et al., 2013).

As visualized in **Figure 2**, the calprotectin heterodimer has two sites (S1 and S2) that can take up metal. Transition metal ions are formed at the interface of the S100A8/S100A9 heterodimer (Fritz *et al.*, 2010). At the S2 site, metal binding is coordinated by two histidine ligands (His83 and His87) from S100A8, and a histidine and aspartic acid ligand (His20 and Asp30) from S100A9. The S1 site can coordinate metals through a tetra-histidine or a hexa-histidine binding motif. In tetra-histidine binding, metal binding is coordinated by four histidine residues, two from S100A8 (His17 and His27), and two from S100A9 (His91 and His95). In hexa-histidine binding two additional histidine residues, His103 and His105, from the C-terminal end of S100A9 contribute. Manganese is bound by the calprotectin dimer at this hexa-histidine ring. Zinc can be bound to both hexa- and tetra-histidine forms at S1, and at the S2 site. Besides depriving pathogens of metal ions, the binding of Zn^{2+} also shields the S100A9 C-terminal tail from proteolytic degradation by proteinase K. Calprotectin is the only known human protein that can chelate Manganese (Nakashige *et al.*, 2016).

A S100A8/A9 heterodimer can be formed in the presence or absence of Ca^{2+}, but the formation of heterotetrameric calprotectin (by two S100A8/A9 heterodimers) is strictly dependent on the presence of Ca^{2+}-or/and Zn^{2+} (Vogl, Gharibyan and Morozova-Roche, 2012; Pruenster *et al.*, 2016).

In-house experiments at Gentian AS, (not part of this thesis) show that the antibodies used in the GCAL® immunoassay can detect and bind to all forms of calprotectin present in a natural sample (α, β, α_2, β_2, αβ, $\alpha_2\beta$, $\alpha\beta_2$, $\alpha_2\beta_2$ and higher oligomeric combinations (where α =S100A8 and β=S100A9). The main forms observed in biological fluids are heterodimeric (αβ) and tetrameric ($\alpha_2\beta_2$) forms.

S100A8 and S100A9 are mainly expressed in neutrophils, but also in other cells such as monocytes, dendritic cells, fibroblasts, mature macrophages, vascular endothelial cells and keratinocytes. In neutrophils, S100A8/A9 constitutes 45% of all cytosolic proteins. In monocytes this is only 1%.

Cell stress or inflammation induces extracellular release of S100A8 and S100A9, where these proteins assemble to calprotectin and bind cell surface receptors RAGE and TLR4. Binding to TLR4 and RAGE activates intracellular pathways via NF-kB, and results in a signaling cascade that regulates inflammation, cell proliferation, differentiation, and tumor development. Binding to TLR4 promotes the expression of proinflammatory proteins (cytokines, chemokines, etc.) that will cause adhesion and trans-endothelial migration of leukocytes. Calprotectin also acts as a chemotactic factor by inducing adhesion of neutrophils, and promotes apoptosis and autophagy in lymphocytes, macrophages, endothelial cells, and tumor cells (Xia *et al.*, 2018, Frosch *et al.*, 2009).

1.2.2. Avian immunoglobulin (IgY)

Antibodies used in immunoassays often are produced in mammalian animals (e.g. rabbits, goats). In Gentian's calprotectin assay GCAL®, avian antibodies (IgY) from chicken are used. These are produced by immunizing hens against human native or recombinant calprotectin. The hens produce antibodies, and a particular form of these, IgY, are transported from the hens' circulation to the egg yolk (Patterson *et al.*, 1962). The antibodies can be extracted from the eggs, without the need for bleeding the animal.

Another advantage of IgY is that the phylogenetic distance between chicken and humans allows successful production of immunoglobulins against antigens that are highly conserved in different mammalian species. IgY shows little cross reaction with mammalian immunoglobulins, do not bind to mammalian Fc receptors, rheumatoid factor (RF), protein A and G, or activate the mammalian complement pathway. This avoids interference that often is observed in immunoassays based on mammalian antibodies (Gassmann *et al.*, 1990; Larsson *et al.*, 1993). Rheumatoid factor (RF) is found in serum samples from patients with rheumatoid arthritis and RF-positive polyarticular JIA (Hinks *et al.*, 2018), but also in in 3-5% of healthy individuals (Munhoz *et al.*, 2014).

Chicken IgY is structurally close to mammalian IgG (**Figure 3**), which is important for antigen recognition. It has similarities with both mammalian IgG and IgE and seems to be the evolutionary predecessor of both. It is phylogenetically derived from IgM, and IgA and has links to IgX (Warr, Magor and Higgins, 1995; Bengtén *et al.*, 2000).

However, IgY also has marked differences. Avian IgY has five domains (V, C1-C4), as opposed to four domains in mammalian IgG. It also has no hinge, but switch regions with limited flexibility at the Cv1 and the Cv3-Cv4 domain interfaces. This limited flexibility offers unique biochemical properties, for example the inability to precipitate antigens at physiological salt concentrations. Also, the two arms may be so closely aligned that they prevent cross-linking of epitopes on large antigens. Chicken IgY occurs mainly in its monomeric form, H_2L_2, with a molecular weight of 180 kDa (Warr, Magor and Higgins, 1995).

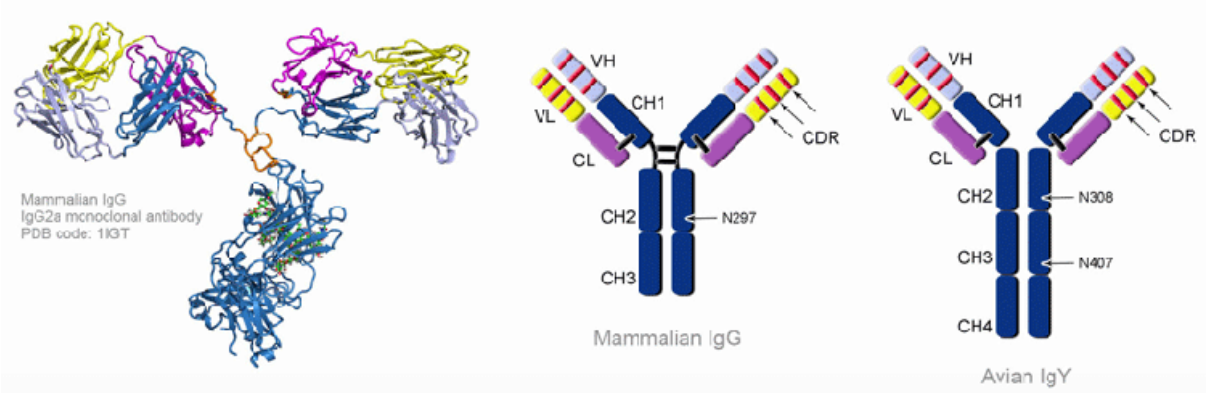


Figure 3 Structure of IgY (avian) and IgG (mammalian). Source: CNC (Center for Neuroscience and Cell Biology)

1.2.3. Juvenile Idiopathic Arthritis

Juvenile idiopathic arthritis (JIA) is the term for a heterogenic group of arthritic diseases of unknown cause, which have an onset in childhood (before 16 years). There are several subclassifications of JIA, each with their own clinical symptoms.

The main subtypes that are of interest are:

- Systemic onset JIA (soJIA) causes inflammation in one or more joints and is often accompanied by a high spiking fever that lasts at least 2 weeks and a skin rash. Other possible signs include inflammation of the heart or lungs; anemia; or enlarged lymph nodes, liver or spleen.
- Oligoarticular JIA causes arthritis in four or fewer joints, typically the large ones (knees, ankles, elbows).
- Polyarticular JIA causes inflammation in five or more joints, often the small joints of the fingers and hands, but weight-bearing joints and the jaw can also be affected.

Besides those, there are also Juvenile psoriatic arthritis**,** enthesitis-related JIA**.** Undifferentiated arthritis is the term used to describe a JIA form that does not fit into any of the above types, or that involves symptoms spanning two or more subtypes.

Systemic JIA is considered an autoinflammatory disease. The other types are considered autoimmune diseases. A healthy immune system will have an appropriate reaction to invading viruses and bacteria. In people with autoimmune or inflammatory diseases, the immune system becomes overactive even when there is no infection to fight, or it mistakenly attacks the organisms own healthy cells and tissues.

Several studies showed that calprotectin is a better marker for the diagnosis of JIA than conventional markers such as C-Reactive Protein (CRP). This is not surprising, as there is strong evidence that there is a direct pathogenic role of calprotectin in chronic inflammation, especially in arthritis and systemic-onset JIA (Frosch *et al.*, 2009b).

A large study (60 patients with soJIA; 85 with systemic infection, 40 with acute lymphoblastic leukemia, 5 with acute myeloblastic leukemia, 18 with NOMIC, and 50 healthy controls) by Frosch *et al.* (2009a) concluded that calprotectin allowed early differentiation between patients with systemic-onset JIA and those with other inflammatory diseases. In contrast to CRP levels, calprotectin concentrations could also distinguish systemic-onset JIA from infections (which is important, as clinical symptoms can be similar in soJIA and severe infections).

In a study in 160 patients with systemic-onset, polyarticular, RF-negative and oligoarticular subtypes of juvenile idiopathic arthritis (JIA), the blood calprotectin levels were higher in patients with systemic-onset subtype of the disease, and differed significantly from levels in healthy children, levels in patients with articular subtypes of JIA, and patients with RF-negative polyarthritis and oligoarthritis. The researchers concluded that blood calprotectin level is useful to confirm the diagnosis of soJIA and to monitor the disease activity and therapy effectiveness (Bojko, 2017).

A recent study in over 300 patients enrolled with inflammatory symptoms, confirmed that calprotectin allows early differentiation of soJIA in patients with fever of unknown origin. It concluded that levels of patients with soJIA were elevated compared to other diagnoses including infections, vasculitis and other autoinflammatory diseases. ('10th Congress of International Society of Systemic Auto-Inflammatory Diseases (ISSAID)', 2019).

All studies were performed with (different) ELISA calprotectin tests, commercial (Bühlmann, PhiCal) or in-house (München) or lateral flow immunoassay (LFIA). No studies were performed with Gentian's Calprotectin Immunoassay (GCAL®).

2. Methods and materials

2.1. Overview experimental setup

First, **sample volume** and the **calibration range** were adjusted to evaluate the effect on the sensitivity of the GCAL® test in low biomarker concentrations. Sensitivity of a diagnostic test is the "true positive rate" or the ability of the test to correctly identify patients (vs healthy).

The adjusted test (hsGCAL) was compared to the reference GCAL® assay and tested for hook/security zone, recovery, and linearity on a turbidimetric clinical analyzer (Abbott Architect c4000).

To gain more insight on the anti-calprotectin antibodies used in the assay, affinity purified IgY was fractionated with size exclusion chromatography (SEC) to be able to select IgY-fractions with mainly a) monomers, b) fragments or c) aggregated antibodies. The affinity of selected antibody fractions for calprotectin was tested by surface plasmon resonance (Biacore X100).

Nanoparticles were coated with different fractions of IgY. The size of the coated particles was analyzed by nanoparticle tracking analysis (Malvern Nanosight NS300), and their affinity for calprotectin was studied by establishing calibration curves (Mindray BS400).

To evaluate clinical relevance of the adjusted assay, serum, EDTA plasma and synovial fluid from children with JIA and controls from Karolinska Institute (Stockholm, Sweden) were tested with both the reference calprotectin assay (GCAL®) and the adjusted method (hsGCAL) with increased sample volume and adjusted calibration range (Abbott Architect c4000).

Gentian Calprotectin Immunoassay (GCA®L)

The Gentian Calprotectin Immunoassay (GCAL®) is used as a basis for the experiments during the entire study. The GCAL® immunoassay kit consists of immunoparticles, buffer, calibrators and controls. In the development of this assay (not part of this thesis), heterodimeric human calprotectin (MRP8/MRP14) from human granulocytes was used to immunize hens to produce anti-human calprotectin avian (IgY) antibodies that were extracted from chicken eggs. Affinity purified avian immunoglobulin fractions (IgY) are covalently attached to uniform polystyrene nanoparticles. As reaction buffer, 3-(N-morpholino) propanesulfonic (MOPS) with pH = 7.2 is used.

Purified calprotectin is also used for the development of calibrators. Purified calprotectin is diluted in a phosphate buffer pH = 7.4 to achieve six calibration levels**:** 0, ~1, ~3, ~6, ~10, ~20 mg/L (depending on the lot)**.** The calprotectin concentration of the stem solution is assigned by the Biuret method (Bioquant™, Merck KGaA, Darmstadt, Germany).

Quality Controls are prepared by adding purified calprotectin to normal human serum. Two levels are available: Control Low at ~1 mg/L and Control High at ~10 mg/L.

2.2. Background on technology and methods used

2.2.1. Particle-Enhanced Turbidimetric Immunoassay (PETIA)

GCAL® is an example of a particle-enhanced turbidimetric immunoassay (PETIA). "Turbidimetry is the measurement of light-scattering species in solution by means of a decrease in intensity of the incident beam after it has passed through the solution", according to the Immunoassay Handbook (Sheehan and Binder, 2013). A particle-enhanced immunoassay uses the classical antibody-antigen binding principle. However, the antibodies to the antigen of choice are bound (coated) to a latex nanoparticle. When antigen is introduced to these antibody-coated nanoparticles, it leads to crosslinking of the particles and the formation of aggregation complexes (**Figure 4**) that can be detected turbidimetrically, for example by a clinical chemistry analyzer such as the Abbott Architect c4000 (Abbott, USA), used in most of the experiments in this thesis.

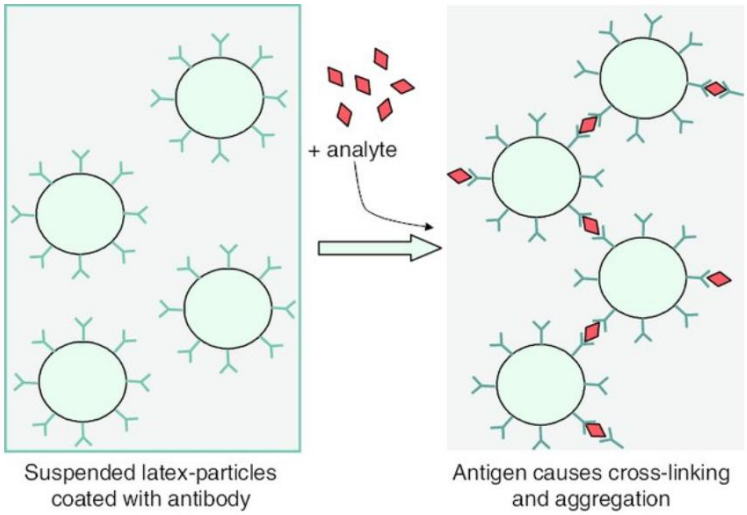


Figure 4 Visualization of agglutination in particle enhanced immunoassays (Gubala *et al.*, 2014)

Clinical chemistry analyzers are automated, usually high-throughput, instruments that are in routine use in central hospital and commercial laboratories for the analysis of a large range of markers. Such instruments offer automatic pipetting of the sample and reagents, results are offered.

2.2.2. Size Exclusion Chromatography (SEC)

In size exclusion chromatography (SEC), a column is packed with resin (solid phase), a porous matrix of chemically and physically stable spherical particles with properties that minimize adsorption of biomolecules. The column is then equilibrated with buffer, which fills the pores of the matrix and the space between the particles. The liquid inside the pores is in equilibrium with the liquid (buffer or mobile phase) outside the particles. The buffer remains the same during the separation. Molecules that are larger than the largest pores eluate together with the void volume as they pass directly through the column. Molecules with partial access to the pores of the matrix are separated and elute from the column in order of decreasing size. Small molecules such as salts have full access to the pores and are not separated when they move down the column. These molecules usually elute slightly before the complete buffer volume has passed through the column.

