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Avian antibodies applied in particle enhanced turbidimetric immunoassay

Developement of serum/plasma calprotectin immunoassay and its clinical performance as a marker for bacterial infections

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Abstract
Nilsen, T. 2018. Avian antibodies applied in particle enhanced turbidimetric immunoassay. Developement of serum/plasma calprotectin immunoassay and its clinical performance as a marker for bacterial infections. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1507. 54 pp. Uppsala: Acta Universitatis Upsaliensis.
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Calprotectin is a cytosolic protein in the granulocytes, consisting of S100A8 and S100A9. On the site of inflammation, the neutrophils release the cytosol as an inflammatory response. The circulating calprotectin concentration increases and can therefore be used as marker for neutrophil activation and inflammation.

To raise specific antibodies, it is crucial to immunize with pure calprotectin antigen. We purified calprotectin from human granulocytes by ion-exchange chromatography, dialysed it towards saline and concentrated it to required levels, suited for immunisation of the hens. The purified antigen solutions were assigned concentration values by the Biuret method and the purity was checked by SDS PAGE and size exclusion chromatography. The yield was approximately 2 mg purified antigens per unit of 450 ml blood.

A prototype calprotectin particle enhanced turbidimetric immunoassay was developed from the purified antigen and the affinity purified antibodies. The antigen was spiked into PBS to prepare calibrators and controls. The antibodies were coated to latex particles to prepare immunoparticles. The performance of the immunoassay was technically tested on a clinical chemistry analyser. LoQ, antigen excess, linearity, precision and calibration stability met the pre-set criteria.

In the production process of immunoparticles there are several factors affecting the performance of the assay. Investigating eight factors applying a Taguchi L12 screening, we experienced that conductivity and pH of conjugate buffer, coating grade and conductivity of dialysis buffer II affected the sensitivity and antigen excess the most.

The assay was used to measure clinical samples. Serum samples from elderly people aged 70+ were collected. Only patients with no infections were included to establish a reference interval for this patient group. The reference interval in serum was 0.3 mg/L to 2.5 mg/L for both genders. Furthermore, the plasma calprotectin immunoassay was tested clinically on critically ill patients to assess the ability of plasma calprotectin as an early marker for detection of bacterial infections. It showed promising results. Calprotectin was a better predictive marker for sepsis than procalcitonin and white blood cell count. Because some patients with an inflammation have low numbers of granulocytes, we examined the correlation between white blood cell count and the calprotectin levels in a group of patients with an inflammation. There was a weak correlation between the number of white blood cells and calprotectin concentration.

Keywords: Sepsis, avian antibodies, calprotectin antigen, particle enhanced turbidimetric immunoassay.

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Nilsen T., Haugen S.H., Larsson A. (2018) "Extraction, Isolation, and Concentration of Calprotectin Antigen (S100A8/S100A9) from Granulocytes." *Health Science Report*, https://doi.org/10.1002/hsr2.35.

II Nilsen T., Sunde K., Larsson A. (2015) "A new turbidimetric immunoassay for serum calprotectin for fully automated clinical analysers." *Journal of Inflammation* 12:45

III Nilsen T., Larsson A. "Influence of 8 parameters when coating calprotectin antibodies to latex particles". - in manuscript

IV Nilsen T., Sundström J., Lind L., Larsson A. (2014) "Serum calprotectin levels in elderly males and females without bacterial or viral infections." *Clinical Biochemistry* 47 (12):1065-8.

V Jonsson N., Nilsen T., Gille-Johnson P., Bell M., Martling, C-R., Larsson, A., Mårtensson J. (2017) "Calprotectin as an early marker of bacterial infections in critically ill patients: An exploratory cohort assessment." *Critical Care and Resuscitation: Journal of the Australasian Academy of Critical Care Medicine* 19 (3): 205-13.

Contents

Abbreviations

EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
HAMA	human anti-mouse antibodies
HNL/NGAL	human neutrophil lipocalin/neutrophil gelatinase-associated lipocalin
ICU	intensive care unit
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IgY	immunoglobulin Y
IQR	interquartile range
LoQ	limits of quantitation
MOPS	3-(N-morpholino) propanesulfonic
MRP14	myeloid-related protein 14
MRP8	myeloid-related protein 8
PCT	procalcitonin
PETIA	particle enhanced turbidimetric immunoassay
RF	rheumatoid factor
RIA	radioimmunoassay
RM-ANOVA	repeated measures analysis of variance
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SIRS	systemic inflammatory response syndrome
STD	standard deviation
TRIS	tris-(hydroxymethyl)aminomethane
UV	ultra violet

Introduction

Immunoassays utilise the immune complex formation when antibodies and antigens react.

The first sensitive quantitative immunoassay is credited to Yalow and Berson and their work on insulin measurements in humans published in 1960 (Yalow and Berson 1960). This immunoassay was a competitive radioimmunoassay (RIA) where antigens were labelled with a radioactive isotope and mixed with the sample.

The first latex immunoagglutination assay was described in 1956 by Singer and Plotz. It was applied for diagnosis of rheumatoid arthritis. The latex particles reacted with rheumatoid antibodies and precipitated. The results were categorized from negative to plus four and was read by the naked eye (Plotz and Singer 1956).

The development of immunoassays evolved and improved through the next decades. The detection label was coupled to the antibodies and new types of detection techniques was developed. A popular heterogeneous immunoassay type was developed by Perlman and Engvall at University of Stockholm in the 70s (Engvall and Perlmann 1971). It was the enzyme-linked immunosorbent assay (ELISA).

The ELISA has been widely used in clinical and research laboratories since the innovation in 1971 (Lequin 2005). A search on PubMed for ELISA gives more than 240.000 hits in October 2017.

The sandwich ELISA technique physically immobilises the antigens in a solution by capturing-antibodies attached to the walls in the tube. To detect the antigens, specific enzyme-labelled antibodies are added, where an enzyme-substrate reaction is changing the optical characteristics, which is detected by a spectrophotometer (Engvall, Jonsson, and Perlmann 1971) (Engvall and Perlmann 1972). This technique requires multiple steps of separation (washing) and incubation, which makes it time consuming and labour intensive, even with partly automated solutions.

Because homogeneous immunoassays do not require separation steps, these types of assays were developed to adapt immunoassays to automated analysers (Wu 2006). Homogeneous particle enhanced turbidimetric immunoassays (PETIA) are today widely used for quantitative measurement of proteins, pathogens and other molecules in body fluids, such as plasmas, serum, urine etc.

The advantages of PETIA on fully automated clinical analysers over ELISA are several: all pipetting steps are done by the instrument, eliminating the human error of the pipetting process, which improves the precision of the tests. Compared to free antibody assays, the antigen excess characteristics and sensitivity are better.

Most clinical chemistry analysers are random access instruments, which means that samples can be measured continuously as they arrive at the laboratory, reducing turnaround time compared with traditional ELISA of the microtiter plate type. It is cost-effective due to low reagent consumption and low labour costs due to limited handling times during the measuring procedures. It takes approximately 10 minutes from initiation of the measurement until the results are ready, applying the PETIA technology.

Particle enhanced turbidimetric immunoassay

The immunoparticles are prepared by binding the antibodies to the polystyrene particles. The antibodies are absorbed to the surface or covalently bound to a surfactant molecule, such as a methyl-group. Instead of a radioactive isotope or a coloured substrate to detect the formed immune complexes, the detection technique is based on the change of opaqueness of the solution containing sample, assay buffer and immunoparticles. This optical change is caused by aggregation of the particles. The antibodies on the polystyrene particles form immune complexes with the antigens present in the sample. Because the antibodies are coated on the whole surface of the immunoparticles, several antigens are bound to the same immunoparticle (Newman, Henneberry, and Price 1992). Each of the antigen can react with antibodies attached to other polystyrene particles to form aggregates of immunoparticles bound together by the antigen (**figure 1**). The change in optical characteristics is measured by the absorption of monochromatic light through the solution. Absorption is measured at a point before the reaction starts and at a point after the reaction has declined. The difference in absorption is referred to as the signal. A quantitative measurement of an unknown sample is performed in relation to a calibration curve. A six-point calibration curve is established from six known samples, known as standards with different concentrations. A regression line is calculated using the signal and concentration from each sample.

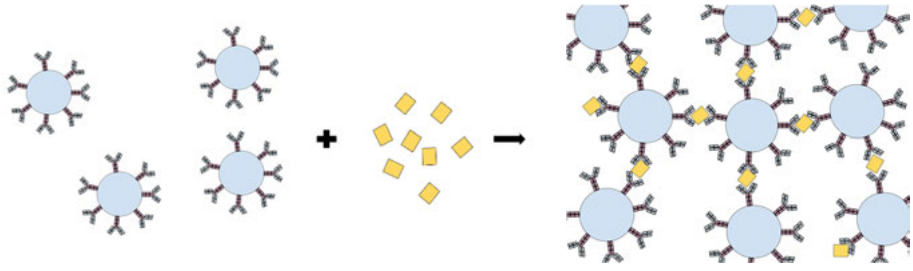

Figure 1. Particle enhanced immunocomplex: Schematic drawing of aggregates of immunoparticles, formed by immune reaction between antibodies conjugated to polystyrene particles and antigen.