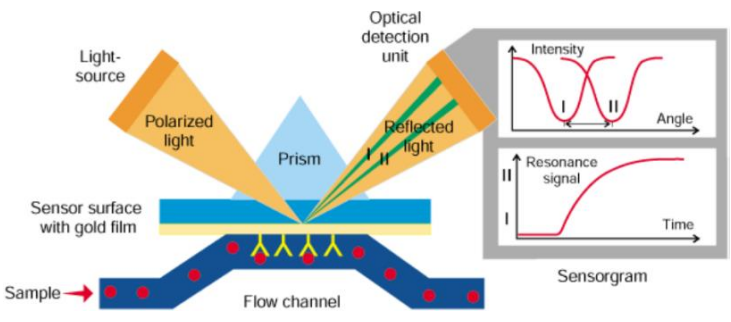
Results from SEC are expressed as a chromatogram (elution profile) that shows the variation in concentration of sample components as they elute from the column in order of their molecular size, with the largest molecules eluting first (Fekete *et al.*, 2014; GE Healthcare, Size Exclusion Chromatography, principles and methods. 18102218).

2.2.3. Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) is an optical technique used to monitor real-time binding interactions between two different molecules (in which one is mobile and the other one fixed on a thin metal film, usually gold) and provides information on binding, kinetics, affinity, specificity, and active binding concentration without the need for labels.

SPR occurs when polarized light hits a metal film at the interface of media with different refractive indices, coated with an electrically conducting film. The SPR technique excites and detects collective oscillations of free electrons (known as surface plasmons).

Figure 5 Refractive index (source: Encyclopedia Brittanica)



In the Biacore system used in this thesis, the sensors' surface consists of a glass slide coated with a thin gold film. A (dextrane or other) matrix covered by an electrically conducting film (e.g. gold) acts as a substrate to which ligand molecules (antibodies to calprotectin, in this experiment) can be attached, and provides a hydrophilic environment for the interaction. The analyte (calprotectin in this experiment) is injected in a continuous flow of solution.

An evanescent wave created by total internal reflection interacts with free electron clouds in the metal causes a drop in the intensity of light reflected at a specific angle from the glass side of the sensor surface (position I in **Figure 5**). As analyte molecules bind to the ligand molecules on the sensors' surface, the refractive index (measure of the bending of a ray of light when passing from one medium into another) close to the surface changes, altering the angle of minimum reflected intensity (position II in **Figure 5**). The angle is monitored continuously as the SPR signal, and a plot of the signal against time. The change is SPR angle is proportional to the material (mass) bound. The result from the detection of change in the refractive index is displayed as a **sensorgram**, where the binding response on the y-axis is plotted against time on the x-axis. It shows the changes in refractive index as sample binds to and dissociates from the surface. Since light does not penetrate the sample, analysis can be performed on colored or opaque samples. (Tang, Zeng and Liang, 2010)

Interpreting the results

K_D is the equilibrium dissociation constant, a calculated ratio of K_d/K_a, between the antibody and its antigen. The association constant (K_a) is used to characterize how quickly the antibody binds to its target. The dissociation constant (k_d) is used to measure how quickly an antibody dissociates from its target.

Affinity is defined as the strength of binding of the antibody to its ligand (antigen). A high-affinity interaction is characterized by a low K_D, rapid binding to the target (high K_a) and strong stability of formed complexes (low K_d).

A low-affinity antibody will show a K_D in the micromolar range (μM, KD between 10^{-4} to 10^{-6}), while high-affinity antibodies are in the picomolar (pM, KD between 10^{-10} to 10^{-12}) to femtomolar (fM, KD between 10^{-13} to 10^{-15}) range. The nanomolar (nM, KD between 10^{-7} to 10^{-9}) indicates medium affinity.

However, it is important to evaluate K_d and K_a in addition to K_D, as two antibodies can have the same affinity while one may have both a high K_d and K_a, while the other may have a low K_d and K_a. (Source: *KD value: a quantitative measurement of antibody affinity | Abcam*, 2019)

2.2.4. Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analysis (NTA) makes use of the properties of both light scattering and Brownian motion to detect the size and size distribution of extremely small particles (10nm to 2000nm). For this work, NanoSight NS300 was used.

As is illustrated in **Figure 6, a** laser beam illuminates the particles, which are then detected individually by a standard microscope. A camera captures a video of each individual particle's Brownian movement over time. The software then analyses the movements and calculates the hydrodynamic diameter of each particle (Source: *Nanoparticle Tracking Analysis NTA*).

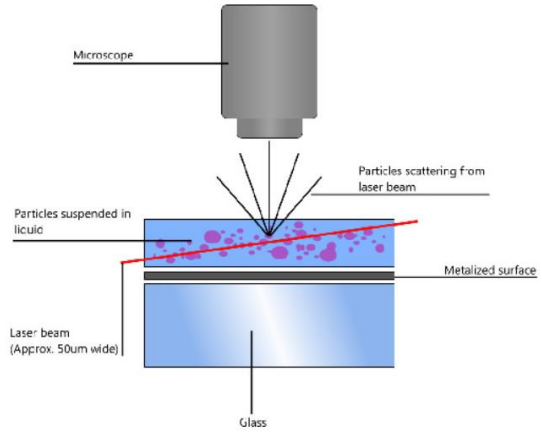


Figure 6 Schematic view of the Nanoparticle tracking Analysis technology used in the NanoSight NS300 system. Source: Malvern Panalystical

2.3. Assay parameter adjustments

2.3.1. Calibration range

In this experiment, the standard calibration range ("Current") was compared to an adjusted calibration range ("Test") (**Table 1**) to assess the effect on sensitivity. It was expected that the adjusted calibrator point would be more sensitive than the current calibrator set and could be used to measure lower blood calprotectin concentrations (both serum and Lithium-Heparin plasma). The parameters tested were plasma vs serum, and the two different calibrator sets.

It should be noted that the GCAL® assay is validated for plasma and serum, however it was only CE-marked for serum.

Table 1. Values of current and new calibrator set (lot number 1703429)

Channel	Calibrator set	Point 1 (ml/L)	Point 1,5 (ml/L)	Point 2 (ml/L)	Point 3 (ml/L)	Point 4 (ml/L)	Point 5 (ml/L)	Point 6 (ml/L)
1	Current	0.00	-	1.27	2.73	5.42	10.84	**21.67**
2	Test	0.00	**0.64**	1.27	2.73	5.42	10.84	-

To obtain the new calibrator point (point 1,5) for the adjusted calibration, 250 µL of calibrator 1 and 250 µL of calibrator 2 from the current calibrator set were measured on a scale and mixed. The new point was assigned the average value of point 1 and 2 ((0,00+1,27)/2= 0,635 mg/L). Calibrators and controls were not used for more than 5 calibrations to avoid evaporation effect, though the same lot number was used throughout the experiment. Likewise, the same lots of reagents (**Table 2**) were consistently used throughout the experiment.

Table 2. List of equipment and materials (calibration range)

Controls	Article No.	Lot No.	Lower range	Upper range
CGAL Quality Control Low (QC low)	1220	1703417	0.84 mg/L	1.26 mg/L
CGAL Quality Control High (QC high)	1221	1703418	8.35 mg/L	12.53 mg/L
Calibrator set	Article No.	Lot No.		
Calibrators 1-6		1703429		
Reagents	Article No.	Lot No.		
Gentian Plasma calprotectin R1/Assay buffer	1207	1703421		
Gentian Plasma calprotectin R2/Immunoparticles	1214	1703432		
Gentian Plasma calprotectin calibrators	1251	1703429		

Lithium Heparin plasma and serum samples were taken from four healthy volunteers (with informed consent), who were assumed to have a low calprotectin concentration. Gel collection tubes were used, as this is the common type of tube used in hospitals. The serum samples were left to rest for 1 hour in RT before they were centrifuged at rps 3000 for 10 minutes. The supernatant was aliquoted into new tubes, and the pellet discarded.

The standard plasma calprotectin application was applied on the Abbott Architect c4000. Two instrument channels were programmed according to **Table 3**, one for each lot of calibrator set, as it is preferable that the samples are measured simultaneously for both calibrator sets to avoid additional variables.

Table 3. Instrument settings used on Abbott Architect c4000

Parameter	Settings
R1: Assay buffer Volume [µL]	180
Sample Volume [µL]	4
Dilution factor	1:10
R2: Immunoparticles [µL]	30
First reading time [cycle]	12-15
Second reading time [cycle]	21-23
Primary wavelength [nm]	604

The channels were calibrated, one with the Current and one with the Test calibrator set. To validate the calibration curve, the two quality controls (QCs) were measured and confirmed within validity criteria after calibration and prior to measurement of the samples.

Reagents, calibrators and controls were stored at T=2-8°C until the start of the study. When the study was ongoing, the reagents were stored in the instrument (cooled), while the calibrators and controls were capped and stored at T=2-8°C immediately after use.

The blood samples were measured in 10 replicates on both applications simultaneously (same run), using the same reagent/calibrator lots.

MS Excel was used to calculate average value (**Equation 1**), SD (**Equation 2**) and CV (**Equation 3**) from all replicates for each sample.

Equation 1

$$\bar{X} = \frac{\sum_{i=1}^{n} x_i}{n}$$

n= number of replicates

Equation 2

$$SD = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{X})^2}{n-1}}$$

n Number of replicates

$\bar{X}$	Mean	From Equation 1

Equation 3 $$CV\ [\%] = \frac{SD}{\bar{X}} \times 100\%$$

$\bar{X}$	Average concentration	From *equation 1*
SD	Standard Deviation	From *equation 2*

2.3.2. Calibration and sample volume

After determining the effect of the calibration range, the sample volume was increased (from the current 4 µL) to 6µL and 8µL to see if any specific combination of calibration set and sample volume would further increase sensitivity. The 8 µL was only tested with the adjusted calibrator set, because a valid calibration curve could not be achieved on the Abbott Architect c4000 with this sample volume.

The instruments and materials used were identical to the calibration range experiment, described in **Table 2.** To test if the CV (%) would decrease with increased sample volume and if the adjusted calibrator set would perform better, serum and plasma blood samples from the two healthy donors were measured in 10 replicates each on five applications with calibrator set (current and test) and sample volumes (4µL, 6µL and 8µL) as variables, as in **Table 4**. The five channels were programmed according to the instrument settings in **Table 5**. The same lots of reagents, calibrators and controls were used as in the first experiment (**Table 2**).

Table 4 Calibration and sample volume parameters programmed on Abbott Architect c4000

Channel /application	Calibration range / set	Sample volume (µL)
1	Current	4
2	Test	4
3	Current	6
4	Test	6
5	Test	8

Table 5. Instrument settings

Parameter	**Settings channel 1+2**	**Settings channel 3+4**	**Settings channel 5**
R1: Assay buffer Volume [µL]	180	180	180
Sample Volume [µL]	4	6	8
Dilution factor	1:10	1:10	1:10

R2: Immunoparticles [µL]	30	30	30
First reading time [cycle]	12-15	12-15	12-15
Second reading time [cycle]	21-23	21-23	21-23
Primary wavelength [nm]	604	604	604

MS Excel was used to calculate average value (**Equation 1),** SD (**Equation 2**) and CV (**Equation** 3) from all replicates for each sample.

The assay combinations mentioned in **Table 4** were further assessed with regards to how they would perform in measuring very low calprotectin values. For this, 4 plasma samples with different levels of calprotectin were diluted with HEPES Casein (to mimic the natural matrix) to levels of ~0.3 mg/L and ~0.15mg/L. Each original sample was measured in triplicates to assess the initial calprotectin value, and the dilutions to ~0.3 mg/L and ~0.15 mg/L were measured in ten replicates on each of the five channels in **Table 4**.

MS Excel was used to calculate average value (**Equation 1**), SD (**Equation 2**) and CV (**Equation** 3) from all replicates for each sample. To assess accuracy of the measurements, the recovery (%) was calculated (**Equation 4**).

Equation 4 Recovery (%)

$$Recovery\ [\%] = \frac{\bar{X}}{\overline{X_{theoretical}}} \times 100\%$$

$\overline{X_{theoretical}}$	Expected value of the sample	From Equation 5
$\bar{X}$	Average measured concentration	From Equation 1

2.3.3. Security zone

The purpose of this experiment was to determine the security zone of the of the adjustments of the assay on Abbott Architect c4000. Security zone is defined as the antigen concentration range that will be accurately reported by the instrument. The security zone usually extends beyond the highest calibrator point. Higher values will be “flagged” by the instrument as outside of the calibration range, and a rerun (a second measurement) can be performed after a dilution either by the instrument (1:10 dilution in dH20) or manually by the user.

The reason for this experiment was that, with an increased sample volume (=more antigen), it is possible you will get false low results as there will be more antigen (calprotectin) molecules in the sample than available antibodies in the reaction cuvette. This is called “antigen excess” and can occur if the antigen is present at such high concentrations that it interferes with the antigen-antibody linking, for example because of steric hindrance or monovalent (instead of multivalent) binding. This results in the formation of smaller, less stable immunoparticle-antigen complexes and causes the instrument to underestimate the concentration of the antigen in the sample and give a false low result as visualized in **Figure 7.** Antigen excess is a common issue in turbidimetric immunoassays as well as other immunoassay methods (Jacobs *et al.*, 2015).

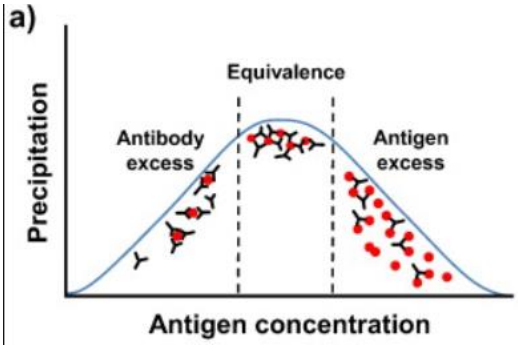


Figure 7 Antigen excess (source: Jacobs et al., 2015)

2.3.3.1. Security Zone

The experiment was performed by measuring a dilution series of a serum sample which was spiked with calprotectin to 62.1 mg/L, to determine until which concentration the instrument (Abbott Architect c4000) will correctly “flag” the result as being over the highest calibration point. This is called the security zone.

The same equipment and materials were used as in **Table 2**. A normal and an adjusted calibrator set was prepared as in **Table 1** and the setting on instrument Abbott Architect c4000 ware set up as in **Table 3.** The samples were prepared according to **Table 6.**

Samples	**Article No.**
Human serum samples	N/A
Saline	2138
Calprotectin native antigen	2117
HEPES-casein buffer	2155

Table 6 List of materials used to sample preparation

Antigen stock solution

To prepare the sample HIGH, calprotectin antigen (with a concentration of 1700 mg/L) was diluted with HEPES-casein buffer (Gentian art.no 2155) to a final concentration of antigen solution ~400 mg/L (antigen stock solution). The antigen stock solution concentration was determined by preparing a 1:50 dilution with HEPES-casein buffer and the calprotectin concentration was measured in triplicates for all sample volume/calibrator combinations in this experiment.

Dilution series

The dilution series was prepared using human serum.

Sample HIGH (SZ1): Human serum samples with elevated calprotectin concentration were pooled to obtain a volume of 3 ml. The pooled sample was spiked with native calprotectin antigen (Gentian, 2117) to obtain a sample of calprotectin antigen concentration of ~75 mg/L as a sufficiently high natural sample was not available.

Sample LOW (SZ10): Human serum with low calprotectin concentration was pooled to prepare a sample with a volume of approximately 4 ml. The concentration of the low pool samples was intended to be < 1.0 mg/L.

Five different channels were programmed according to **Table 4,** including correct calibrator values (according to **Table 1**). The channels were calibrated using the relevant calibrator kit (current or test). The calibration curves were verified with the two QC controls in singlet.

After this evaluation, the samples SZ1 and SZ10 were combined in various volumes to obtain the dilution series according to **Table 7.**

Table 7 Dilution series preparation

Sample ID	**% of sample SZ1**	**% of sample SZ10**	**SZ1 volume [µl]**	**SZ10 volume [µl]**	**Total volume [µL]**
SZ1 (high)	100	0	500	0	500
SZ2	80	20	400	100	500
SZ3	70	30	350	150	500
SZ4	60	40	300	200	500
SZ5	55	45	275	225	500
SZ6	50	50	250	250	500
SZ7	25	75	150	450	600
SZ8	12.5	87.5	75	525	600
SZ9	6.25	93.75	75	1125	1200
SZ10 (low)	0	100	0	500	500

The samples SZ1-SZ10 were measured in triplicates on all 5 channels. An automatic diluted rerun was requested for all samples expected to be outside of the calibration range, whether flagged or not flagged.

Theoretical concentration was calculated with the following equation:

Equation 5 Theoretical concentration

$$X_{theoretical}\left[\frac{mg}{L}\right] = \frac{\bar{X}_{SZ1} \times V_{SZ1} + \bar{X}_{SZ10} \times V_{SZ10}}{V_{SZ1} + V_{SZ1}}$$

$\bar{X}_{SZ1}$	Concentration of sample SZ1	
$\bar{X}_{SZ10}$	Concentration of sample SZ10	
V_{SZ1}	Volume of sample SZ1	From **Table 7Table** 7
V_{SZ10}	Volume of sample SZ10	From **Table 7Table** 7

Recovery (discrepancy in % from measured value compared to the theoretical value) was calculated from the theoretical value to evaluate linearity of instrument diluted rerun**.**

The measured mean concentration was plotted against the theoretical concentration (calculated according to equation **Equation 5**, both before the re-run with dilution ("normal run" in **Figure 12**) in and after re-run ("Diluted rerun" in **Figure 12**). The results were inspected to see if/where the theoretical concentration of the highest calibrator hits the no-rerun curve (y-value), in other words at which concentration the instrument doesn't give a warning that the concentration is above the highest calibrator, while it should have given this warning. The corresponding x-value corresponds to the higher end of the "security zone", i.e. the highest tested calprotectin concentration which the assay still correctly identifies. Concentrations above this level will be reported as below the highest calibrator, and not flagged for re-run (false low values).

2.3.4. Linearity

The purpose of the linearity study is to evaluate if the adjusted hsGCAL assay is linear in the range 0.2 – 10,84 mg/L (on Abbott Architect c4000).

Two methods were applied to review linearity. In formal assay development at least one of the methods must pass the acceptance criteria for the study to pass.

1. Recovery method

Acceptable deviation ≤0.1 mg/L from theoretical value, or 80-120 % recovery from theoretical value.

2. Emancipator-Kroll method

Acceptable deviation from linearity (DL) ≤ 0.1 mg/L or 20 %.

In addition, when evaluating the results, the coefficient of variation (CV) for sample measurements are preferably <10 % for samples with calprotectin concentration >1.0 mg/L, and <20 % for samples with calprotectin concentration ≤ 1.0 mg/L.

Materials used in samples preparation are listed in **Table 2**.

SAMPLE HIGH (L1): to prepare the high starting sample, 1.7 mL of serum was spiked to a calprotectin concentration slightly below the concentration of the highest calibrator (to avoid auto-dilution on the instrument).

SAMPLE LOW (L10): to prepare the low starting sample, 4.9 ml of serum was prepared with a calprotectin concentration < 0.2 mg/L.

Sample L1 was diluted with sample L10 according to **Table 8.**

Table 8 Linearity dilution volumes

Sample ID	Dilution factor	L1 volume [µl]	L10 volume [µl]	Total volume [µL]
L1	100	500	0	500
L2	80	400	100	500
L3	60	300	200	500
L4	40	200	300	500
L5	20	100	400	500
L6	10	50	450	500
L7	5	25	475	500
L8	2.5	20	780	800
L9	1.25	20	1580	1600
L10	0	0	500	500

Calprotectin assay parameters were set up on two channels according to **Table 9** was performed using the calibrator kit “Current” or “test”, with calibrator values according to **Table 1**. The QC controls were run and the calibration curve was checked for validity. Then samples L10 to L1 were run in triplicates.

Table 9. Parameters on the Abbott Architect c4000 for the linearity study

Parameter	Settings Channel 1 (CURRENT)	Settings Channel 2 (TEST)
R1: Assay buffer Volume [µL]	180	180
Sample Volume [µL]	4	8
Dilution factor	1:10	1:10
R2: Immunoparticles [µL]	30	30
First reading time [cycle]	12-15	12-15
Second reading time [cycle]	21-23	21-23
Primary wavelength [nm]	604	604

Recovery was calculated from the theoretical value, and bias and linear trend was calculated according to the Emancipator-Kroll method.

Recovery

Mean (**Equation 1**), SD (**Equation 2**) and CV (**Equation 3**) were calculated. It was checked if CV was within the validity criteria.

Then the dilution factor and mean concentration of L1 and L10 samples were used to calculate the theoretical concentration of each level ($C_{theoretical}$ of samples L1-L10) according to **Equation 6**.

Equation 6. Theoretical concentration

$$C_{theoretical}\left[\frac{mg}{L}\right] = \frac{\overline{X_{L1}}\, x\, V_{L1} + \overline{X_{L10}}\, x\, V_{L10}}{V_{total}}$$

$\overline{X_{L1}}$	Average concentration of sample L1 determined in first run	From *equation 1*
$\overline{X_{L10}}$	Average concentration of sample L10 determined in first run	From *equation 1*
V_{L10}/ V_{L10}	Volume of sample L1/L10	From **Table 8**
V_{total}	Total sample volume	From **Table 8**

Emancipator-Kroll

Recovery and bias of the dilution range were calculated using **Equation 6** and **Equation 7**.

Equation 7. Bias

$$Bias\ \left[\frac{mg}{L}\right] = |\bar{x} - C_{\text{theoretical}}|$$

$\bar{X}$	Average sample concentration	From *equation 1*
$C_{theoretical}$	Theoretical concentration	From *equation 6*

Analyse-it for excel ® was used analysing linearity according to Emancipator-Kroll method. This method fits the data to first, second and third order polynomials and obtain the corresponding coefficients by using an appropriate regression software where expected concentration is on the x-axis and corresponding measured mean concentration (mg/L) on the y-axis. The t values are then compared with values at the given degrees of freedom in a two-sided test with α =0.05. If all t-values are lower than the table value, the dataset is considered linear. If the calculated t- values (absolute values) exceed the t-value in the table, then the dataset is statistically non-linear. The data set need to be further investigated to evaluate the if the non-linearity is clinically relevant.

In the formal immunoassay validation process for the plasma calprotectin assay, acceptable deviation from linearity (DL) is ≤ 0.1 mg/L or 20 %. If acceptance criteria are fulfilled, the linearity is acceptable even if statistically significant non-linear effect has been detected. If the nonlinear concentration (that exceeds the linearity criteria) is at either end, the data point is removed, and the statistical analysis is rerun. This will reduce the linear range which can be claimed. Linearity range is then stated based on samples that passed acceptance criteria for this method. For this study, an indication of linearity for the adjusted assay suffices.

2.4. Adjusted coating with different IgY fractions

Nanoparticles were coated with different antibody fractions to examine the effect on the sensitivity of the assay.

2.4.1. Fragmentation with SEC

Size Exclusion Chromatography (SEC) was used to separate anti-calprotectin IgY by molecular size (molecular weight and shape). While other separation methods are available (such as SDS-PAGE), SEC has the advantage that it exercises minimal impact on the conformational structure of the molecules. Because the molecules of interest, in this case antibodies, do not attach to the resin as they flow through the column, they do not need to be eluted under harsh conditions. This way, they retain their native, folded structure and, importantly, their function for binding antigen. To evaluate the binding kinetics of the different fractions obtained with SEC, plasmon surface resonance was used.

1.8L of Superdex™ 200 prep grade solid phase (GE 17-1043-01) were packed in a XK50/100 column (GE 28-9889-65). The packed column was connected to an ÄKTA explorer 100 Air system to separate and purify large amounts of affinity purified chicken IgY. Prior the purification, the column was equilibrated with PBS (0.1M, 0.15M NaCl, 0.9% NaN3, pH7.2 (mobile phase) and approximately 1g of affinity-purified polyclonal chicken-anti-human-calprotectin IgY antibodies (Getica, lot 1210014) was dialyzed into PBS (Gentian, lot nr 1803705) on Labscale^{TM} TFF Filtration system (Millipore, 12811676) with a Pellicon XL 30Dka ultrafilter (Millipore, PXB030A50).

The column was calibrated using the ÄKTA explorer 100 Air, in PBS 0.1M, 0.15M NaCl, 0.9% NaN3, pH7.2 (mobile phase) at a controlled-flow pressure of 0.25MPa (flow between 12 and 15ml/mn) at room temperature.

100µl of each of the following four calibrators (at 5 mg/ml) was used.

1. Thyroglobulin (bovine) – 667kDa
2. B-Amylase (sweet potato) – 200kDa
3. Alcohol Dehydrogenase (Saccharomyces Cerevisiae) – 150kDa
4. Serum Albumin (bovine) – 66kDa

The dialyzed chicken-anti-human-calprotectin IgY was pumped through the ÄKTA system at a controlled-flow pressure of 0.25MPa (flow between 12 and 15ml/mn) at room temperature. and collected in 80 fractions. Absorbance was continuously monitored at OD_{280} for an indication of the size distribution, peaks and concentration of the antibodies in the different fractions. The fractions were stored in 2-4˚C.

2.4.2. Coating

The coating procedure used is based on Gentian's standard operational procedures (ROP12B and ROP12C), with the difference that the total IgY was divided into fractions with Size Exclusion Chromatography, and then combined into different antibody lots for coating.

Calprotectin antibody fractions were combined into pools, consisting of 50mg antibody each (defined spectrophotometrically). In the fragments peak, there was not enough antibody concentration to be able to coat a lot with them.

Table 10 Coating lot composition (AGGR, MONO, ALL)

Name of coating lot	**Composition of IgY in pool**	**SEC fractions in pool**
AGGR	Aggregate, precipitates, oligomers	A6-D6
MONO	Monomer peak	E3-G2
ALL	IgY composed of all fractions	A4-N3

Each pool was treated similar, as follows:

Preparation of antibody/ovalbumin mixture

The antibodies were concentrated using ultrafiltration (UF) to a minimum of 4 mg/mL and then pH-shocked by adding borate buffer (at 150% of antibody volume). The pools were concentrated back to original volume by UF, dialyzed in a 1:10 borate buffer (overnight) and then in a 1:20 borate buffer (for 2,5 hours) in a 10K MWCO dialysis cassettes (Thermo Scientific, 66810) to obtain optimal pH and ionic strength conditions needed for coating.

After dialysis, the antibody solution was mixed with ovalbumin (Norwegian Antibodies, B101-OVA lot 101-Moer-61) to obtain a better distribution of antibodies on the nanoparticle surface and to reduce nonspecific binding by blocking the hydrophobic or charged binding sites on the particle surface. If needed, additional 1:20 borate buffer was added to dilute the mixture to an antibody concentration of 2,13 mg/ml, which from experience (within Gentian) seems to be the optimal antibody concentration for coating purposes.

Preparation of nanoparticles

Latex 94nm nanoparticles (4111 lot 1212006) were prepared for coating by dilution in distilled water to equal the antibody solution, and sonication (60 seconds).

Coating process

The nanoparticle and antibody/ovalbumin solutions were combined (poured equally and simultaneously into a third beaker) on a magnetic stirrer and were left on the stirrer for 1 hour at RT to ensure thorough mixing. The coated particles were incubated at 37°C for 3 days.

Glycine buffer (Gentian, 2103) was added to obtain a 10nM glycine concentration and the mixture was incubated at 37°C for 2 hours. Glycine (MW 75,1 Da) has the same purpose as ovalbumin (MW 42.7 kDa) but occupies different sites on the nanoparticle surface, mainly because of its smaller size. Then particle dilution buffer with ovalbumin (2104, lot nr. 1802729) was added at a volume of 30% of the particle suspension volume. The large surplus (1mg/ml) of albumin, which is negatively charged at pH 9.25, prevents aggregation of the coated immunoparticles by ensuring enough albumin is available to maintain the equilibrium (bound/unbound albumin) while the coating process is ongoing.

Blocking

The immunoparticles were incubated at 37°C for 2 days, and then left at RT for 9 days before dialysis into a blocking buffer (Gentian, 2042), to end the coating process and wash away any surplus of free antibodies. After this, the coated nanoparticles were dialyzed into a TRIS-based storage buffer with extra albumin (1mg/ml). ProClin 950 (Gentian, 4114) was added to hinder microbial growth. Finally, the mixture was left at RT overnight and then kept at 2-4°C until further use.

2.4.3. Evaluation of binding interactions between calprotectin and anti-calprotectin IgY

Surface Plasmon Resonance (SPR) technique using the instrument Biacore X100 (GE Healthcare, instrument ID 2128933) was used to evaluate the binding interactions between calprotectin and different fractions of IgY (not the coated nanoparticles, only IgY fractions in different combinations) as shown in **Table 11**.

Recombinant Calprotectin (Novoprotein, NP 10459) was diluted to 0,5 µM in 10 mM sodium acetate buffer (pH 5.0) and immobilized for 7 minutes on CM5 Sensor Chip (GE Healthcare, BR100399) containing carboxymethylated dextran covalently attached to a gold surface using a Biacore amine coupling kit (GE healthcare, BR-1000-50).

The chip was inserted into the surface plasmon resonance system Biacore X100 (GE Healthcare, instrument ID 2128933) and the system/chip was equilibrated with HBS P+ (GE Healthcare, BR-1006-71) as a buffer. For the regeneration cycles between each sample/test, 10 mM glycine pH 1,5 (GE healthcare, BR100354) was used.

To monitor the association and dissociation rates, the analyte is injected in increasing concentrations (0,370 nM, 1,13 nM, 3,33 nM, 10 nM, and 30 nM). In multi-cycle analysis, the system is regenerated in

between the different concentrations. In single-cycle analysis, the concentrations are injected sequentially (without regeneration).

Because the dispersion of the antigen bound to the surface of the chip is calculated based on the expected (given) concentration of analyte in the sample, it was tested if the assigned molecular weight (and thus concentrations prepared) had influence on the result, the same sample with anti-calprotectin antibodies from the F2 fraction containing mainly monomeric IgY (**Table 11**) was run twice. Once with its "true" concentrations (based on molecular weight of 180 kDA) programmed (0,370 nM, 1,13 nM, 3,33 nM, 10 nM, and 30 nM) and once programmed with "false" concentration (1/10^{th} of the actual concentrations, 3nM; 1 nM; 0,33nM; …).

As it appeared that especially the association rate constant (k_a) and the equilibrium dissociation constant K_D, differed substantially between those two runs ("false" and "true"), it was decided that rather than using the heterodimer MW of 180 kDa in the calculations (Equation 8) for preparing all sample dilutions, the MW for each of the fractions would be estimated based on the SEC fraction chromatography profile (**Figure 16**).

Equation 8 Concentration

$$C = (A/\varepsilon m)/MW$$

Where, C= concentration in M, A= Absorbance, εm = molar extinction coefficient *(= 1,32 $M^{-1} \cdot cm^{-1}$)* and MW= molecular weight (Da or g/mol).

As shown in **Table 11**, the samples (composed of one single fraction, or several fractions combined) were diluted in HBS P+ (GE Healthcare, BR-1006-71) to above mentioned concentrations based on their assumed molecular weight and measured absorbance, using **Equation 8**. Single-cycle analysis (no regeneration in between the injections with different concentrations) was programmed.