Important factors designing particle enhanced turbidimetric immunoassay

Many factors need to be considered when designing a PETIA, a few of them will be addressed here.

The assay buffer must ensure optimal reaction conditions for the formation of the immunocomplexes. Factors to be considered are pH, ionic strength, buffer capacity etc.

When designing immunoparticles there are several important factors, such as characteristics of the antibodies, colloidal stability and particle size, that must be optimized. Characteristics of the antibodies relies on the purity of the antigen used for immunizing. This is crucial. Contamination in the immunogen will raise antibodies against the contaminants, creating unspecific signal. It may also cause sepsis in the vaccinated animals. It is also important that the immunogen is as similar as possible to the native protein present *in vivo* and in the samples that the assay is intended for. Colloidal stability is related to particles size, concentration of particles, pH and ionic strength in the storage buffer. To avoid unintentional aggregation the mentioned factors must be combined optimally (Molina-Bolívar and Galisteo-González 2005).

Calibrators and controls are stored in a protein solution matrix. The best choice of calibrator and control matrix is usually the same matrix as the intended sample material. However, there are factors such as stability of antigen and risk reduction related to transferable diseases, which has to be considered when choosing a suitable matrix. Non-biological buffer matrices are required for some markets. To ensure accurate measurements, a correct value of the calibrator is essential. If a standardized reference material is available a value transfer protocol should be applied, such as Blirup-Jensen et al (Søren Blirup-Jensen et al. 2008).

Technical criteria for PETIA

In addition to the measuring range of the assay and the calibration range there are several features that are required from a PETIA before it can be applied for clinical purposes. A PETIA must be substantially tested to ensure required performance characteristics, both technically and clinically. There is a well-known technical limitation for this technology: It is not possible to measure both very high concentrations in addition to measure accurately and precisely at very low concentrations with the same assay. The clinical purpose of the PETIA determines the design and which criteria the PETIA must meet. Commercially available CRP PETIAs illustrates this. CRP is measured for two clinical purposes; as an inflammation marker and as a cardiac risk marker (Ridker et al. 2002). The different purposes require different performance characteristics, therefore the manufacturers usually provide two different products with different measuring ranges, measuring the same molecule. Because concentrations of acute phase proteins tend to increase dramatically as a response to inflammation, CRP may increase from <1 mg/L up to >400 mg/L, with a cut off value of 40 mg/L often used as a threshold for bacterial infections and for antibiotic treatment (arkiv.www.unn.no) ("Nasjonal Brukerhåndbok I Medisinsk Biokjemi"). As a tool for detection and monitoring of infections a CRP assay does not require a quantitation limit below 1 mg/L, but an ability to detect concentrations up to 500 mg/L and even higher is desired. As a cardiac risk marker, it is crucial to measure CRP concentrations accurately and precisely in the lower concentration range, because CRP levels from <1 mg/L to > 3 mg/L distinguish between low risk to high risk of developing a cardiovascular disease ("hsCRP-Nasjonal Brukerhåndbok I Medisinsk Biokjemi"). Hence, the limit of quantitation (LoQ) must be much lower (< 0.5 mg/L), and the ability to measure high concentrations is not necessary for such an assay. The differences in measuring range may require two different assays due to the limitations in the technology. It is challenging to measure both accurate and precisely in the lower range in addition to measure extremely high concentrations. In the market, CRP and high-sensitive CRP are often available as two different products but are measuring the same molecule.

Clinical and Laboratory Standards Institute (CLSI), develops guidelines for laboratories and manufacturers for methods evaluations, covering quality and performance aspects. Performance such as security zone, detection capability, linearity, precision, interference and method comparison among others must be tested and documented when developing and evaluating assays. CLSI provide guideline documents on how to design these studies. To achieve the required criteria from these studie's technical aspects such as background signal and span of calibration curve should be evaluated.

Two main features of a PETIA are lower quantitation limit and antigen excess characteristics in comparison with assays using free antibodies. These should be considered carefully. The LoQ is defined as the lowest concentration which can reliably be detected and should be below the lower reference interval limit. The hook effect occurs at the lowest concentration where the observed turbidimetric response is below the value of the turbidimetric response of the highest calibrator.

For an optimally designed PETIA all results higher than the highest calibration standard will force the instrument to flag the sample and the user will be alarmed to start a diluted rerun if needed.

Applications on clinical chemistry analysers

An optimised PETIA is a combination of optimised reagents and an optimised application specific for the instrument. A range of parameters is usually possible to adjust to find the best application. These parameters are sample volume, assay buffer volume, immunoparticles volume, wavelength, reading time and regression line for the calibration curve. In addition, most instruments have different options in assay type, which is different ways of reading and calculating the absorbances. Examples of assay types are end-point, fixed time and rate. In addition, some instruments have different blanking options to reduce background noise.

Chicken antibodies

Traditionally mammalian immunoglobulin G (IgG) antibodies are the most widely used antibodies in immunoassays. However, there is an increasing interest in the use of avian antibodies, immunoglobulin Y (IgY), which may improve the performance of the immunoassay in many cases. Specific avian antibodies are produced by immunizing hens with the specific antigen multiple times (Carlander 2002). The IgY is transferred from the serum of the mother hen into the egg yolk (Losch 1996). The egg yolk is then separated from the egg white, and the preferred IgY can be isolated by affinity chromatography or precipitation techniques.

Characteristics of chicken antibodies

For antibodies found in eggs, immunoglobulin M (IgM) and A (IgA) in eggs are present in the egg white, while IgY is only present in the yolk (Rose, Orlans, and Buttress 1974). Functionally IgY is the chickens equivalence to the mammalian IgG (Warr, Magor, and Higgins 1995), however there are differences in size, immunological and physical-chemical characteristics (Sun et al. 2001).

Like IgG the chicken IgY consists of two heavy chains and two light chains, bound together with a disulphide link. Where the IgG has a flexible hinge region, IgY has a shorter and less flexible hinge (Warr, Magor, and Higgins 1995). Because the heavy chain of IgY is longer, it has a greater molecular weight, 167.5 kDa compared with the IgG's 160 kDa (Sun et al. 2001), **figure 2**. The pI is lower than its mammalian equivalence, 5.7-7.6 compared with 6.1-8.5 (Dávalos-Pantoja et al. 2000).

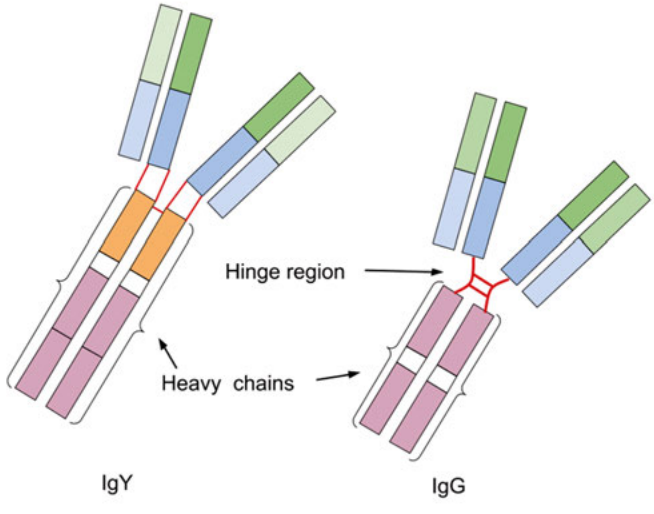

Figure 2. IgY vs IgG. The main differences in structure are illustrated. The hinge region is more flexible in the IgG molecule and the heavy chain is longer for IgY.

Advantages of chicken antibodies over mammalian antibodies

Avian antibodies were used in this experiment because of its advantages compared to mammalian antibodies, which are several. In the production of avian antibodies the egg yolk is collected and affinity purified, which avoids the painful blood collection of animals and in this way reducing animals suffering

(Hau and Hendriksen 2005). IgY based immunoassays are expected to be associated with less interference from human samples as the human complement system is not activated by the chicken antibodies (Larsson et al. 1992). In addition there will be no reaction between the chicken antibody and human anti-mouse IgG antibodies (HAMA) or rheumatoid factor (RF) which are considered to be a major problem in immunoassays (Carlander and Larsson 2001), (Larsson and Sjöquist 1988). IgY coated polystyrene particles seems also to be more colloidal stable compared to its IgG counterpart (Dávalos-Pantoja et al. 2000). And applied in particle enhanced turbidimetric immunoassays, the IgY gives a higher signal then IgG (Hansson et al 2008.)

In this project polyclonal antibodies raised in hens immunised with purified calprotectin were used. The antibodies are expected to detect both monomers and dimers and even oligomers of the calprotectin subunits.

Inflammation, neutrophil activation and its biomarkers

Inflammation can be caused by several stimuli; physical impact (heat, cold, radiation, etc.), chemical impact (irritation or corrosive tissue damage), autoimmune processes or infections (viral, bacterial or fungal) (Roald 2016).