Table 11. Assigned molecular weight (kDa) and absorbance (OD_{280})

SEC Fraction(s)	Anticipated content of the fraction(s)	Contents (SEC fractions)	Assigned molecular weight (kDa)	Absorbance (OD_{280})	Original concentration (nM)
B3	Aggregates	B3	1000	0.454	343.94
C4	Precipitates	C4	600	0.685	1092.1
D8	Oligomers	D8	400	0.426	806.82
A4-N3	All IgY fractions together	A4-N3	180	0.416	1746.6
E1	Monomer peak (start)	E1	290	0.416	1086.2
E3-G2	Monomer peak (all)	E3-G2	180	1.456	6127.9
F2	Monomer peak (top, right)	F2	180	1.580	664.98
L8b	Fragment peak (top)	L8	90	0.181	1522.2
K3-L2	Fragment peak (all)	K3-L2	50	0.133	2015.1

Changes in the refraction index were measured (response in Resonance Units (RU) with 1000 RU corresponding to 1ng/mm^2 of protein on CM5 chip) and the analysis was performed with Biacore X100 Evaluation Software 2.0.1 Plus Package using a 1:1 binding model (the assumption that one molecule of immobilized antigen binds to one antibody molecule).

2.4.4. Evaluation of nanoparticle size

The size of the immunoparticles coated with the three different fraction pools AGGR, MONO, ALL, see (**Table 10**) were examined by nanoparticle tracking analysis (NTA) using Malvern Nanosight NS300. In order to correctly assess size, uncoated nanoparticles were also examined.

The nanoparticles and each of the fraction pools were diluted 1:20 000 in dH20.

Of each of the diluted fraction pools, as well as the uncoated nanoparticles, 1ml was pumped through the Malvern Nanosight system with a syringe pump at a flow speed of 0.05ml per second. In between the different samples, the system was cleaned with buffer (dH2O) and a new, clean syringe was used for both cleaning and each new sample. The system was checked for air bubbles before starting the syringe pump.

For each measurement, the following steps were followed:

First, the image was optimized by running an initial live image. The camera level was adjusted until all of the particles in the sample can be seen clearly but no more than 20% are saturated (colored pixels). Then it was checked if the particle concentration was in the range of ~107-109 particles/ml

(approximately 20-100 particles in the field of view). A too high sample concentration may prevent accurate particle tracking. Lower concentrations require longer capture and analysis time to produce statistically significant results. Then the Laser Beam Position was corrected if needed so that the illuminated particles would fill the field of view. Focus was adjusted to achieve a uniform spherical focus with clearly distinct particles.

Then the measurement was taken in three (3) captures for each sample, with a capture duration of 30 seconds each.

The reports were collected for further visual inspection of the size distribution profile data charts. Mode values of the different samples were compared. Mean and SD given by the instrument were used to calculate CV% (Equation 3).

2.4.5. Response /signal

Because the Abbott Architect c4000 was out of order, the last experiment was performed on another qualified (validated) turbidimetric clinical analyzer, Mindray BS380.

The three lots coated with different fractions of anti-calprotectin IgY: AGGR (mainly aggregates/precipitates/polymers), MONO (mainly monomers) and ALL (all fractions combined) were used to set up a calibration curve, to assess the signal strength of the three lots. A verified production lot of calprotectin (also with all fractions included) was used as a control. The strength of the signal and the form of the curve might provide information about the binding abilities of the selected antibody fractions to calprotectin. For example, a stronger signal could mean that a particular coating lot has a better ability to form the immunoparticle-antibody conglomerates necessary for measuring on a turbidimetric instrument.

2.5. Clinical testing

In this part of the study calprotectin in blood serum samples received from Astrid Lindgren Children's Hospital (Stockholm, Sweden), was measured using both the CE marked Gentian Calprotectin Immunoassay (GCAL®) and the highly sensitive calprotectin immunoassay (hsGCAL), developed as part of this thesis. The purpose of this part of the study was to compare values measured by hsGCAL and GCAL®, and to study if one of the assays would show a better correlation with clinical and laboratory parameters.

Samples

In total 54 blood serum samples (200 µL) were received from the Astrid Lindgren Children's Hospital in Sweden. Of these, 44 samples were from patients (children) with various forms of Juvenile Idiopathic Arthritis (JIA) and Rheumatic Arthritis (RA), including active Oligo-arthritis, active Polyarthritis, and active systemic-onset JIA. The remaining 10 serum samples were from a healthy (non-RA/JIA) control group. Samples were stored frozen (<-80°C).

GCAL® / hsGCAL

The Gentian Calprotectin Immunoassay (GCAL®) is a particle enhanced turbidimetric immunoassay (PETIA) for quantitative analysis of total calprotectin in serum or plasma. The assay can be applied on a wide range of automated clinical chemistry analyzers. The GCAL® assay is validated for both serum and plasma, but CE marked only for plasma. The GCAL® assay is currently validated to be used on Abbott Architect c4000, Mindray BS400, Roche Cobas c501 and Beckmann Coulter AU400. The specifications/results in this report only relate to Abbott Architect c4000. The GCAL® assay is linear in the range 0.5 - 20 mg/L with a security zone up to 95 mg/L.

The high sensitivity calprotectin immunoassay (hsGCAL) application is based on the GCAL® assay but has an adjusted calibrator set and increased sample volume compared to the reference GCAL® assay.

As shown earlier in this master thesis, the hsGCAL assay is linear in the range 0.05 – 10.40 mg/L, with a security zone up to at least 60 mg/L (highest concentration tested) and shows improved/lower CV compared to the reference assay (GCAL®).

The same lots of reagents and controls were used for both applications (**Table 12**). Calibrators for both applications were based on the same lot. Reagents, calibrators and controls were stored at T=2-8°C until the start of the study. While the study was ongoing, the reagents were stored in the instrument, while the calibrators and controls were capped and stored at T=2-8°C immediately after use. Two instrument channels on the Abbott Architect c4000 were programmed as in **Table 13**. Values assigned to the two calibrator sets are summarized in **Table 14**.

Table 12. List of instruments and materials used in the analysis of clinical blood samples from Karolinska Institute.

Materials	Article No.	Lot No.	Lower range (mg/L)	Upper range (mg/L)
Abbott Architect c4000	Z-459	n/a	n/a	n/a
Lab shaker	Z-309	n/a	n/a	n/a
CGAL Control Low	1220	1703417	0.84	1.26
CGAL Control High	1221	1703418	8.35	12.53
Gentian Plasma calprotectin R1/Assay buffer	1207	1703421	n/a	n/a
Gentian Plasma calprotectin R2/Immunoparticles	1214	1703432	n/a	n/a
Gentian Plasma calprotectin calibrators	1251	1703429	n/a	n/a
54 serum blood samples from Astrid Lindgren's	n/a	n/a	n/a	n/a

Table 13. Instrument settings used on Abbott Architect c4000

Parameter	Settings GCAL	Settings hsGCAL
R1: Assay buffer Volume [µL]	180	180
Sample Volume [µL]	4	8
Dilution factor	1:10	1:10
R2: Immunoparticles [µL]	30	30
First reading time [cycle]	12-15	12-15
Second reading time [cycle]	21-23	21-23
Primary wavelength [nm]	604	604

Table 14. Values of reference/GCAL and test/hsGCAL calibrator set (lot number 1703429)

	Std 1 (mg/L)	Std 1,5 (mg/L)	Std 2 (mg/L)	Std 3 (mg/L)	Std 4 (mg/L)	Std 5 (mg/L)	Std 6 (mg/L)
Calibrator set 1 (reference/GCAL®)	0,00	-	1,27	2,73	5,42	10,84	21,67
Calibrator set 2 (test/hsGCAL)	0,00	0,64	1,27	2,73	5,42	10,84	-

Measuring procedure

Two channels on the Abbott Architect c4000 were programmed as in **Table 13,** and calibrated with the relevant calibrator set (calibration sets reference/ GCAL® and test/hsGCAL). QC High and Low were run for both applications and checked against the analytical value sheet (**Table 12**). The samples were thawed at room temperature, and vortexed. All samples were measured in two replicates each for both applications (GCAL® and hsGCAL). If calprotectin values were above calibration range (and flagged as such by the instrument), a rerun with a 1:10 automatic dilution in saline was requested to obtain the calprotectin value. After measurement, the samples were immediately stored in the freezer (< -80°C).

Both methods, hsGCAL and GCAL®, were compared with regards to average calprotectin values and CV (%).

Levels of calprotectin were tested for correlation with available clinical and laboratory parameters by Karolinska Institute. All parameters were not available for all patients. For statistics, unpaired t-test and Pearson´s correlation (coefficient of determination, R_2) were used, and $P<0.05$ was considered significant.

3. Results

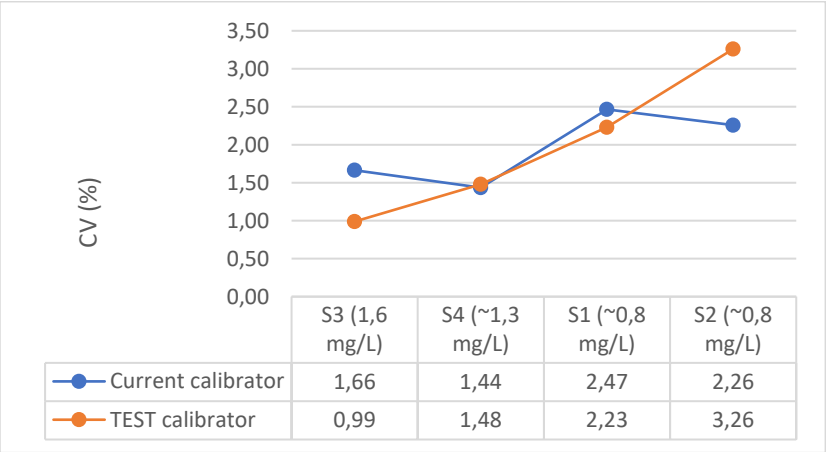
3.1. Results assay parameter adjustments

In this section, the results from the assay parameter adjustments (calibration set and sample volume) will be presented.

3.1.1. Calibrator adjustment

The CV (%) of the Current and TEST calibrator set are plotted against each other in **Figure 8** for serum samples (S1-S4) and **Figure 9** for Li-Hep plasma samples (P1-P4) from the same donors. The samples are ordered in decreasing average calprotectin concentration measured.

Figure 8 shows the TEST calibrator set has a lower or same CV for serum samples S3, S4 and S1, but a higher CV for serum sample S2. Notice the increased CV as the calprotectin concentration in the samples gets lower, for both the Current and the TEST calibrator set.



	S3 (1,6 mg/L)	S4 (~1,3 mg/L)	S1 (~0,8 mg/L)	S2 (~0,8 mg/L)
Current calibrator	1,66	1,44	2,47	2,26
TEST calibrator	0,99	1,48	2,23	3,26

Figure 8 CV (%) of Current calibrator set (blue) and TEST calibrator set (orange), for serum blood samples S1-S4

Figure 9 shows that the TEST calibrator set has a lower CV compared to the current calibrator set for Li-Heparin plasma sample P1, and a higher CV in P1, P2 and P4. Again, we see a tendency for increased CV as the calprotectin concentration in the samples gets lower, for both calibrator sets.

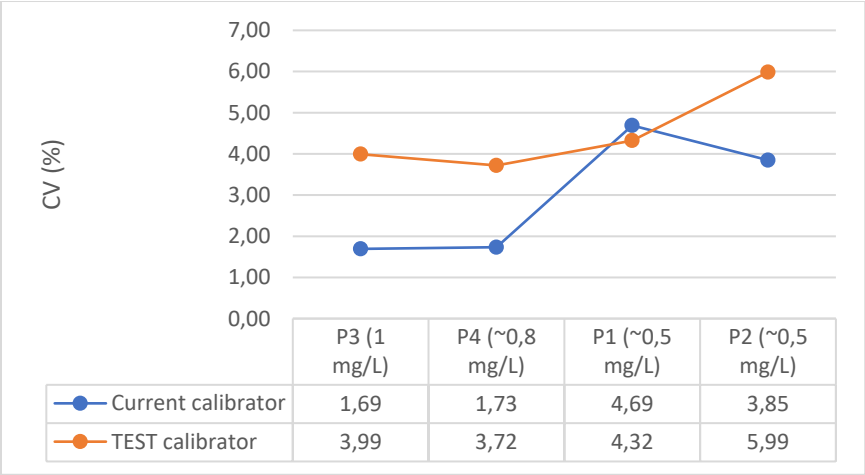


Figure 9 Coefficient of Variation (%) of current calibrator set (blue) and TEST calibrator set (orange), for Lithium-Heparin plasma blood samples S1-S4

3.1.2. Calibrator and Sample volume

Because, based on the results from this experiment (3.1.2), it was not possible to draw conclusions if the adjusted calibrator set gives improved CV (%) when measuring calprotectin concentrations compared to the current calibrator set. A second experiment was set up to evaluate the effect of the sample volume (4µL, 6µL and 8µL) and to test on samples with lower calprotectin values.

As shown in **Figure 10**, the average CV (%) decreases with increasing sample volume for both calibrator sets, and in both plasma and serum. In serum (with relatively high calprotectin values compared to paired plasma samples), the current calibration set (orange line) performed better at all sample volumes than the adjusted calibrator set (blue line). However, in plasma (lower calprotectin concentrations) the adjusted calibration set (grey line) performed as good as the current calibrator set (yellow line).

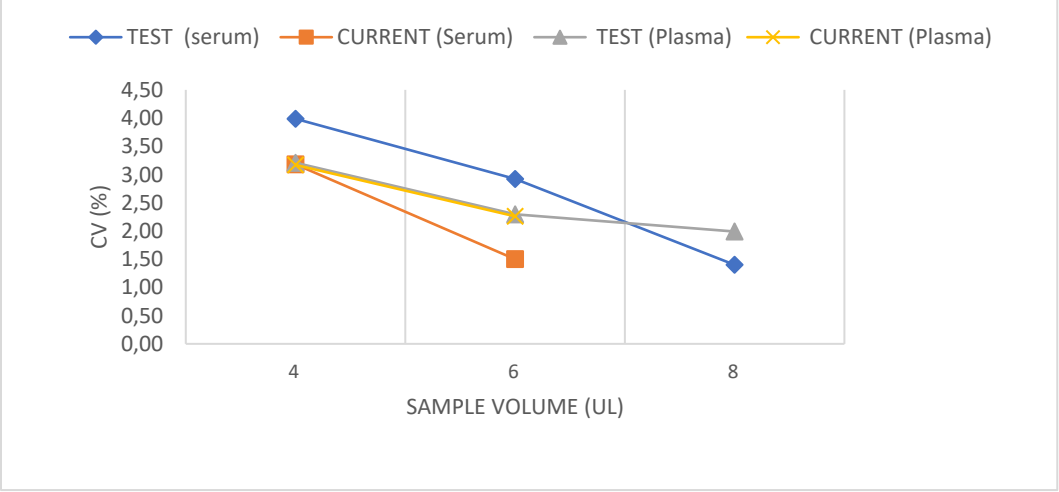


Figure 10 CV (%) for measurements with different calibrator sets and sample volume (4µL, 6µL and 8µL)

Discussion: Because the measured samples still did not have very low calprotectin values, it was decided to use a dilution in HEPES Casein to obtain samples of around 0.3 mg/L and 0.15 mg/L to assess how the adjusted calibrator set would perform compared to the current one.

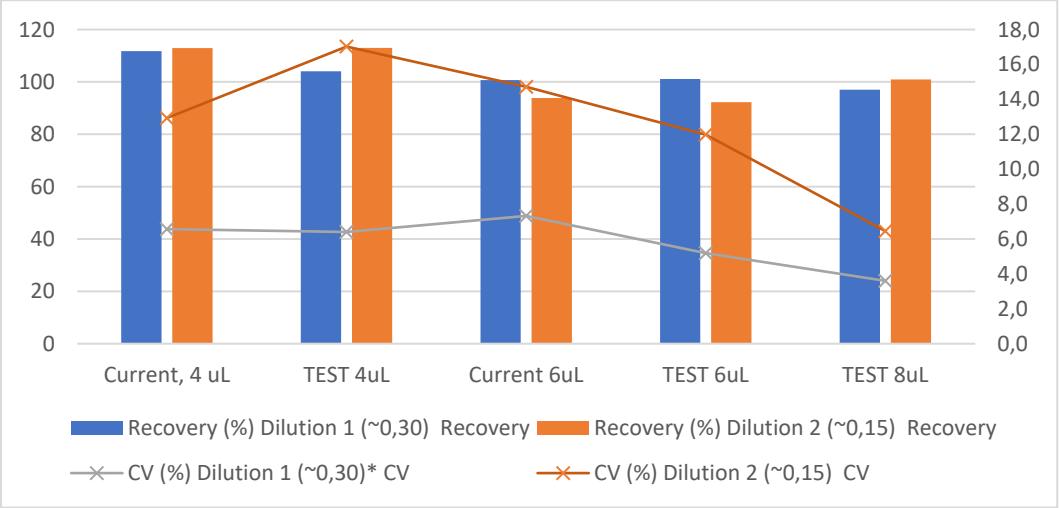


Figure 11 Average Recovery (%) and CV (%) for dilutions to 0.3 mg/L and 0.15 mg/L (plasma)

Figure 11 shows that while the recovery percentage stays relatively constant for all samples and applications, the CV (%) decreases with increasing sample volume. Overall, CV (%) for the samples diluted to ~0.15 mg/L is higher than for samples diluted to ~0.3 mg/L.

3.1.3. Security Zone

For each of the applications 1 to 5 (**Table 4**), a chart has been made to visualize the measured against the theoretical concentrations of calprotectin in the different samples of the dilution series. As can be seen in **Figure 12**, the curve for application 5 with the adjusted calibration set (TEST) and a sample volume of 8µL, the difference between measured concentration and theoretical concentration increases. However, all samples with concentrations above the highest calibration point were still appropriately flagged by the instrument. This means that the highest level in the "security zone" was not yet reached. However, as the curve begins to flatten, it seems that the "equivalence zone" (see **Figure 7**) was reached or almost reached. Similar curves were obtained for the other applications 1 to 4.

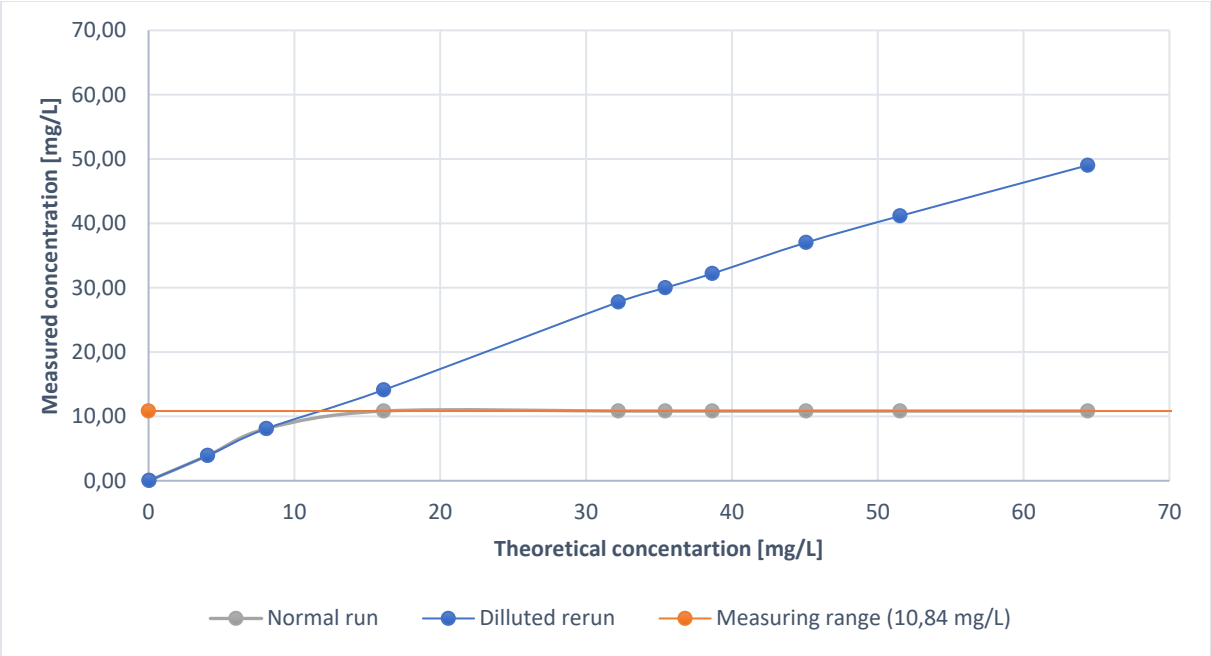
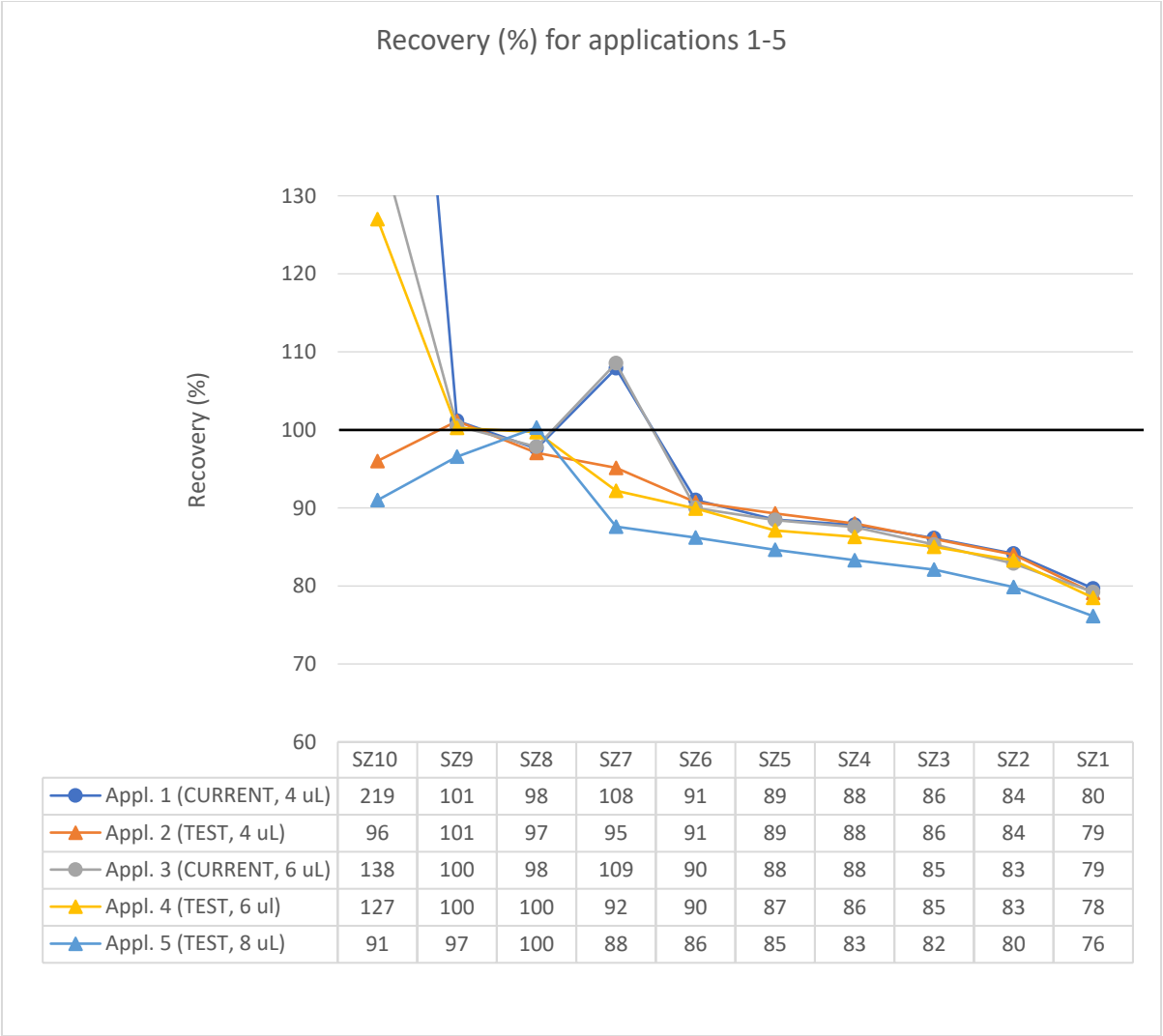


Figure 12 Security Zone. Measured calprotectin concentrations (mg/L) plotted against theoretical calprotectin concentrations (mg/L) for application 5 (calibrator set: TEST / Sample volume: 8µL). *The lines represent the measured concentration vs the theoretical concentration. The orange line is the measuring range /highest calibration point value at 10.84 mg/L. The grey line represents the results for the normal run, without dilution. The grey line flattens when the measurement result is above the highest calibration point and therefore "flagged" by the instrument (= a warning that the measured concentration is above the measuring range/highest calibration point), after which a rerun with a 1:10 saline dilution was requested to obtain the blue line.*

Recovery was calculated for all 5 applications mentioned in **Table 3**, and the results are visualized in **Figure 13**. SZ10 has the lowest calprotectin concentration, while SZ1 has the highest concentration.



	SZ10	SZ9	SZ8	SZ7	SZ6	SZ5	SZ4	SZ3	SZ2	SZ1
Appl. 1 (CURRENT, 4 uL)	219	101	98	108	91	89	88	86	84	80
Appl. 2 (TEST, 4 uL)	96	101	97	95	91	89	88	86	84	79
Appl. 3 (CURRENT, 6 uL)	138	100	98	109	90	88	88	85	83	79
Appl. 4 (TEST, 6 ul)	127	100	100	92	90	87	86	85	83	78
Appl. 5 (TEST, 8 uL)	91	97	100	88	86	85	83	82	80	76

Figure 13 Recovery (%) in dilution series. *100% recovery is achieved when the measured and theoretical concentrations are equal. When the measured concentration is lower or higher than the theoretical concentration, the recovery percentage will be reduced or augmented, respectively. SZ10 has the lowest calprotectin concentration, while SZ1 has the highest calprotectin concentration)*

Recovery is very high at SZ10 (low calprotectin concentration), though much higher for the standard assay (application 1) than for the adjusted applications. With dilutions, there is always a risk of pipetting mistakes, and this may partly account for the high recovery rates in the SZ10 sample, however, it does not explain the difference between the applications as measurements for all applications were performed using the same sample, at the same time and on the same instrument.

For all applications, the recovery rate increasingly diverts from the 100% recovery line (where measured and theoretical values are equal) as concentrations increase (towards SZ1), especially after the measurements are flagged above the highest calibration point, and a dilution is requested. Also note that the application 5 with the highest sample volume (application 5) has more diversion from the 100% recovery line compared to the other adjusted applications with lower sample volumes

(application 2 and 4). This could also mean that these applications are reaching the phase of antigen excess explained in section 2.3.3.

3.1.4. Linearity

The linearity test results measured with the adjusted hsGCAL immunoassay (with adjusted calibrator set and sample volume of 8µL) is linear in the range 0.05 – 10.4 mg/L (on the Abbott Architect c4000) and BS380.

Both methods, hsGCAL and GCAL®passed the Emancipator Kroll test, as well as the Recovery test (acceptable deviation ≤0.1 mg/L from theoretical value, or 80-120 % recovery from theoretical value). The results for hsGCAL are summarized **Table 15** (Recovery), **Table 16** (Emancipator-Kroll) and visualized in **Figure 14** Results for GCAL®can be found in Annex 1.

Recovery method: **Table 15** shows that for the adjusted assay hsGCAL, CV (%) was higher than the allowed 20%, and Recovery (%) was outside the allowed 80-120 % range in the sample with the lowest concentration (0,05 mg/L), but because the difference between measured and theoretical concentration was less than 0,1 mg/L, the test still passed.

Table 15. Results of recovery test for hsGCAL (8µL) on Abbott Architect c4000

Measured concentartion [mg/L]	SD [mg/L]	CV [%]	Theoretical concentartion [mg/L]	Recovery [%]	Difference measured and theoretical concentration
0.03	0.02	74.83	0.05	54	-0.02
0.09	0.017	18.79	0.11	82	-0.02
0.16	0.02	14.37	0.18	89	-0.02
0.27	0.03	9.44	0.25	105	0.01
0.30	0.02	5.04	0.31	99	0.00
0.59	0.01	0.98	0.56	104	0.02
1.09	0.03	2.30	1.08	101	0.01
2.04	0.03	1.30	2.12	96	-0.08
3.98	0.02	0.38	4.19	95	-0.21
5.96	0.02	0.39	6.26	95	-0.30
8.39	0.04	0.50	8.33	101	0.06
10.51	0.03	0.29	10.40	101	0.11

Table 16. Results of the Emancipator-Kroll test, Abbott Architect c4000, hsGCAL (adjusted calibrator set, sample volume: 8µL)

Linear fit	Nonlinear fit (2nd order polynomial)	Nonlinearity	95% CI		Relative nonlinearity	Difference measured and theoretical concentration
0.01091	0.07160	0.06069	0.02901	to 0.0924	556.4%	-0.02
0.07560	0.13014	0.05454	0.02607	to 0.0830	72.1%	-0.02
0.14030	0.18877	0.04848	0.02317	to 0.0738	34.6%	-0.02
0.21793	0.25924	0.04131	0.01974	to 0.0629	19.0%	0.01
0.26968	0.30629	0.03661	0.01750	to 0.0557	13.6%	0.00
0.52846	0.54237	0.01391	0.00665	to 0.0212	2.6%	0.02
1.04601	1.01869	-0.02732	-0.04158	to -0.0131	-2.6%	0.01
2.08112	1.98799	-0.09314	-0.14176	to -0.0445	-4.5%	-0.08
4.15134	3.99316	-0.15818	-0.24076	to -0.0756	-3.8%	-0.21
6.22155	6.08712	-0.13443	-0.20461	to -0.0642	-2.2%	-0.30
8.29177	8.26987	-0.02190	-0.03333	to -0.0105	-0.3%	0.06
10.36199	10.54141	0.17942	0.08575	to 0.2731	1.7%	0.11

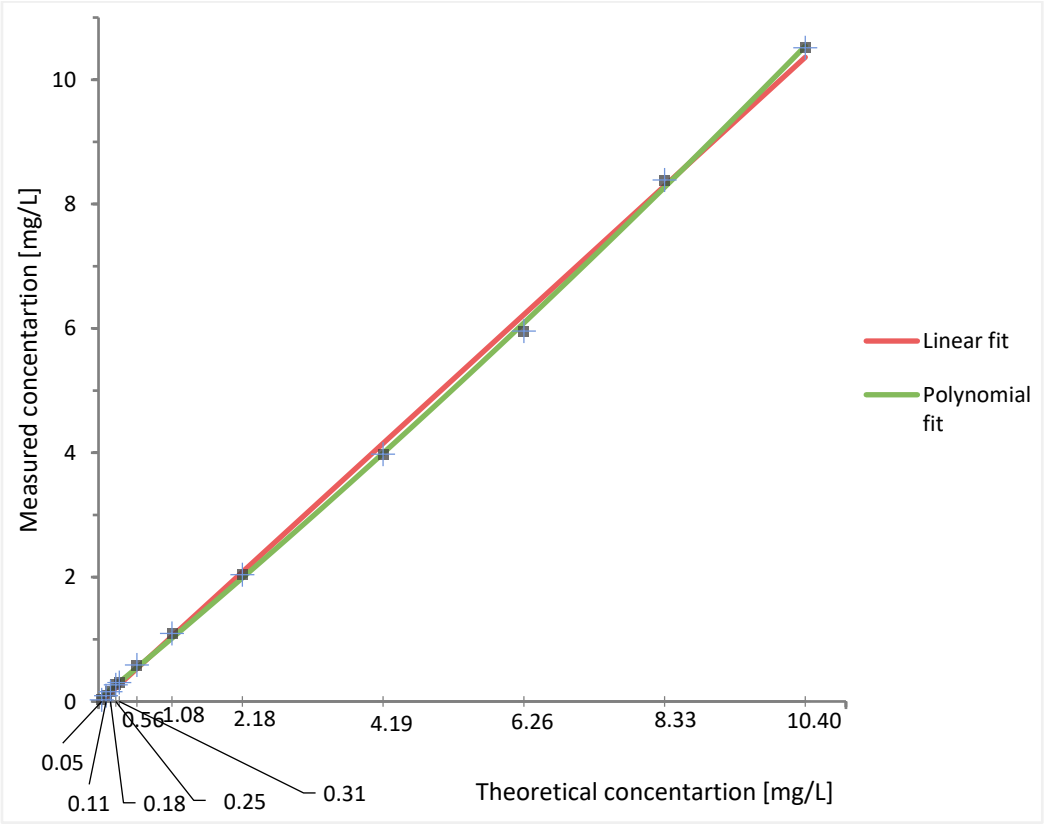


Figure 14. Visual representation of linearity of the hsGCAL assay on Abbott Architect c4000 (Abbott)

3.1.5. Summary results (assay parameter adjustments)

The results from the hsGCAL assay (with adjusted calibrator set and 8 uL sample volume) meet the criteria for linearity in the range 0.05 – 10.4 mg/L (on Abbott Architect c4000) with a security zone up to at least 60 mg/L (highest concentration tested), and shows improved/lower CV (%) compared to the reference assay (GCAL®).