As part of the defence against bacteria, the neutrophil granulocytes are activated. The neutrophil granulocytes attack the invading bacteria by releasing its cytosol at the site of inflammation. A few hours after activation, the number of neutrophil granulocytes can reach a 4 to 5 fold of normal amount, but the number of circulating neutrophils may also decrease as the neutrophils are extravasated. When the neutrophils are activated and leak its cytosol in the process of defeating the infection, a wide range of proteins may be released into the bloodstream (Bjårli, Haug, and Sand 1998). More than 1000 proteins have been detected in human granulocyte cytosol (Kasper et al. 1997). These proteins may be used as biomarkers for activation of neutrophil granulocytes. Examples of neutrophil granulocyte cytosol proteins are human neutrophil lipocalin/neutrophil gelatinase-associated lipocalin (HNL/NGAL) and calprotectin. HNL/NGAL has been shown to be superior to CRP and procalcitonin in distinguish between bacterial and viral infections (Venge et al. 2015).

Traditionally white blood cell count, erythrocyte sedimentation rate (ESR), and CRP among others have been used to detect inflammation. The ESR is a late responder to inflammatory stimuli and thus not rapid enough for acute infections. White blood cell counts are not sufficiently sensitive or specific. Because CRP is synthesized in the liver (Hurlimann, Thorbecke, and Hochwald 1966), a hypothesis is that CRP has longer response time than calprotectin which is stored in the neutrophil granulocytes and are released immediately upon neutrophil activation, see **figure 3,** (Eriksson et al. 1998) (Lipcsey et al., unpublished data).

The increasing problem with antibiotics resistant bacteria also emphasizes the need of improved tools to distinguish between bacterial and viral infections to avoid using antibiotics in patients with viral infections. This will be important in the process of reducing the use of antibiotics in treatment of inflammations.

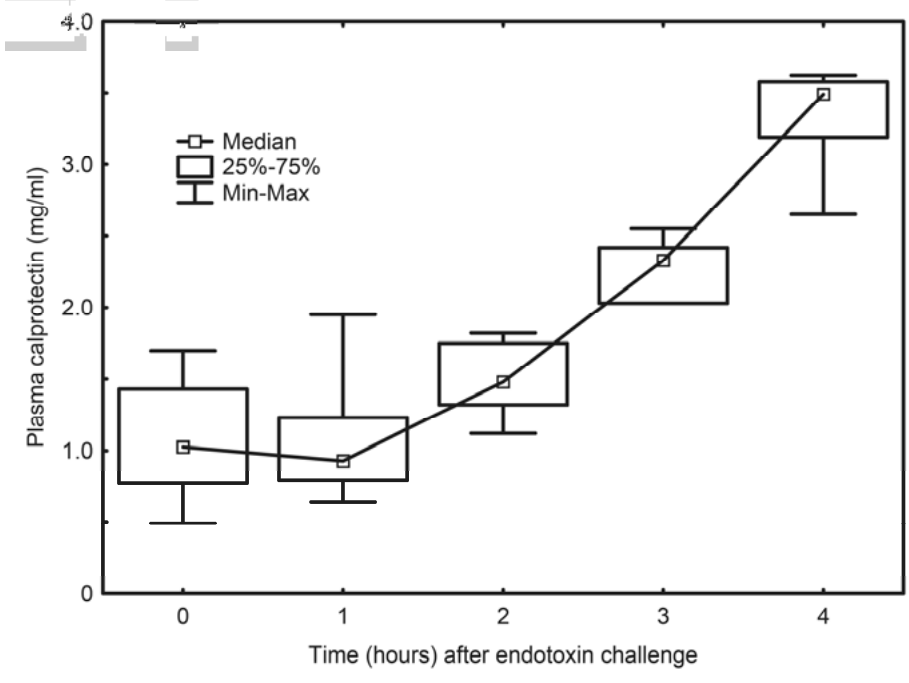

Figure 3. The kinetics of the calprotectin after infection of E. coli in whole blood (*in vitro*) (Lipcsey et al., unpublished data).

Sepsis

The definition of sepsis has varied during the decades. However, *The third international consensus for sepsis and septic shock (Sepsis-3 from 2016)* has lately defined sepsis as a life-threatening organ dysfunction caused by a dysregulated host response to infections (Singer et al. 2016).

In US hospitals, septicaemia is the most expensive condition and counts for 3.6% (2013) of all hospital stays in US (Martin et al. 2016). An early diagnosis of sepsis will reduce the mortality rate (Cannon et al. 2013). It is reported that the mortality rate from sepsis is as high as 15% (Rhee et al. 2017). Because the mortality rate is high and early diagnosis is crucial, fast and reliable tests

for diagnosis of sepsis is required. For diagnosis of sepsis, tests like procalcitonin and CRP are used as a part of the total evaluation of the patient. Calprotectin is also suggested as a sepsis marker (Gao et al. 2015).

Calprotectin

One of the most abundant protein in the neutrophil granulocyte cytosol is calprotectin, where it accounts for approximately 30-40% of the total protein content (Dale, Fagerhol, and Naesgaard 1983). Calprotectin comprises of two subunits, S100A8, also known as myeloid-related protein 8 (MRP8) and S100A9, also known as myeloid-related protein 14 (MRP14). S100A8 contains 93 amino acids and has a molecular weight of 10.8 kDa, while S100A9 contains 113 amino acids and has a molecular weight of 13.2 kDa (Odink et al. 1987), (Wilkinson et al. 1988). S100A9 also exists in an isoform which contains 110 amino acids with a molecular weight of 12.7 kDa. The two isoforms of S100A9 have the same functional properties (Pruenster et al. 2016).

The exact *in vivo* structure of calprotectin is debated in the literature. Three of the mentioned configurations of S100A8 and S100A9 are heterodimer (Edgeworth, Freemont, and Hogg 1989),(Hogg, Allen, and Edgeworth 1989), heterotrimer (Johne et al. 1997) and heterotetramer (Vogl et al. 1999). A combination of all these configurations is also suggested (Teigelkamp et al. 1991).

The function of calprotectin *in vivo* is not fully understood, but the literature agrees about an important role in inflammations caused by infections (Pruenster et al. 2016). Elevated calprotectin concentrations are observed in faeces and blood from patients with inflammatory conditions. The elevated calprotectin levels can be explained by the release of cytosol upon neutrophil activation caused by the infection (Voganatsi et al. 2001). Therefore, calprotectin is considered as an important inflammation marker and a part of the neutrophil mediated inflammation (Yui, Nakatani, and Mikami 2003), (Berntzen and Fagerhol 1990).

Elevated calprotectin concentrations in faeces have been used for diagnosis of intestinal inflammatory diseases, such as inflammatory bowel disease (IBD), Crohn's disease among others (Røseth et al. 1992) for a couple of decades. Faecal calprotectin is widely used to discriminate between IBD and irritable bowel syndrome. Measuring calprotectin values in serum samples is not as widely applied as for faecal samples, however, it is proposed as an inflammatory marker, such as diagnosis of sepsis (Terrin et al. 2011; Gao et al. 2015); (Pepper et al. 2013), for acute appendicitis (Schellekens et al. 2013) and rheumatoid arthritis (Adel 2014) .

In theory, as a neutrophil activation marker, calprotectin will be able to distinguish more precisely between bacterial and viral infections compared to

for instance CRP, because the neutrophils are activated specifically by bacterial infection. It is identified that HNL/NGAL, which is another neutrophil activation marker, distinguishes between bacterial and viral infection (Xu, Pauksen, and Venge 1995). The rapidly growing problem with antibiotics resistance has resulted in demands for more specific use of antibiotics (Laxminarayan et al. 2013). A better tool, which in the early stage of an infection can separate a bacterial infection from a viral infection will possibly reduce the use of antibiotics.

Among several measures in the diagnosis of sepsis is the white blood cell count. This can be misinterpreted because the neutrophils may attach to the endothelium and leave the circulation which reduces the number of white blood cells in the bloodstream (Larsson et al. 1996). A measurement of a protein related to the activation of neutrophils rather than the number of white blood cells in the circulation may circumvent this.

Ethical approvals

Paper I, II and III:

The regional ethics review board in Uppsala approved to use leftover samples from the clinical chemistry laboratory at Uppsala University Hospital for method comparison purposes (ref: Ups01-367).

Paper IV

The study is part of the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study. The study was performed in accordance with the ethical principles for medical research of the Declaration of Helsinki and approved by the ethics review board in Uppsala (Dnr 2005:M-079).

Paper V

The regional ethics review board in Stockholm approved our study. Written informed consent from patients or their next of kin were obtained (Ref:Ethical application and decision from EPN: 2006/1469-31/2 with amendments: 2007/1275-32, 2009/1688-32, 2012/143-32 and 2016/1801-32.