Due to these results, the assay with the adjusted calibrator set and a sample volume of 8µL was chosen as a basis for further assessment of the clinical samples. The assay with these parameters is called hsGCAL (highly sensitive GCAL).

3.2. Results Adjusted coating with different IgY fractions

3.2.1. Size Exclusion Chromatography (SEC)

Figure 15 shows how the calibration of the column used in the SEC experiment. The four peaks correspond to the four substances with known molecular weight, against the background of the results from the injections 1-3 with anti-calprotectin IgY (Ab -injection 1; Ab -injection 2; Ab -injection 3).

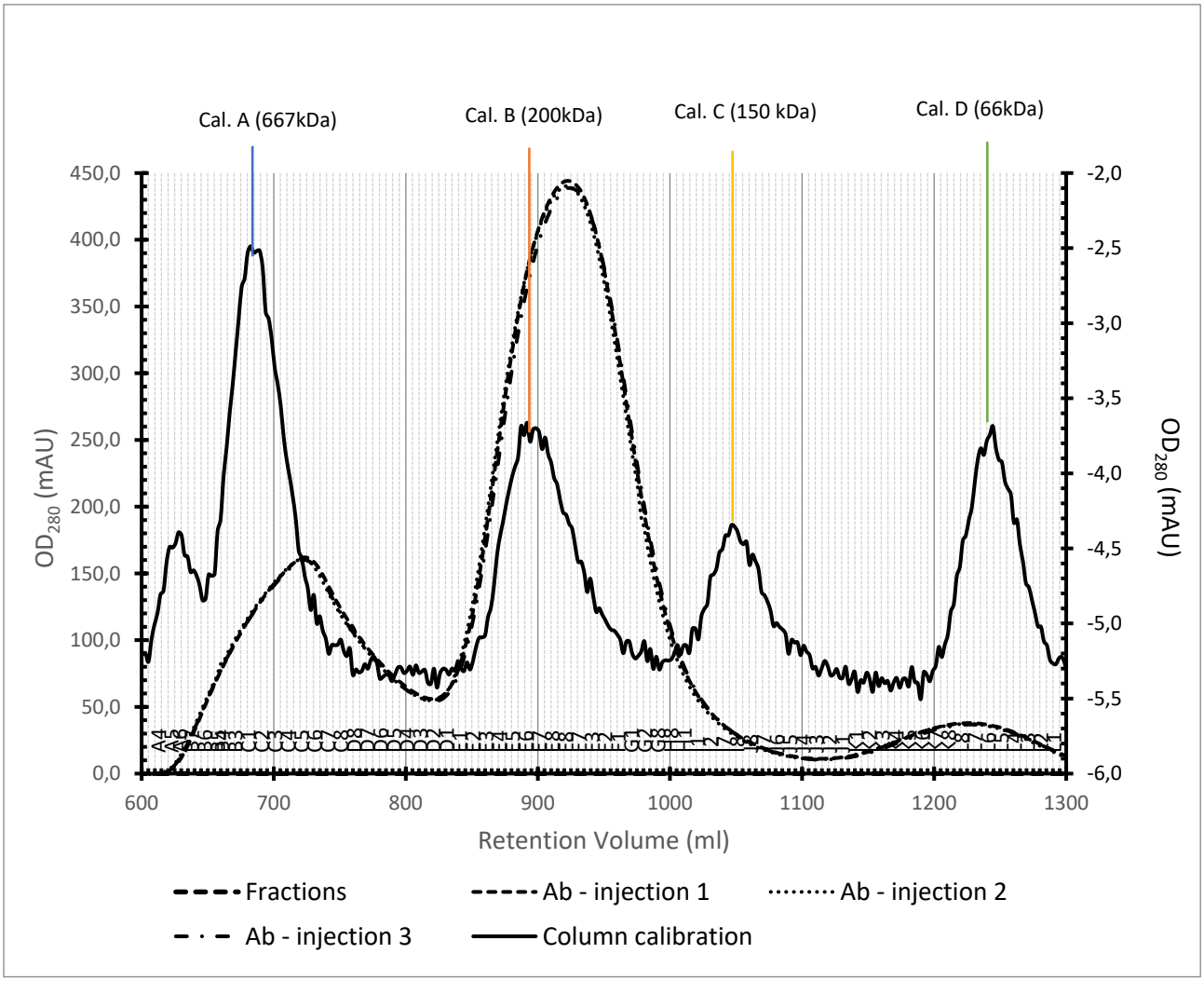


***Figure** 15. **Chromatography profile for Calprotectin IgY (Getica, lot 1210014).** Calibration with four calibrators of different molecular weight, against the results from injection 1-3 of anti-calprotectin IgY. The blue line corresponds to calibrator A with bovine Thyroglobulin (667kDa), the orange line corresponds to Calibrator B with B-Amylase from sweet potato (200kDa), the yellow line corresponds to calibrator C with Alcohol Dehydrogenase/Saccharomyces Cerevisiae (150kDa) and the green line corresponds to calibrator D with Serum Albumin (66kDa).*

The values obtained from the calibration were used to assign MW values to each of the IgY-fractions used in the SPR experiment (**Table 17**).

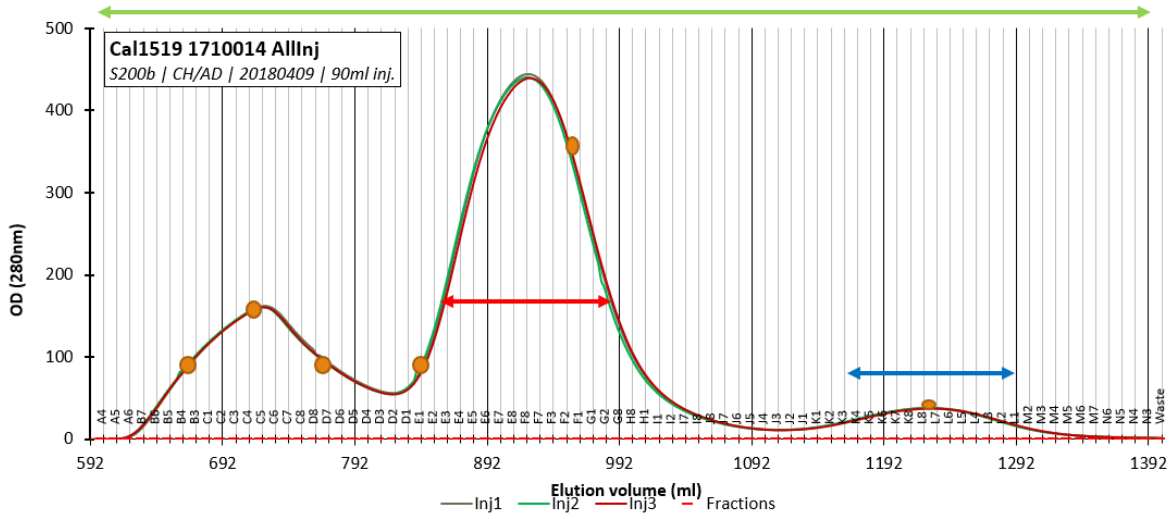


Figure 16 Chromatography profile for calprotectin IgY (Getica, lot 1210014). The orange dots and green, red and blue arrows represent fractions / sets of fractions that were chosen for evaluation with the surface plasmon resonance technique).

The molecular weight in the first peak in **Figure 16** corresponds to oligomers (e.g. aggregates, precipitates, and oligomeric forms of IgY), the molecular weight of the molecules in the second peak corresponds to IgY monomer. The last, smaller peak corresponds to IgY fragments (Fab, Fc etc.).

3.2.2. Binding interactions between calprotectin and anti-calprotectin IgY

Table 17 shows the assigned Molecular Weight (MW) that was used when preparing the fractions for analysis with SPR. Values were based on the calibration of the SEC column.

Table 17. Anticipated content of the SEC fractions, including assigned and corrected Molecular Weight (MW)

SEC Fraction(s)	Anticipated content of the fraction(s)	Assigned MW (kDa)
B3	Aggregates	1000
C4	Precipitates	600
D8	Oligomers	400
A4-N3	All IgY fractions together	180
E1	Monomer peak (start)	290
E3-G2	Monomer peak (all)	180
F2	Monomer peak (top, right)	180
L8b	Fragment peak (top)	90
K3-L2	Fragment peak (all)	50

The kinetics of molecular interaction of the main experiment were described in terms of association rate constant (k_a), dissociation rate constant (k_d), and the equilibrium constant K_D.

When interpreting the results from SPR, the following assumptions were made:

- the optimal concentration of calprotectin antibody (analyte) is set on 30nM to increase likelihood of 1:1 binding.
- preparation of the analyte concentrations is based on assumed molecular weight.

As can be seen in **Table 18,** when antibody concentration in the sample was higher than «expected» by the instrument (BiaCore), it gave a higher association rate constant (K_a) and lower equilibrium dissociation constant (K_D). Lower **K_D** indicates greater binding affinity of the ligand for its target. Therefore, if the assigned molecular weight assigned to a fraction of sample is not correct, this may affect the **Equilibrium Dissociation Constant** (K_D) value, and the **association rate constant** (K_a). In other words, the results may suggest higher affinity between the ligand and the analyte than really is the case.

Table 18 True and False run for F2

	k_a (1/Ms)	k_d (1/s)	K_D (M)
F2 True	349600	0.0002601	7.44E-10
F2 False	7409000	0.0003051	4.11E-11

If you purely look at the Equilibrium Dissociation Constant (K_D) values (the red line in **Figure 17**), it appears that the higher the MW of the analyte molecule is (such as aggregates, precipitates, oligomers), the greater the binding affinity between ligand and analyte is.

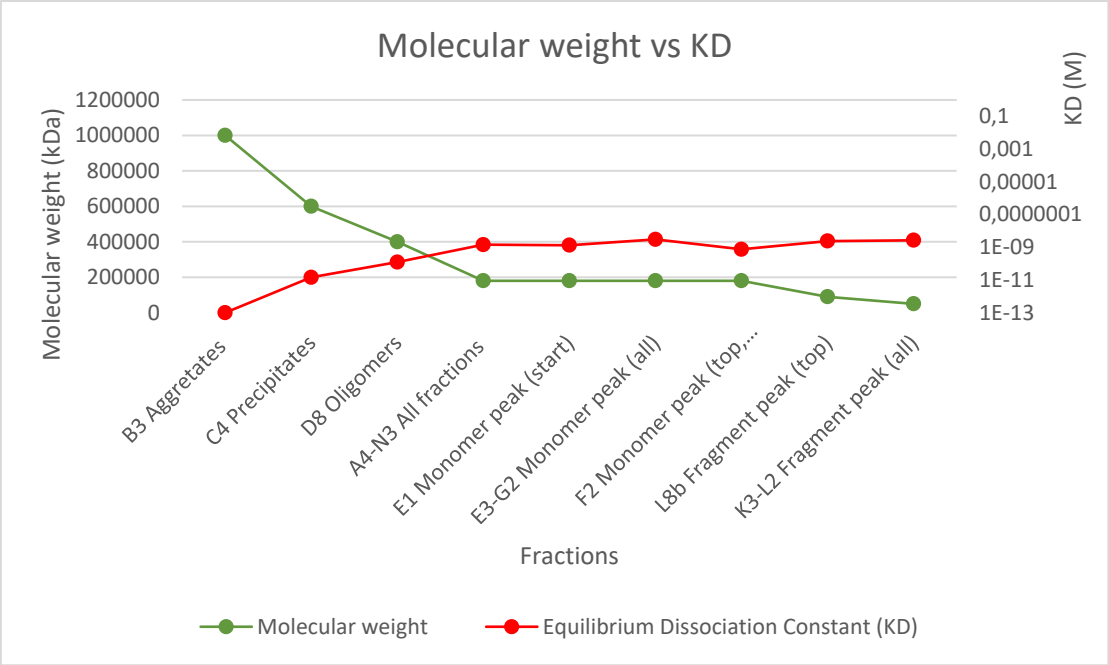


Figure 17 Molecular weight against KD. KD results are plotted on a log10 scale for more clarity.

When plotting the Association and Dissociation Rate Constant results in numbers, as was done in **Figure 18**, this gave a similar picture.

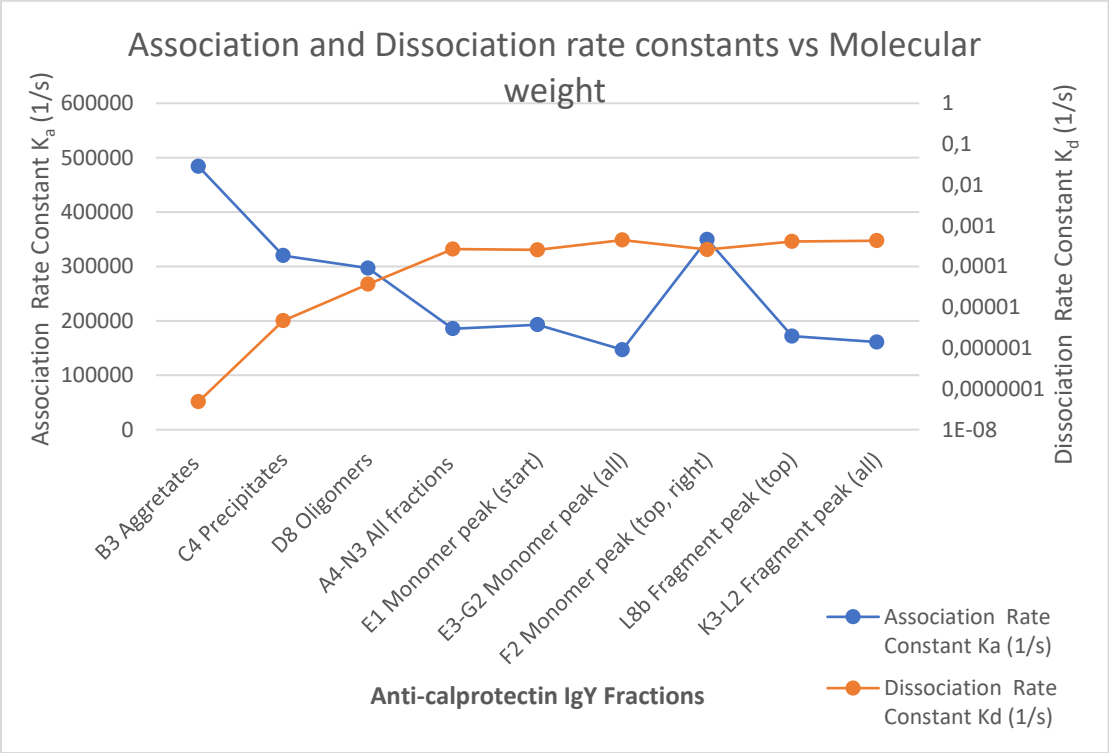


Figure 18 Association and dissociation rate constants. KD results are plotted on a log10 scale for more clarity.

Fractions ordered in decreasing association response: B3 Aggregates, F2 Monomers (top), E1 Monomers (start), C4 Precipitates, L8 Fragments, A4-N3 All fractions combined, D8 Oligomers, E3-G2 Monomers (all), K3-L2 Fragments (all).