Aims

The main goal in this project was to develop a prototype turbidimetric immunoassay measuring serum or plasma calprotectin and test its technical performance on a fully automated clinical chemistry analyser. For clinical testing the aim was to evaluate calprotectin as a marker for inflammation caused by infection, such as sepsis. To achieve the overall goals, several intermediate objectives were defined:

Antigen production (Paper I):

Establish a method for purifying calprotectin antigen from human granulocytes to be used for immunisation of hens to raise antibodies reacting specific to calprotectin and to be used in calibrators and controls. The amount of purified antigen from this method should be sufficient to supply a potential commercial production line.

Prototype assay (Paper II):

To design a turbidimetric immunoassay for measuring serum calprotectin on Mindray BS-380 and test the technical performance according to pre-set criteria.

Improving sensitivity and security zone (Paper III):

The scope of this study was to see which parameters made significant impacts on the output. The output was chosen to be sensitivity and security zone.

Reference interval of calprotectin in elderly males and females (Paper IV):

To establish a reference interval for calprotectin in elderly people.

Evaluate clinical relevance (Paper V):

Evaluate the diagnostic value of calprotectin as a marker for sepsis.

Materials and Methods

Extraction of granulocyte cytosol (Paper I)

To lyse the red blood cells, buffy coat from a 450 ml blood bag were mixed with 250 mL of a lyse buffer (8.3 g/L ammonium chloride, 0.85 g/L sodium hydrogen carbonate) and left to incubate for 5-6 minutes. The granulocytes were forced to precipitate by careful centrifugation at 65xG for 12 minutes at room temperature, and the supernatant was carefully removed before mixing with a washing buffer (9 g/L NaCl, 1 g/L dipotassium EDTA dihydrate, pH 7.0). The granulocytes were gently mixed into solution before centrifuged at 55xG for 12 minutes, and the supernatant was again decanted, and the granulocytes were re-suspended in binding buffer (18.75 mM 5,5-Diethylbarbituric acid, 0.623 mM dipotassium EDTA dihydrate, pH 7.4). After at least 24 hours in the freezer (-55ºC), the solution was brought to room temperature and was ultra-sonicated to release the cytosolic proteins. The supernatant was collected after 20 minutes of centrifugation (4000xG) and diluted with purified water (resistivity > 1 MΩ/cm, Elix(R) Gulfstream C35, EMD Millipore, Merck KGaA, Darmstadt, Germany) to a required conductivity.

Purifying calprotectin antigen (Paper I)

The calprotectin was purified by applying the granulocyte extract to an anion exchange column, with 30 mL gel (Sepharose DEAE™, GE Healthcare, Uppsala, Sweden). The gel was equilibrated against a diemal buffer (18.75 mM 5,5-Diethylbarbituric acid, 0.623 Dipotassium EDTA, pH 7.4), before the extract was applied. The volume of the mixed extracts from 4 donors was typically 40 mL after adjusting the conductivity to similar level as the binding buffer. A washing buffer (85 mM diemal buffer, pH=8.6) was then rinsed through the column until pH= 8.6.

The 2 ml fractions were collected when a diemal buffer (18.75 mM diemal, 0.623 mM EDTA, 10 mM $CaCl_2$, pH 8.6), was used to elute the bound calprotectin. The high concentration calprotectin fractions were pooled after measurement on the Mindray BS-380. The purification procedure is in accordance with the method described in the patent from 1989, held by Fagerhol (Fagerhol, Dale, and Naesgaard 1989).

The pooled antigen solution was then concentrated and the elution buffer was replaced with 0.9 % saline without any preservatives, applying Amicon(™) Ultra centrifuge filters Ultracel(™) 3K from Merck Millipore (Tullagreen, Ireland).

Electrophoresis (Paper I)

The purified antigen solution was analysed with a two-dimensional sodium dodecyl sulphate polyacrylamide electrophoresis (SDS PAGE) (Amersham(™) ECL(™) Gel Box system from GE Healthcare, Uppsala Sweden, using Amersham(™) ECL(™) Gel Running Buffer x10 and Amersham(™) ECL(™) Gel 4-20%). The samples were mixed with a reducing buffer (Lane Marker Reducing Sample Buffer x5 from Thermo Scientific) before heated for 5 min at 95ºC and then applied to the gel. The separation conditions were 160 V and 160 mA for approximately 60 minutes. The gel was stained overnight (PAGE blue(™) protein Staining Solution from Thermo Scientific (Rockford, USA)), and then first rinsed in purified water then soaked in purified water until required contrast between stained bands and non-stained gel. As a molecular weight reference (ladder) Protein Marker II (6.5 - 200 kDa), pre-stained, AppliChem, Darmstadt, Germany was applied.

The gel was analysed by UN-Scan-it(R) from Silk Scientific, Inc. UT 84059 USA to establish a profile of each lane based on pixel density.

Size exclusion chromatography (Paper I)

Size exclusion chromatography (SEC) of the antigen was performed on a Äkta FPLC system connected to a fraction collector (Frac-950), where 100 µL of the antigen solution at a concentration of 1.57 mg/mL was injected onto the column (GE HC Superdex(TM) 75 10/300 GL). 40 fractions of 500 µL were collected applying an elution buffer (0.1M PBS pH 7.4: 30 mM NaH_2PO_4 + 138 mM Na_2HPO_4, 150 mM NaCl at 0.4 mL/min). The fractions were analysed by UV on the Äkta system and with the turbidimetric calprotectin immunoassay on Mindray BS-380.

Biuret Method (Paper I)

0.33 mL of the purified antigen was mixed with 0.66 mL of BIOQUANT(TM), Merck KGaA, Darmstadt, Germany. After an incubation time of 30 minutes the absorbance was measured at 546 nm on a spectrophotometer, Shimadzu UV-1650PC, Kyoto, Japan. The absorbance was compared

to an eight-point calibration curve to calculate the concentration in the samples. The calibration curve was established from a seven-point bovine serum albumin standard curve (Pre-diluted protein standards, Kit, Thermo Scientific, Rockford, USA) and purified water was used as the zero standard. As blank sample, 0.9 % saline with no preservatives was used, which is the same matrix as the antigen was dialyzed into after concentration. The standards were prepared and measured the same way as described for the sample.

Clinical chemistry analysers (Paper I, II, III, IV, V)

The fully automated clinical instruments Mindray(TM) BS-380 and BS-400 (Mindray Medical International, Shenzhen, China) were used for all calprotectin measurements.

Applications/assay procedures on Mindray BS-380 (Paper I, II, IV, V)

The applications optimised for the calprotectin immunoassay were for Mindray BS-380: sample volume=3 µL, R1 volume=200 µL and the R2 volume=30 µL. The wavelength was 605 nm and the reading times 38/39-55/56, which equals to 204 seconds as each cycle counts 12 seconds, and for Mindray BS-400: sample volume= 2 µL, R1 volume= 200 µL, R2 volume= 40 µL and reading time = 43-55 (12 x 9 sec = 108 sec).

Turbidimetric calprotectin immunoassay reagents (Paper I, II, III, IV, V)

Assay buffer (Reagent 1): 3-(N-morpholino) propanesulfonic (MOPS) buffer adjusted to pH=7.2.

Immunoparticles (Reagent 2): Purified polyclonal chicken antibodies raised against human calprotectin were covalently bound to uniform polystyrene particles and stored in a trisaminomethane (TRIS) buffer.

Calibrators: Phosphate buffer pH=7.4 spiked with high concentration calprotectin solution. A calibrator kit contained 6 levels: 0, 1, 3, 6, 15, 30 mg/L. The purified calprotectin solution was assigned with Biuret method (Bioquant™, Merck KGaA, Darmstadt, Germany).

Controls: Spiking normal human serum with the purified calprotectin solution. Two levels were prepared; 1.2 mg/L and 8.9 mg/L.

The performance studies (Paper II):

All studies were based on CLSI protocols, however some of them modified.

Lower quantitation limit

Lower limit of quantitation (LoQ) was defined as the calprotectin level of the sample with lowest concentration where the total coefficient of variation was lower than 20%. To define this level for the calprotectin assay, 4 samples in the range 0.2 to 0.6 mg/L were measured in triplicate for 3 days in a row, in total 36 measurements for each sample. A new calibration curve was established every day. The samples were prepared by diluting a normal serum sample in the calibrator matrix until required concentrations and stored at -55ºC until the day of measurement. The undiluted samples were measured in 12 replicates the first day to calculate the theoretical concentration of the diluted samples. The total error was calculated using the calculated standard deviations and biases in the root mean square method.

Antigen excess

A high concentration sample was prepared by spiking normal serum with granulocyte extract to required concentration (50-60 mg/L). The spiked sample was used to prepare a dilution series of nine samples with concentrations ranging from 2.5 to 100% of its original concentration. The dilution series was measured in triplicate. The lowest concentration level measured to be lower than the highest calibrator level is where the antigen excess starts.

Linearity

A high calprotectin sample was prepared by spiking a normal serum sample with a high concentration granulocyte extract to a required concentration of calprotectin (approximately 20-30 mg/L). A saline diluted normal serum sample (0.1-0.2 mg/L) was used to prepare a dilution series from the high calprotectin sample. The series consisted of 10 samples with concentration range from 100% to 0% of the high calprotectin sample. To be able to calculate the theoretical values of each sample in the series via the dilution factors, the high and low samples were assigned values by measuring them in triplicates. Then all samples were measured in duplicates, and recoveries from expected values were calculated. The acceptable deviation from the theoretical values were set to be less than 10%.