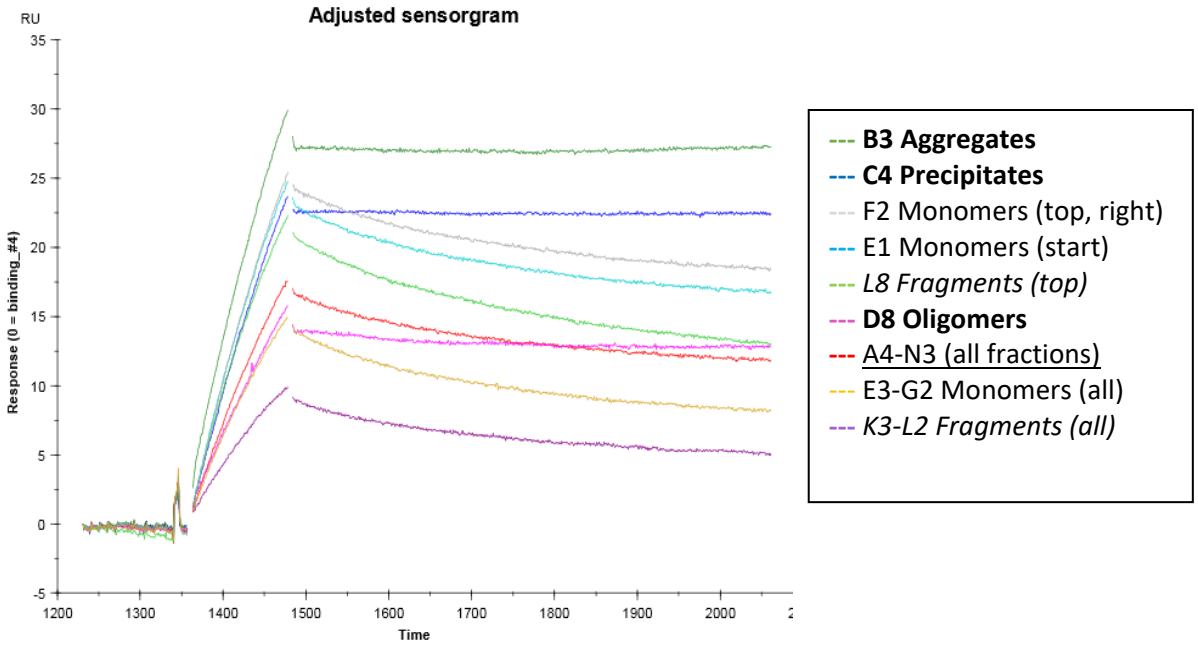


Figure 19 RPS Sensorgram of the biding process between calprotectin and anti-calprotectin IgY

The results are also presented in a sensorgram visualizing the binding process of the ligand with analyte in association and dissociation curves (**Figure 19**). The sensorgram curves reveals that fractions with larger molecular weight (aggregates, precipitates) show high association rates and nearly no dissociation (flat line from ~1500 sec). Monomers and fragments show various degrees of association, but all have a faster dissociation rate than the fractions with larger MW.

Different parts of the monomer peak give a different response. While the fractions E1 (start of the monomer top) and F2 (top of the monomer top) show high association rates, E3-G2 or the "overall monomer peak" show much lower association rate.

3.2.3. Nanoparticle size

The size distribution of the three lots of nanoparticles coated with different antibody fractions (paragraph 2.4.2), as well as a reference lot with uncoated nanoparticles were examined with nanoparticle tracking analysis. The results are visualized in **Figure 20.** A narrow peak indicates homogeneous particle size (as in A. NANO), while a broader peak or different peaks indicates that the particle size is more diverse (as in C. AGGR).

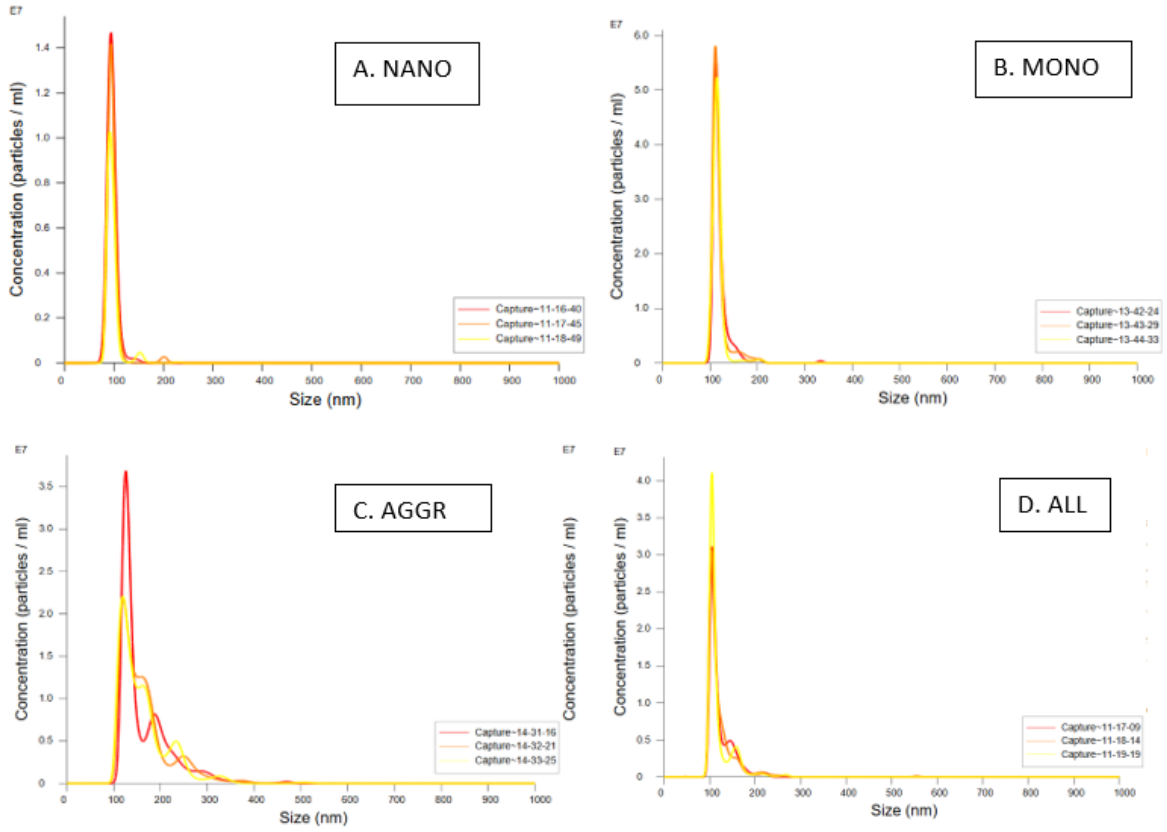


Figure 20. Distribution of particles by size and concentration *for A. Uncoated nanoparticles (NANO); B. nanoparticles coated with mainly monomers (MONO); C. nanoparticles coated with aggregates, precipitates and polymers (AGGR); and D. nanoparticles coated with all fractions of IgY combined.*

The CV (%) for each of the measurements is presented in **Table 19**, based on the mean, Mode and SD values given by the instrument.

Table 19. Mean (nm), Mode (nm), SD (nm) and CV(%) for the uncoated nanoparticles, and nanoparticles coated with different fractions of IgY

No	Lot name	Mean (nm)	Mode (nm)	SD (nm)	CV (%)
A	NANO	96.7	**93.4**	14.1	14.60 %
B	MONO	118.6	**112.6**	18.9	15.90 %
C	AGGR	163.4	**124.4**	53.7	32.90 %
D	ALL	115.1	**106.0**	24.1	20.90 %

A. NANO: Uncoated nanoparticles

The nanoparticles used to coat the three lots of antibodies (ALL, MONO and AGGR) had a diameter of 94 nm according to the producer. The results show a mode of 93.4 nm and low CV at 14.6%, indicating that the size distribution narrow.

B. MONO: Nanoparticles coated with monomers

This lot was coated with fractions from the monomer peak. As the nanoparticles used have a diameter of 94nm and the height of an IgY monomer is typically ~10 nm, the expectation was that nanoparticles coated with pure monomers would typically have a diameter around 114 nm. The results show a mode of 113 nm, and a mean of 118.6 nm. The measurement has a low CV % of 15.9%.

C. AGGR: Nanoparticles coated with IgY fractions of the first peak (aggregates, precipitates, dimers).

Visually, **Figure 20 (C)** shows large distribution in sizes for the lot coated with combined SEC fractions from the "aggregates" peak. This is confirmed by a higher CV at 32.9%, compared to the other lots. Both mean particle size at 163.4 nm and mode particle size at 124.4 nm are larger than for the other lots.

D. ALL: Nanoparticles coated with all IgY fractions combined

According to the IgY profile retrieved from the SEC experiment (**Figure 16**), the IgY-lot using all the fractions include mostly monomeric IgY but also IgY molecules with larger size than monomeric IgY (aggregates, precipitates, dimers) and smaller sizes (antibody fragments such as Fc and Fab). It was therefore expected that the results from this lot would show higher variability than the lot coated with only monomers (MONO). Based on the results from this experiment, this does not seem to be the case as CV is still reasonably good at 20.90 %. Both mode particle size (106.0 nm) and mean particle size (115.1 nm) are smaller than for the lot coated with only monomer (MONO), which was unexpected.

It is possible that something went wrong during the coating process. However, when it was tested for turbidimetric response the "ALL" lot gave results similar to the reference production lot (also using total IgY), so it is expected that the coating went reasonably well. Perhaps that the nanoparticles in this lot are covered with mainly monomers, some fragments and virtually no polymers (aggregates, precipitates or dimers), but this was not tested.

3.2.4. Response (turbidimetric)

To check the coating process and compare the different lots of coated nanoparticles in terms of response to the antigen (calprotectin), calibration curves were established on a turbidimetric clinical analyzer (Mindray BS-380).

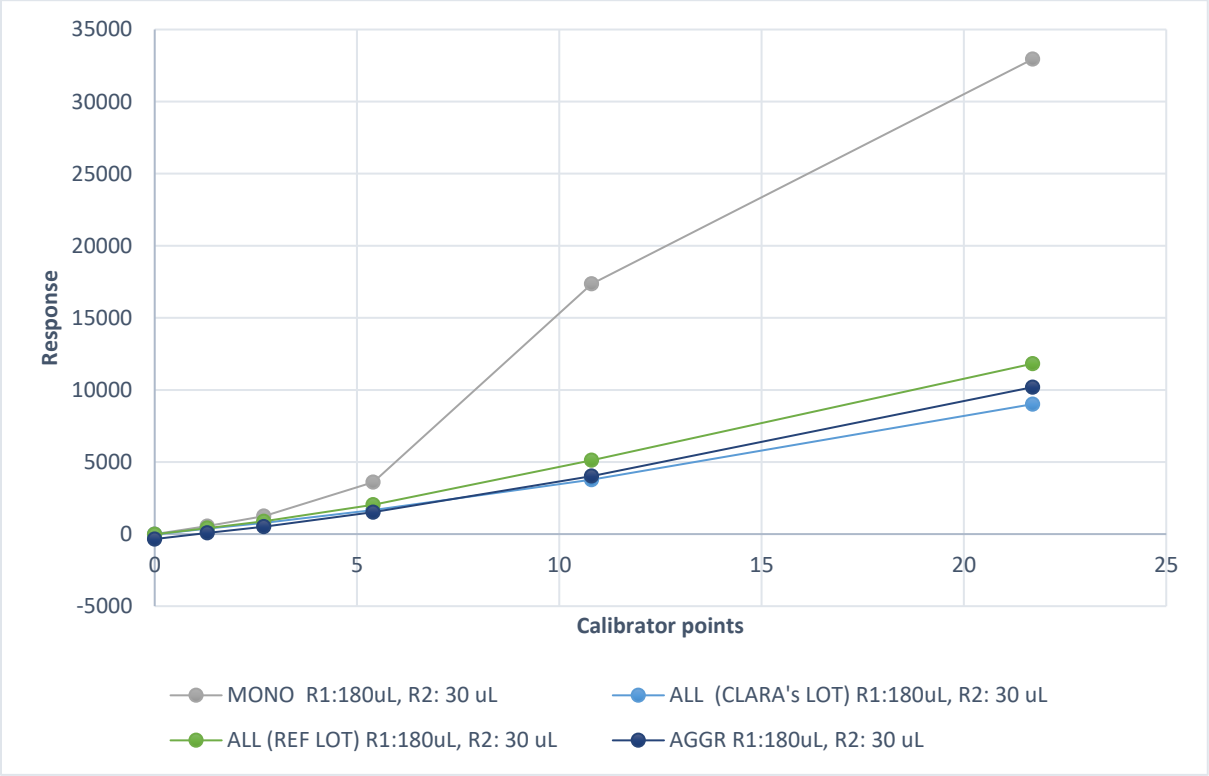


Figure 21. Response / Calibration curves for immunoparticle lots ALL, MONO and AGGR, as well as a reference lot. *R1=buffer (volume), R2=immunoparticle (volume). Performed on turbidimetric clinical analyzer Mindray BS380.*

As **Figure 21** shows, the response for both the lot with aggregates, precipitates and polymers (AGGR, dark blue line) and the lot with all fractions combined (ALL, CLARA's LOT light blue line) is overall lower than the reference lot (ALL, REF LOT, green line).

However, the calibration curve for the immunoparticles coated with mainly monomers (MONO) shows increased response, especially from the third calibration point, compared to the other lots. The calibration curve is far from linear, and in addition such a high response would be unacceptable as it could lead to an early "hook effect" due to antigen excess. In a proper immunoassay development, this would have to be optimized, for example by altering the instrument assay parameters such as the volume of immunoparticles (R2) and buffer (R1), time allowed for the reaction or when the results are measured. However, for the purposes of this study it was enough to see that what type of response the particles coated with different IgY lots would show.

3.2.5. Summary results (adjusted coating with different IgY fractions)

The nanoparticle lot coated with mainly IgY monomers (MONO) gives a much higher turbidimetric response, compared to the lots coated with total IgY and the lot coated with polymers. The results from SPR show a somewhat diffuse picture, with the fractions from the start and the top right of the monomer peak showing good binding kinetics (high association rates), while the combined fractions from the peak do not show the same (low association rates).

Total IgY (ALL) gives a lower response on the turbidimetric instrument than the lot coated with monomers (MONO), consistent with average association rates of total IgY compared to fractions with monomers seen in SPR.

Both total IgY (ALL, CLARA's LOT) and nanoparticles coated with aggregates, precipitates an polymers (AGGR) give lower turbidimetric response compared to the reference lot (ALL, also total IgY).

3.3. Results clinical samples

Serum samples from children with different forms of JIA received from Karolinska Institute were analyzed turbidimetrically on Abbott Architect c4000 (Abbott, USA), and the two methods GCAL® and hsGCAL were compared with regards to laboratory and clinical parameters routinely used by clinicians at Karolinska Institute. Both methods, hsGCAL and GCAL®, were compared with regards to average calprotectin values and CV. Results are summarized in **Table 20**.

Table 20. Average CV (%) for hsGCAl and GCAL® assays in samples grouped according to their calprotectin concentrations (under 1 mg/L, between 1-2 mg/L, between 2-3 mg/L and over 3 mg/L).

Calprotectin values measured	Average CV (%) for hsGCAL	Average CV (%) for GCAL®
< 1mg/L	2.47	2.94
1-1.99 mg/L	0.89	1.31
2-2.99 mg/L	0.36	0.87
≥ 3 mg/L	0.14	0.18
All samples	**1.16**	**1.50**

The Passing-Bablok method was used to determine analytical accuracy of hsGCAL against the reference GCAL assay, resulting in the equation y = -0.08802 + 1.027 x (with y=hsGCAL and x= GCAL®). The results were visualized using a Bland-Altman difference plot (**Figure 22)**, showing higher bias in the low concentration areas. To better show bias in the lower range, values above 5 mg/L (4 samples) are not included in **Figure 22**, though they were included in the Passing-Bablok analysis above.