Precision and calibration curve stability

Three samples spanning the calibration curve, and two controls, were stored at -55ºC in 14 aliquots à 300µL. The calibration curve was established at day one. The samples were brought to room temperature, vortexed and measured immediately in duplicates. For ten subsequently working days this procedure was repeated on the same calibration curve. The reagents were stored onboard during the study period. Total coefficient of variations (CV) were calculated.

The established calibration curve was further applied to measure the same samples every week for up to 4 more weeks after the last day of the precision study to investigate the calibration curve stability of the immunoassay.

Recovery

In order to test the recovery, a pair of test samples was prepared. The purified antigen was added to one of the samples and the same volume of saline into the other sample as described by Westgard (Westgard 2008). By measuring the pair of samples, the difference between the test and control samples was observed, and the observed difference in amount of antigen was calculated. This was the amount antigen observed added in the test sample. This amount was compared to the actual amount added and then the percentage recovery was calculated. The amount of antigen added was aimed to increase the concentration from within the reference range as found in an earlier study (Nilsen et al. 2014), to a level significantly higher than the upper bound of the reference interval. Recombinant calprotectin from two suppliers were tested.

Interference

The calprotectin turbidimetric immunoassays was tested for interference with intralipid, haemoglobin or bilirubin on Mindray BS-380. Test samples with 10 g/L intralipid, 5 g/L haemoglobin, and 400 mg/L bilirubin were prepared and measured in triplicates. The results were compared with a control samples prepared for each sample.

The intralipid sample was prepared by adding 0.125 ml intralipid into 2.3 ml of serum and the control sample was prepared by adding 0.125 ml of pure water into 2.3 ml of serum. The haemoglobin samples were prepared by adding 0.106 ml of 94 g/L hemolysate to 1.894 ml of serum. The control sample were prepared by adding 0.106 ml of saline in 1.894 ml of serum. The bilirubin sample was prepared by adding 0.050 ml of bilirubin dissolved in 1M NaOH into 2.3 ml of serum and the control sample was prepared by adding 0.050 ml of 1M NaOH into 2.3 ml of serum.

The aim was to verify that there was no clinically significant difference between the mean of the test samples and control samples. No interference is

defined as no statistical significant difference between test and control samples. If a statistical difference is observed, we allow for an observed difference of 10 %.

Method Comparison

60 samples were measured on both calprotectin turbidimetric immunoassay and Bühlmann MRP8/MRP14(TM) ELISA (Bühlmann Laboratories AG, Schönenbuch Switzerland) the same day, starting with the turbidimetric immunoassay. The samples analysed were distributed over the ELISA calibration curve (0.4 to 24 mg/L) with a predominance of samples from within the reference interval up to approximately 6.5mg/L. The results were analysed by a Passing Bablok regression analysis with linear fit in Analyse-It(TM) for MS Excel(R) (Analyse-It Software, Ltd, Leeds UK). Observed deviations greater than 25% between duplicates on the ELISA method were defined as outliers and removed from the data set.

Lot variation

Two immunoparticles batches were prepared with two different antibody batches, where the antibodies were raised using two separately prepared antigens. 80 samples were measured on both batches and evaluated by a Passing Bablok linear regression analysis in Analyse-It for MS Excel.

Materials and methods used in preparation of immunoparticles (Paper I, II, III, IV, V)

Affinity purified polyclonal avian antibodies raised against human calprotectin. The hens raising the antibodies were immunised with the antigen prepared as described in this thesis. The antibodies were covalently coated to 96 nm latex particles from Lifediagnostics. The coated latex particles were dialysed against two buffers, dialysis buffer 1 and 2 applying a Labscale(TM) TFF system, Merck Millipore KGaA, Darmstadt, Germany, with Pellicon(R) XL filter 500 kDa and 30 kDa, Merck Millipore KGaA, Darmstadt, Germany. The latex particles were tested on the automated clinical chemistry analyser, Mindray BS-400, Mindray, Shenzhen, China.

Buffers

The antibodies were kept in conjugate buffer prior to coating onto the latex particles. The conjugate buffer, which was a borate buffer, was tested in four versions; 1) low pH and high conductivity, 2) low pH and low conductivity, 3) high pH and high conductivity and 4) high pH and low conductivity. The

low pH buffer had a pH value 0.5 closer to the pI of the antibodies. The low conductivity buffers were prepared by diluting the high conductivity buffers 1:10.

A glycine buffer adjusted to pH=9.3 was tested in 2 versions; undiluted for high concentration of glycine and diluted 1:2 for low concentration of glycine.

Undiluted particle dilution buffer, pH= 9.3, was used as the high concentration tween 20 buffer, and the same buffer diluted 1:2 was used as the low tween concentration buffer.

The dialysis buffer I was a borate buffer with adjusted pH to 9.2. No variations of this buffer were applied in the experiment.

The dialysis buffers II were TRIS buffers. Two similar buffers with a difference in pH value of 0.3 were used undiluted or diluted 1:10, to vary ionic strength. In total 4 versions of the Dialysis buffer II were applied; 1) low pH and high conductivity, 2) low pH and low conductivity, 3) high pH and high conductivity, and 4) high pH and low conductivity.

Statistical model (Paper III)

The process of coating latex particles with antibodies includes a high number of parameters which potentially may affect the performance of the assay if varied. Eight parameters were chosen to be variables in this experiment. High and low values were tested for of each of the following parameters; pH of conjugate buffer, conductivity of conjugate buffer, coating grade, concentration of ovalbumin, concentration of glycine, concentration of tween 20, pH of dialysis buffer II and conductivity of dialysis buffer II. The experiment was designed according to the Taguchi L12 screening method as described in the textbook "Understanding Industrial Designed Experiment" by Schmidt and Launsby 4th edition (Schmidt and Launsby 1994). The study design with its orthogonal array was created in the Microsoft Excel add-in, DOE KISS Pro XL 2010 (R) (v2.40.100 SigmaZone.com/Air Academy Associates LLC, Colorado Springs, US).

The analysis of the data was also done in DOE KISS pro XL. Marginal means plots were created to visualise the magnitude of each effect.

Preparation procedures (Paper I, II, III, IV, V)

The antibodies were dialysed into the conjugate buffer on the Labscale TFF system (30 kDa Pellicon filter) prior to adding the ovalbumin solution. The protein solution was mixed with the particles in suspension for coating. To make sure there was no initial aggregation of the particles, ultrasonication was given prior coating. The coated particles were placed in 37ºC for two days and then added first glycine buffer and then particle dilution buffer 4-6 hours later. The solution was mixed well after each step. Then the particle solution was dialysed in two steps on the Labscale TFF system with a 500 kDa Pellicon XL filter. The particle solution was dialysed against dialysis buffer I, applying a volume equivalent to 10 times the particle solution volume and then against dialysis buffer II applying a volume equivalent to 10 times the particle solution volume. The parameters were varied by carefully following the combination matrix created in DOE KISS pro XL.

Testing of all batches for sensitivity and antigen excess (paper III)

For all batches prepared, a six-point calibration curve was established on Mindray BS-400. The output in the statistical model was defined as sensitivity (std2 - std1) and security zone. Security zone was tested by measuring a high sample (spiked to approximately 60 mg/L). The higher value the instruments returned the better. The security zone was considered as acceptable if the instrument returned a value higher than the highest calibrator value. The data was analysed according to the Taguchi L12 design to find any significant factors affecting the output. Within-run precision were also tested by measuring a low sample (approx. 0.2 mg/L) in 20 replicates to see if CV is lower with high sensitivity than with low sensitivity.

Patient selection for reference interval (Paper IV)

All subjects living in Uppsala aged 70 years were eligible for the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study (Lind et al. 2005). The subjects were chosen from the register of community living and was invited in random order (april 2001 - june 2005). 1016 of the 2025 invited participated. 821 of these subjects were reinvestigated at age 75, where serum samples were collected and stored in multiple tubes at -80°C. The calprotectin levels were analysed in 2013 while the other markers were analysed in 2010. Only subjects with CRP < 20 mg/L were included in the study to exclude subjects with inflammations. The calprotectin levels were compared to 25 frequent analysed biomarkers. For the full list of biomarkers tested, see paper IV.

The reference interval was calculated from a bootstrap estimation and met the recommendation from the International Federation of Clinical Chemistry applying RefVal(R) 4.0, Department of Clinical Chemistry, Rikshospitalet, Oslo, Norway. Spearman rank correlation was calculated to investigate associations between calprotectin and other markers studied. Mann-Whitney U test was applied to compare calprotectin values in patient groups with a disease with patients without the disease. The disease groups investigated were diabetes, stroke, cardiac failure and myocardial infarction. Both, Spearman Rank correlation and Mann-Whitney U test were calculated in Statistica(R), StatSoft, Tulsa, OK, USA.

Patient selection and determination of likelihood of infection (Paper V)

All patients admitted to the general intensive care unit (ICU) and expected to stay overnight, were assessed for eligibility. Patients with acute or chronic kidney disease (eGFR < 60 mL/min/1.73 m^2) and patients with ongoing antibiotic therapy upon ICU admission was excluded. The first sample of each patient was collected as soon as possible after arrival at ICU. The next samples were collected daily thereafter.

The patients were categorised into two groups: non-infected group and infected group (probable, possible or confirmed infection). The likelihood of infection was determined according to the In-criteria (Calandra, Cohen, and International Sepsis Forum Definition of Infection in the ICU Consensus Conference 2005). A patient, meeting three or more criteria for systemic inflammatory response syndrome (SIRS) (Bone et al. 1992/6) qualified to the infected group.

The EDTA plasma was prepared by centrifuging the EDTA blood at 2000 rpm for 10 minutes. The supernatants were transferred into new vials and stored at -80ºC until analysis. The samples were analysed with the calprotectin PETIA on Mindray BS-380 by qualified personnel blinded to the clinical patient data. Procalcitonin was analysed with an enzyme-linked immunosorbent assay on Cobas(R) EE, Roche Diagnostics, Mannheim, Germany. CRP was analysed by turbidimetry on Architect(R) Ci8200, Abbott Laboratories, Abbott Park, IL. Creatinine was analysed by alkaline picrate colorimetry on LX/DxC(R) 800, Beckman-Coulter, CA.

Statistical analysis (Paper V)

STATA® version 12, Stata Corporation, College Station, TX was used for the data analysis. Continuous variables were expressed as median and interquartile range (IQR) and compared using Mann-Whitney U test. Chi-square test or the Fisher's exact test were applied to assess the categorical variables summarised as n (%).

The changes in biomarker levels over time were tested by repeated measures analysis of variance (RM-ANOVA). The number of days from admission to ICU was set as the repeated measures variable (independent).

An interaction variable between group and time was introduced to the RM-ANOVA model for comparison of change over time between groups.

To assess the independent association between calprotectin levels and infection, a multivariable logistic regression analysis was applied. Only statistically significant variables from the univariable analyses ($p < 0.10$) were included in the multivariable model.

Predictive and diagnostic values were assessed by calculating the area under the receiver-operating characteristics curve (AUROC)

The nonparametric approach developed by De Long et al (DeLong, DeLong, and Clarke-Pearson 1988) was applied to test the equality of ROC areas.

Optimal cut-off levels were determined using Youden's index calculations together with the ROC curve analysis (Bewick, Cheek, and Ball 2004).

A two-sided P-value below 0.05 was considered statistically significant.

Results and Discussion

Extraction and purification of calprotectin antigen from human granulocytes (Paper I)

After haemolysis of erythrocytes and removal of the supernatant after gentle centrifugation, the resuspended granulocyte preparations varied from nearly white to red in colour. Because the extract was applied on the ion exchange chromatography column the colour should not affect the purification of calprotectin. The colour is assumed to be haemoglobin from the lysed erythrocytes, mixed within the precipitated granulocytes. Removal of the supernatant did not remove this haemoglobin.

The extract measured between 1000 mg/L and 1800 mg/L of calprotectin on the PETIA prior to purification. Variation in biological material will cause variation in calprotectin concentrations.

Because any contamination may induce an immune response that will reduce the specificity of the polyclonal antibodies used in the final assay, purity of the antigen solution is very important. In order to investigate the purity of the antigen we made a SDS PAGE analysis and a size exclusion chromatography analysis.

The purified antigen was dialysed into pure saline and concentrated before analysed on a SDS PAGE with acryl-amid gel density of 12% and 20%. Two bands were clearly visible at approximately 10 kDa and 14 kDa. This correspond to the known sizes of the two subunits S100A8 (10.8 kDa) and S100A9 (13.2 kDa) of calprotectin. The gel was analysed by UN-Scan-it, a software analysing the pixel density in a picture of the gel. To ensure all details was visible on the image, the gel was placed on a backlit surface prior to photographing. The two expected bands from S100A8 and S100A9, consisted of more than 96% of all the pixels, showed the pixel analysis of the lanes. A purity of more than 95% was considered as sufficient for this purpose. The software did not flag for saturated pixel density, which leads to the assumption that the pixel density profiles were identified correctly by the software.

As another test of purity, the purified calprotectin solution was applied to a size exclusion chromatography column. The fractions were measured with both the spectrophotometer and the turbidimetric method. When comparing the two profiles in Excel they were found to be similar in shape, meaning the two methods recognise the same molecule. However, both methods identified two peaks in the calprotectin chromatogram, which were the fractions at 9.5

ml and 10.5 ml. The two peaks may be explained by different configuration of the molecule. The subunits, S100A8 and S100A9 forms a heterodimer which will build tetramers if calcium is present (Vogl et al. 1999). The two peaks in the profiles can therefore be explained by content of both tetramers and dimers in our calprotectin solution. The UV profile of the fractions also has a peak at 15 ml. This peak may be monomers or a different molecule not detectable in the turbidimetric system. Because the peak is small compared to the other peaks the contribution from this molecule is expected to be neglectable.

We could assume a pure calprotectin solution from the gel analysis and size exclusion chromatography. With this assumption, the calprotectin concentrations were assigned by total protein concentration with Biurets method. The amount of calprotectin purified was calculated from these concentrations and was calculated to be equivalent to 1.5 mg to 2.6 mg per donor giving a yield of 46% to 77% from the extracts.

The number of neutrophil granulocytes in a 450 ml blood bags may vary by donors due to several factors. However, in literature the reference range in 1 litre of blood is given as 1.6-8.3 x 10^9 ("Nasjonal Brukerhåndbok I Medisinsk Biokjemi"). Using these values estimates a range from 0.72-3.74 x 10^9 neutrophil granulocytes in each of these bags (per donor). Hence, the average amount of calprotectin per neutrophil granulocyte is in the range 0.6 piko gram to 3.1 pikogram.

Freeze and thaw stability of the purified antigen was tested. After the first freeze and thaw cycle the recovery from the start concentration was 88.5%, after the second cycle the recovery was 89.3%, after the third cycle it was 87.1% and 79.9% for the fourth cycle. Only one preparation was tested. The drop in the concentration during the first freeze and thaw cycle, can be explained by disassociation of the molecule structure or aggregation of the molecules creating new molecules which the antibody has lower specificity to. Another explanation can be that some of the antigen was removed from the solution because it was adhering to the walls of the containers.

In this project we assigned the concentrations of the purified calprotectin by the Biurets Method, assuming the calprotectin solution is not containing other proteins. However, in a market with several calprotectin products, there is a need for a standardised calibrator material. Most calprotectin products in the marked are for faecal measurement to diagnose, monitor and determine treatment of inflammatory bowel disease. Results from Equalis AB, a Swedish provider of external quality assessment for clinical laboratories investigations, reports up to 50% differences in values between faecal calprotectin methods. The clinicians use the same cut-off values for F-calprotectin regardless of the calibration of the specific method used. This may cause confusion among the clinicians in the field, leading to erroneous decisions and treatments, which emphasises the need for a standardisation.

Further investigation of the purified protein solution may be a dry mass determination. The Procedure is described in the Blirup-Jensen publication from 2001 (Blirup-Jensen 2001). The observed amount can be used for value transfer to a calibrator via for instance radial immunodiffusion.

Validation of performance characteristics (Paper II)

The validation studies were designed according to CLSI protocols with some modifications. For instance, the LoQ was identified by calculation of total error from between run CV and bias from the theoretical value based on assigned value to the undiluted sample, which was used to prepare the LoQ material. According to the protocol the undiluted material should have been assigned value with a standardized method. Because there was no standardized method for calprotectin measurements in serum/plasma available we assigned a value to the sample with our own method. The results are summarised in **table 1**.

Table 1. Summary of performance studies. The table summarises the results from the validation studies.

Parameter	**Results on BS-380**
LoQ	0.35 mg/L
Security zone	> 54 mg/L
Linearity	0.32 - 24.7 mg/L
Imprecision	< 1.5 %
Interference	Haemoglobin (5 g/L): No Intralipid (10 g/L): No Bilirubin (400 mg/L): No
Method comparison	Passing Bablok fit (without factor): y = -0.04 + 0.32x Passing Bablok fit (factor = 3): y = -0.17 + 0.96x
Calibration curve stability	> 6 weeks

Limit of quantitation

The 0.35 mg/L sample was the lowest sample with a total error of 15.7 %. Because the criterion was a total error less than 20%, a sample with lower concentration would probably pass the criterion and a lower LoQ could be stated. In paper V we identified the reference interval for serum samples to be from 0.5 mg/L to 2.5 mg/L (Nilsen et al. 2014), leaves a LoQ of 0.35 mg/L sufficiently low for clinical purposes, especially as the main use of calprotectin assays is to detect elevated levels.