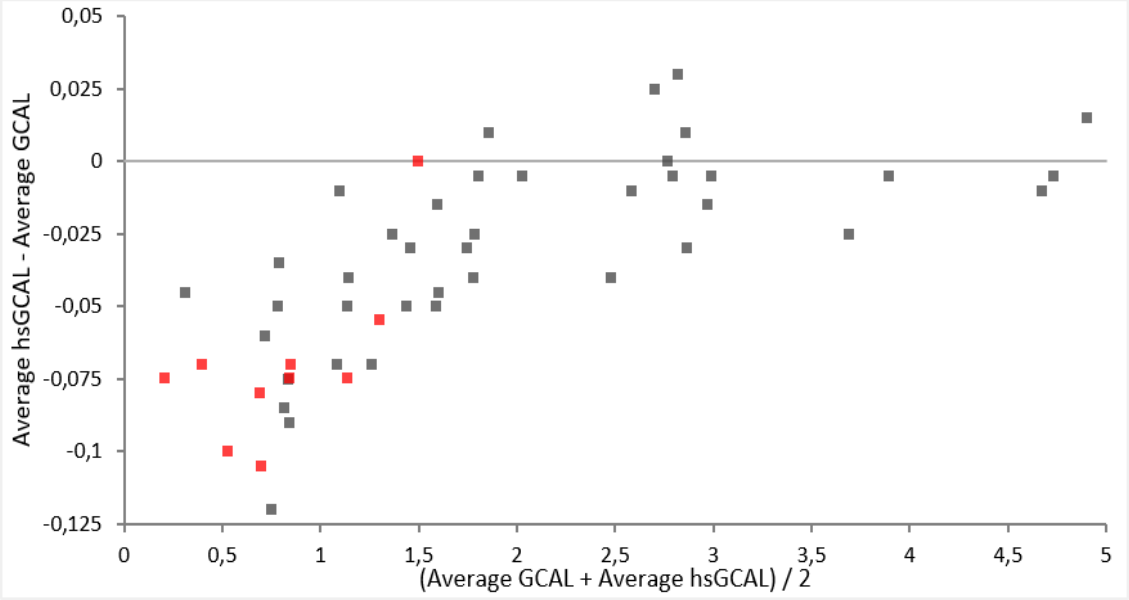


Figure 22. Bland-Altman difference/bias plot of the average values of the hsGCAL assay versus GCAL® assay average values. In red: control group. In grey: patient group. Values above 5 mg/L are excluded from the graph.

As was expected, the values measured by the hsGCAL assay are generally lower compared to the reference GCAL® assay, and bias increases when measuring calprotectin concentrations under 2 mg/L. This confirms earlier indications that hsGCAL is more reliable in the lower concentration area than the measurements by the GCAL® assay because of the lower CV observed when using the hsGCAL assay in these measurements (**Table 20**). Also, hsGCAL generally showed better recovery in previous experiments, especially in the lower concentration areas.

The differences in means between the control and patient group for GCAL® and hsGCAL are summarized in **Table 21** and visualized in **Figure 23**. To better visualize the difference in means between the groups, values above 5 mg/L (4 samples) were not included in the figure, though they were included in the calculations leading to the table.

Table 21. Comparison between patient and control groups for GCAL® and hsGCAL, with regards to average calprotectin concentrations (mg/L), standard deviation (mg/L) and Coefficient of Variation (%).

	hsGCAL			GCAL®		
	Calprotectin concentration (mg/L)	STD (mg/L)	CV (%)	Calprotectin concentration (mg/L)	STD (mg/L)	CV (%)
Patient group	4.45	0.02	0.91	4.42	0.02	1.26
Control group	0.78	0.01	2.12	0.85	0.02	2.09

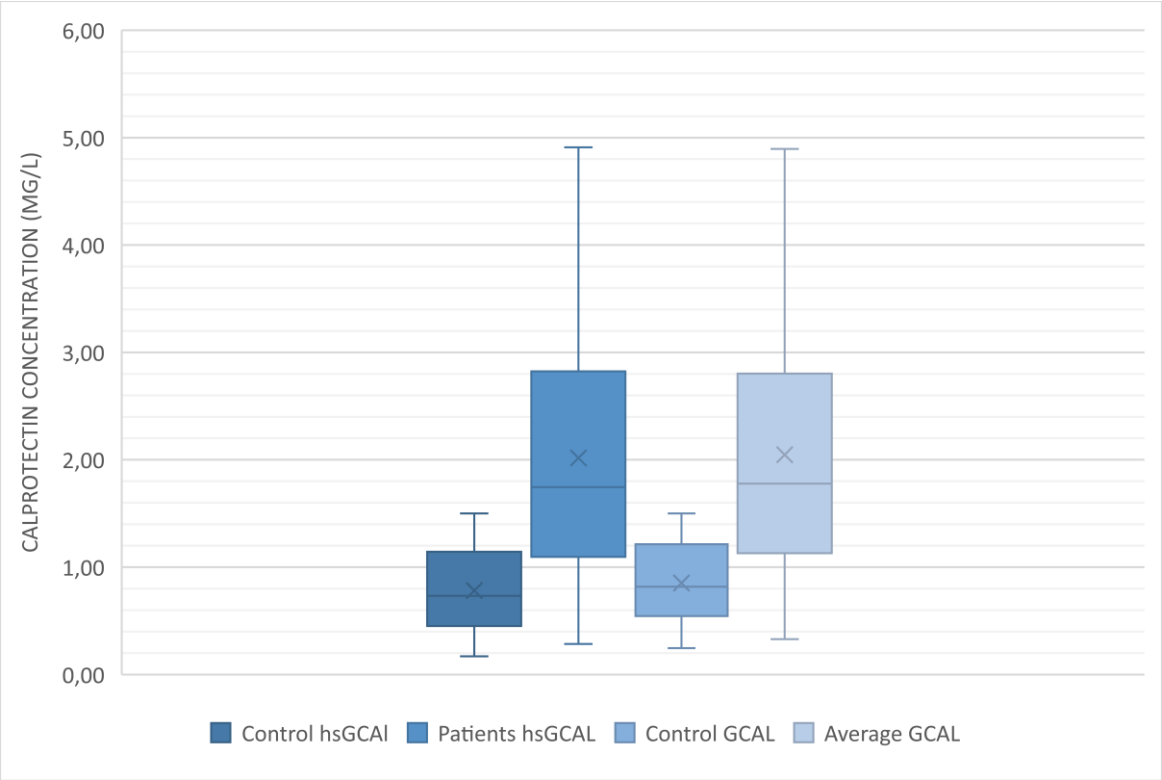


Figure 23. Comparison between patient and control group for GCAL® and hsGCAL. The figure shows difference in means between control and patient groups for hsGCAL and GCAL®. Values above 5 mg/L are excluded from the visualization.

Statistical analysis was performed by Karolinska Institute using a Student's t-test and Pearson's correlation, where $p<0.05$ was considered significant. The results of the statistical analysis, summarized in

Table 22, indicate that the two methods GCAL® and hsGCAL perform almost identically with regards to the clinical and laboratory parameters.

Table 22. Results for statistical method comparison between GCAL® and hsGCAL for the most important laboratory and clinical parameters

Parameter group	**Parameter**	**Nr of patients**	**GCAL®**		**hsGCAL**	
			R^2	**p**	**R^2**	**p**
Autoantibodies	**Rheumatoid Factor, RF (positive or negative)**	23		0.42		0.44
	Anti-nuclear antibody (positive or negative)	36		0.97		0.94
	Anti-citrullinated antibody (positive or negative)	24		0.54		0.55
Joint Status	**Nr of affected joints**	33	0.01	0.56	0.13	0.09
	Nr of joints cortisol-treated at visit	21	0.13	0.09	0.13	0.09
	Sedimentation rate, SR (mm/h)	32	0.68	<0.0001	0.68	<0.0001

Inflammation markers	**CRP (mg(L)**	32	0.54	<0.0001	0.54	<0.0001
Patients own estimation	**Disease effect on life (VAS scale)**	29	0.002	0.8	0.003	0.76
	Pain (VAS scale)	29	8x10-5	0.76	0.0003	0.76
Clinicians estimation	**Global Assessment Disease activity, GAD (VAS scale)**	26	0.48	<0.0001	0.48	<0.0001
Subtypes	**All JIA patients vs. controls**	43		0.07		0.06
	Oligo arthritis vs. controls	32		0.009		0.01
	Poly arthritis vs. controls	26		0.06		0.06
	Systemic onset JIA vs. controls	15		0.002		0.002
	Oligo arthritis vs. poly arthritis	37		0.08		0.08
	Oligo arthritis vs. systemic onset JIA	27		0.23		0.24
	Poly arthritis vs. systemic onset JIA	20		0.60		0.62

4. Discussion

The results show that the hsGCAL assay (with adjusted calibrator set and 8 uL sample volume) meets the criteria for linearity in the range 0.05 – 10.4 mg/L (on Abbott Architect c4000) with a security zone up to at least 60 mg/L (highest concentration tested), and shows improved/lower CV (%) compared to the reference assay (GCAL®).

Nanoparticles were coated with different fractions of IgY. One lot was coated with total IgY, one with mainly monomers, and one with aggregates/precipitates/oligomers. The lot with mainly monomeric IgY gave a higher signal/response on the turbidimetric instrument, even compared to the (optimized) reference GCAL® assay. This is an indication that using monomeric instead of total IgY in the calprotectin assay may help increase sensitivity, as the formation of antibody-antigen complexes is detectable turbidimetrically already at low calprotectin concentrations. However, the curve was not linear, the observation should be confirmed and studied further.

The above results indicate that the adjustments performed and tested in this thesis (increased sample volume, different calibration range, and/or using immunoparticles coated with calprotectin monomers only) have the potential to increase the sensitivity of the GCAL® assay.

However, there are some limitations that should be noted. The tests performed in this thesis are only a few of all the tests that would be performed in the official process of developing or adjusting an immunoassay. The results of this study can therefore only be seen as an indication that the adjustments may lead to a more sensitive calprotectin assay.

Also, the results from the patient samples did not show any clinical benefit of the method with increased sensitivity (hsGCAL) compared to the reference method (GCAL®). This was partly because calprotectin values in pediatric healthy controls as well as in Juvenile Idiopathic Arthritis patients were not as low as anticipated. The control group consisted of just 10 children. A larger control group would have made the comparison between the two methods more reliable, possibly lead to lower mean calprotectin values in the control group and therefore potentially providing a different picture when analyzing with a more sensitive method.

It is possible that a more sensitive method will prove to have more clinical relevance when measuring calprotectin in EDTA plasma instead of serum, because of the generally lower calprotectin levels in EDTA plasma. As noted by Nordal *et al.*, 2018, EDTA plasma is the preferred matrix for measuring calprotectin in adult arthritis patients. In addition, Pedersen *et al.*, 2018 found that pre-analytic variation such as temperature seems to less pronounced in EDTA plasma when measuring calprotectin. Measuring in EDTA plasma might therefore also be the best matrix of choice in JIA. Also, as argued in

Foell, 2010, calprotectin may be useful in the monitoring or predicting the effect of starting/stopping treatment and predict upcoming flares. In addition, a more sensitive assay may be useful in detecting these, possible smaller, changes in calprotectin concentration.

There may be use for a turbidimetric particle-enhanced calprotectin immunoassay with increased sensitivity for other diseases. If the concentration calprotectin levels between healthy and patient samples, or the levels of calprotectin in different diseases stages differ little, an assay that could detect these minor differences better, might have added (clinical) value. However, it is also possible that the improvements in sensitivity are not strong enough to provide benefit in clinical use of the test compared to the GCAL® method.

5. Conclusions and recommendations

The goal of this study was to improve sensitivity of the Gentian Calprotectin Immunoassay (GCAL®), to allow accurate measurements of calprotectin concentrations under 0.5 mg/L in human blood.

Research questions that were addressed in this thesis are:

- What is the effect of varying assay parameters, such as sample volume and calibration range, on the sensitivity of the GCAL® assay?
- Do different fractions of IgY show higher affinity for calprotectin? Does this result in higher sensitivity?
- Does increased sensitivity of the GCAL® assay lead to a better predictive value in JIA (clinical significance)?

In order to answer these questions, the following adjustments were made to the Gentian Calprotectin Immunoassay (GCAL®): the calibration range has been decreased and an extra calibration point was added between point 1 and 2, and the sample volume has been increased from 4µL to 6µL and 8µL to assess the effect on the sensitivity of the assay.

To test the effect of the adjusted calibrator set and sample volume on sensitivity, dilution series (with very low calprotectin concentrations) were tested, and CV and recovery were calculated. Linearity of the most promising combination (8µL and adjusted calibrator) was tested and confirmed in the range 0.05 – 10.4 mg/L (on Abbott Architect c4000). Security Zone up to 60 mg/L was tested and confirmed.

Fractions of IgY were obtained by size exclusion chromatography, and affinity of those fractions towards calprotectin was investigated by surface plasmon resonance. Three lots of nanoparticles coated with different IgY fractions (total IgY; mainly monomers; and aggregates/polymers) and response was measured turbidimetrically on a clinical analyzer. The size of the coated and uncoated nanoparticles was assessed with nanoparticle tracking analysis.

Taking into account the limitations as outlined in the discussion section, the conclusion after performing the work described in this thesis is that, although the changes to the GCAL® assay did not lead to a better predictive value in the analysis of calprotectin in serum of JIA patients, the adjustments do have the potential to lead to increased sensitivity of the GCAL® assay.

To further assess this potential, it is recommended to further research the use of monomeric IgY in the GCAL® assay, to test calprotectin in EDTA plasma, to assess the use of the sensitive calprotectin assay in treatment monitoring and flare prediction of JIA, and to assess clinical use in other patient groups.

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Annexes

Annex 1

Table 23. Recovery test. Abbott Architect c4000, GCAL® Reference method (current, 4 uL)

Measured concentartion [mg/L]	SD [mg/L]	CV [%]	Theoretical concentartion [mg/L]	Recovery [%]	Difference measured and theoretical concentration
9,94	0,03	0,29	9,83	101	0,12
7,82	0,04	0,46	7,87	99	-0,05
5,78	0,04	0,61	5,91	98	-0,13
3,95	0,03	0,81	3,95	100	0,00
2,00	0,02	0,76	1,99	101	0,01
1,09	0,02	1,40	1,01	108	0,08
0,56	0,02	3,57	0,52	107	0,04
0,24	0,02	9,49	0,28	87	-0,03
0,24	0,02	8,33	0,23	105	0,01
0,11	0,02	21,32	0,16	72	-0,04
0,06167	0,04875	79,06	0,09	65	-0,03
-0,0233	0,06	-260,92	0,03	-70	-0,06

Table 24. Emancipator Kroll test. Abbott Architect c4000, GCAL® Reference method (current, 4 µL)

Linear fit	Nonlinear fit (2nd order polynomial)	Nonlinearity	95% CI		Relative nonlinearity
0,01876	0,00044	-0,01833	-0,04156	to 0,0049	-97,7%
0,08014	0,06725	-0,01288	-0,03252	to 0,0068	-16,1%
0,14151	0,13383	-0,00768	-0,02391	to 0,0086	-5,4%
0,21516	0,21342	-0,00175	-0,01422	to 0,0107	-0,8%
0,26426	0,26629	0,00203	-0,00822	to 0,0123	0,8%
0,50976	0,52848	0,01872	0,01087	to 0,0266	3,7%
1,00075	1,04277	0,04202	0,01707	to 0,0670	4,2%
1,98273	2,03715	0,05442	0,00861	to 0,1002	2,7%
3,94670	3,93833	-0,00837	-0,05270	to 0,0360	-0,2%
5,91066	5,81658	-0,09409	-0,13724	to -0,0509	-1,6%
7,87463	7,78449	-0,09015	-0,13060	to -0,0497	-1,1%
9,83860	9,95465	0,11606	0,06111	to 0,1710	1,2%

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