Security zone

As calprotectin is suggested as an inflammation marker and the fact that it is abundant in the neutrophil cytosol, the assay was designed to have a security zone covering concentrations up to 20 times the normal values. There were no observed antigen access measuring the dilution series prepared from a 54 mg/L sample. The assay will therefore not give any false low results for samples up to 54 mg/L.

Linearity

The linearity was tested in the range from 0.3 mg/L to 24.7 mg/L by preparing a dilution series from the high concentration sample diluted with the low concentration sample. Expected values for each of the sample in the series was calculated. The observed values deviated less than 5% in the range tested. Because we wanted to prepare the linearity series from natural samples, and that samples above 20 mg/L is hard to find, the linearity range observed was 0.3 - 24.7 mg/L. However, there is no indication of non-linearity and the assay is expected to be linear in the entire calibration range, 0.3 - 30 mg/L. It is important that an analytical method is linear in a clinical aspect, because a doubling of the true amount of an analyte is expected among clinicians to be reflected in the measured value (NCCLS 2003).

Imprecision

The 10 days within laboratory precision study showed CVs lower than 1.5% for all 5 samples ranging from 1.17 mg/L up to 22.14 mg/L. The samples were measured in duplicates twice a day on 10 subsequent working days. The method was re-calibrated on day 6. These results represent a repeatability of the method, which should not be confused with reproducibility. Reproducibility is an outcome from a very similar study but must include several laboratories and operators (NCCLS 2004).

Interference

We tested if the method interferes with haemoglobin, intralipid or bilirubin. Neither of the three substances interfered significantly at tested levels, which were 5 g/L of haemoglobin, 10 g/L of intralipid and 400 mg/L of bilirubin. The maximum allowed difference between the test and control samples were decided to be 10% from the value of the control samples. This difference is reflecting a clinical irrelevant variation, because calprotectin as an inflammation marker will increase more than 10 % in a patient with inflammation (paper IV).

Method Comparison

The Passing Bablok linear regression fit from the method comparison data between the turbidimetric immunoassay and the Bühlmann MRP8/MRP14 ELISA was:

PETIA = -0.04 + 0.32 x ELISA. However, with a factor 3 applied on the results from the PETIA method, the regression fit was: PETIA = -0.17 + 0.96 x ELISA. This implies that these two methods are basically commutable but have different calibrations.

Calibration curve stability

We measured the same samples every week for 6 weeks on the same calibrations curve. The CVs for each sample based on the observed data were less than 2%. Concentrations of the samples were 1.17 mg/L, 1.22 mg/L, 6.82 mg/L, 8.78 mg/L and 22.65 mg/L at baseline. The data implies a calibration curve stability of at least 6 weeks. In a routine lab it is convenient for the workflow to implement methods which do not require recalibration too often.

Lot variation

For manufacturer it is important to minimise the variation between batches to supply the customers with a stable method to ensure reliable results from the laboratory to clinicians. The two batches of antibodies were coated onto polystyrene particles in two separate preparations, Lot1 and Lot2. A calibration curve was established with these two lots of immunoparticles. The calibrators and assay buffer were the same. From the results of a set of measured samples on both lots, a Passing Bablok linear regression fit was calculated as a measure on the variation between the two preparations. The regression fit was:
Lot2 = -0.09 + 1.0 x Lot1. This equation means that the same results will be reported to the clinicians independent of the lot used.

The results from the method validation studies identifies this PETIA as a robust and qualified method for measurement of calprotectin in serum/plasma.

Influence of 8 parameters when coating calprotectin Antibodies to latex particles (Paper III)

The eight factors included in the analysis were conductivity of conjugate buffer, pH of conjugate buffer, coating grade, ovalbumin concentration, glycine concentration, tween concentration, conductivity of dialysis buffer II and pH of dialysis buffer II for both sensitivity and antigen excess as output.

The suggested regression model estimated that 4 of the 8 parameters had strong significant influence on the sensitivity, in addition to the constant, which was not important for this purpose. The four significant parameters were conjugate buffer (p= 0.000), pH conjugate buffer (p= 0.0037), coating grade (p=0.0001) and conductivity of dialysis buffer II (p=0.0086). These factors influenced the sensitivity the most for the finalised immunoparticles. However, some of the other factors, such as pH of dialysis buffer II also had an influence and should be considered in the optimisation of sensitivity.

In the regression analysis for antigen excess as the output, there were 5 parameters which were strongly statistically significant in addition to the constant. The five parameters were pH of conjugate buffer (p=0.0000), conductivity of the conjugate buffer (p=0.0001), coating grade (p=0.0062), amount of ovalbumin (p=0.0113) and conductivity of the dialysis buffer II (p=0.0385).

A visual presentation of the values and dimension of the parameters are shown in the marginal means plot, **figure 4** and **figure 5**. The plots depict the effects in size. It also gives information of which value of the parameters giving the required response.

The low concentration sample was measured in 20 replicates on each batch. The data were sorted into two groups based on the sensitivity data; low sensitivity (standard2-standard1 < 350 absorbance units) and high sensitivity (standard2-standard1 > 550 absorbance units). For the low sensitivity group, the mean CV of the low concentration samples was 7.8% with standard deviation (std) = 1.91, and for the high sensitivity group, the mean CV was 2.9% with std = 0.31. As expected the low concentration sample had lower CV measured in 20 replicates with the group of batches with high sensitivity than the group of batches with lower sensitivity.

High conductivity in the conjugate buffer was the factor with most impact on sensitivity and antigen excess. However, high conductivity in conjugate buffer gave good sensitivity, but also worse antigen excess characteristics. This seems always to be the case. Designing a turbidimetric immunoassay with

wide security zone seems to lower the sensitivity. In opposite situation where the high sensitivity the security zone range will be narrower.

According to the literature less ionic concentration in solution causes stronger hydrophobic properties of the antibodies. This will cause more aggregation between antibody-antibody (unintended) and between antibody-latex (coating) (Dávalos-Pantoja et al. 2000).

From this statement we can argue that more antibodies will be coated to the latex particle under low ionic strength, while the opposite will happen under high ionic strength. We would then expect that the particles coated under low ionic strength condition could have better antigen excess characteristics, as more antibodies are available in the reaction forming the immunocomplexes. This agrees with what we observed. The particles coated under low ionic strength conditions gave an assay with good antigen excess characteristics. The high concentration sample (approximately 55 mg/L) measured above 30 mg/L (mean=33.1 mg/L, std= 5.5 g/L) on all particles coated under these conditions. On the opposite side, the particles coated under high ionic strength conditions was expected to coat less antibodies onto the particles, measured below 10 mg/L (mean=9.8 mg/L, std=3.7 mg/L) for the high concentration sample, giving false low results.

The sensitivity of the assay was also affected by the ionic strength of the conjugate buffer. The highest sensitivity was observed for the particles prepared under high ionic strength in combination with a high coating grade of antibodies. These particles had an average difference between standard 1 and standard 2 of 580 absorbance units (std=42), while the other particles had an average difference between standard 1 and 2 of 241 absorbance units (std=41).

pH values of the conjugate buffer and dialysis buffer II affected the sensitivity and antigen excess characteristics too. These effects can be explained by the fact that in pH values similar to pI, where the average charge of the antibody molecules are neutral, there are low repulsions between the antibody molecules, which causes aggregation due to hydrophobic properties (Van Oss 1995). In polyclonal avian antibodies, which constitute of a range of slightly different molecules, the pI ranges from 5.7 to 7.6 (Dávalos-Pantoja et al. 2000), (Gee et al. 2003).

If these effects can be utilised to prepare more sensitive immunoparticles for low antigen concentration quantifications, the optimal combination of pH and conductivity of conjugate buffer, coating grade and conductivity of dialysis buffer II, should be explored and established. The effects should also be compared to the effect of using bigger particles. Once the optimal for sensitivity and antigen excess is established the challenge to obtain a colloidal stability will be a challenge, which is crucial in order to avoid unintended aggregation. Unintended aggregation is always a challenge and must be avoided (Molina-Bolívar and Galisteo- González 2005).

Ovalbumin concentration and conductivity of dialysis buffer II were both significant factors impacting the sensitivity and antigen excess. They are not further discussed in this work but should be taken into consideration in an optimisation process.

The purpose of this study was to investigate which of the parameters influenced the output based on a screening method, and not to find final optimal parameter values. The results obtained will be valuable for future work optimizing immunoassays with avian antibodies. It is also worth keeping in mind that an optimal PETIA is a combination of optimised reagents and an optimised application in a specific instrument to achieve the required performance. Because the instrument settings may influence sensitivity, antigen excess and imprecision among other features, this must be established on each instrument the assay will be applied on.

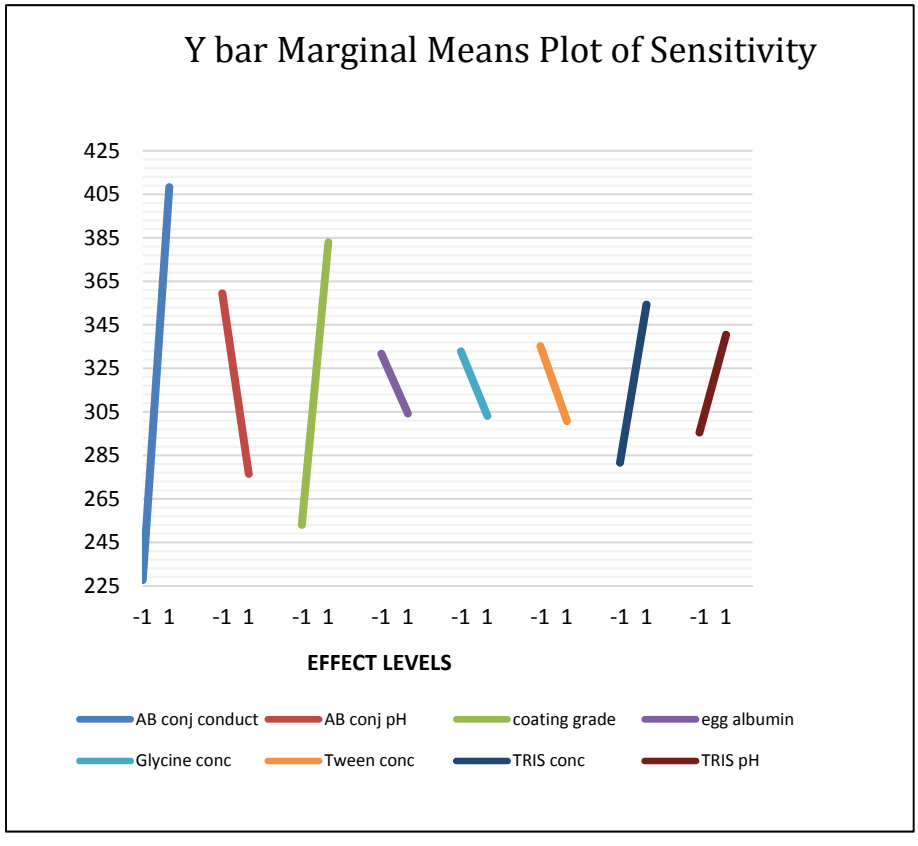

Figure 4. Effects. The marginal means plot illustrates the size and the level of the parameters that influence the sensitivity.

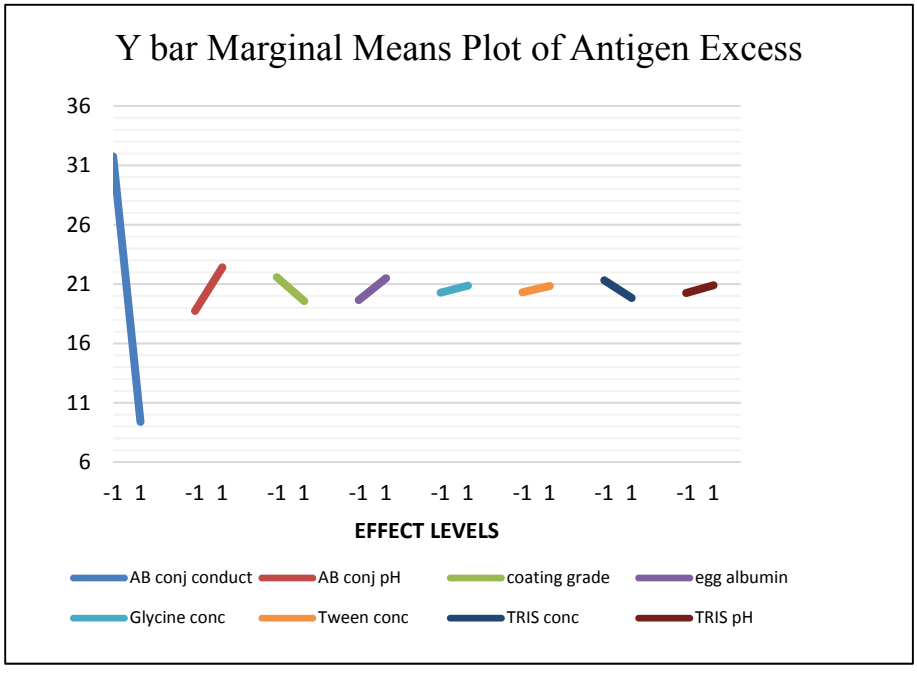

Figure 5. Effects. The marginal means plot for the antigen excess effect shows that a high conductivity of the conjugate buffer was the most influencing parameter when it comes to giving the highest security zone.

Reference interval in elderly healthy males and females (Paper IV)

731 serum samples were collected, where 713 were qualified meeting a criterion of CRP levels < 20 mg/L. No significant difference in calprotectin levels were observed between patients with and without cardiac failure, diabetes, stroke or myocardial infarction applying Wilcoxon U test.

The reference interval for female subjects was 0.3 - 2.6 mg/L (n=366) and for male subjects 0.3 - 2.6 mg/L (n = 347). There was no significant difference in the calculated reference intervals between females and males ($p = 0.61$, Wilcoxon U test). The reference interval for all subjects was 0.3 - 2.6 mg/L (n = 713).

The correlation (Spearman Rank) was significant (defined as $p < 0.001$) between calprotectin and alkaline phosphatase ($R_s = 0.19$; $p = 0.000001$), CRP ($R_s = 0.29$; $p = 0.000001$), direct HDL cholesterol ($R_s = -0.14$; $p = 0.0018$) and apolipoprotein A1 ($R_s = -0.13$; $p = 0.00089$). A weak positive Spearman Rank correlation was observed between calprotectin and body mass index ($R_s = 0.09$; $p = 0.02$).

To establish a reference interval is important to interpret the results correctly to recognise the patients who require further treatment. A reference interval should be representative for the patient groups for whom they are intended for. Patients seeking health care are mostly elderly persons. However, reference intervals often are based on subjects from a younger part of the population, and rarely subjects older than 65 years are included. Therefore, we think it was useful to establish a reference interval for calprotectin based on the samples from +70 years subject in the population. To exclude subjects with CRP levels higher than 20 mg/L in the reference interval calculations, we intended to exclude subjects with subclinical infections affecting the calprotectin values. Despite exclusion of these subjects there were a strong association between calprotectin and CRP, which was expected because both markers increase in response to proinflammatory cytokines (Mørk et al. 2003). The reference interval calculated in this project was based on serum samples. Because there is a shift in values between serum and plasmas (results not shown here), it is recommended to establish a reference interval for the different plasma types.

Calprotectin as an early biomarker of bacterial infections in critically ill patients: An exploratory cohort assessment. (Paper V)

110 patients were qualified to enter the study, where 52 (47.3%) patients did not have infection (non-infected group) and 58 (52.7%) patients had initiated antibiotic therapy within 2.6 (1.0, 4.0) days because of suspected or confirmed infection (infected group). 8 of the 56 blood cultures taken was positive. 24 (41%) patients developed severe sepsis and 25 (43%) patients developed septic shock in the ICU.

3755 calprotectin samples were measured from admission to discharge or death. For the infected group the calprotectin levels were higher than in the non-infected group for samples taken upon admission (1.5 mg/L (0.69, 3.3) vs. 0.78 mg/L (0.38, 1.6), $p = 0.042$). The values are presented as median values with interquartile range in brackets. The calprotectin levels in the infected group were significantly higher than in the non-infected group on the day before initiation of antibiotic therapy (2.2 mg/L (0.83, 3.8) vs. 1.1 mg/L (0.67, 2.0), $p < 0.001$), and on the day of initiation of antibiotic therapy the difference (3.8 mg/L (1.4, 6.3) vs. 1.3 mg/L (0.77, 2.3)). The values also remained higher in the infected group during the first week in ICU ($p < 0.001$).

CRP levels were significantly higher in the infected group than in the non-infected group upon admission (1.5 mg/L (0.69, 3.3) vs. 0.78 mg/L (0.38, 1.6), $p < 0.007$), the day before initiation of antibiotic therapy (135 mg/L (8, 122)

vs. 26 mg/L (8, 43), p = 0.007) and on the day of initiation of antibiotic therapy (202 mg/L (128, 286) vs. 129 mg/L (76, 168), p = 0.003).

Procalcitonin levels were significantly higher in the infected group than in the non-infected group on the day of admission to the ICU (0.9 µg/L (0.2, 3.4) vs. 0.4 µg/L (0.2, 1.8), p = 0.02). There were no significant difference between the infected group and the non-infected group the day before initiation of antibiotic therapy (0.8 µg/L (0.1, 1.3) vs. 0.4 µg/L (0.2, 1.6), p = 0.99)) or on the day of initiation of antibiotic therapy (1.0 µg/L (0.4, 8.6), 0.6 µg/L (0.2, 1.6), p = 0.09).

There were no significant differences in white blood cell count between the non-infected group or the infected group upon admission (p = 0.89), values on the day before initiation of antibiotic therapy (p = 0.35) or the day of initiation of antibiotic therapy (p = 0.43).

The calprotectin values increased with increase in severity of the sepsis (p < 0.001), and in the multivariable logistic regression analysis calprotectin was a independently associated with infection (odds ratio = 2.0, 95% CI 1.32 - 3.14).

The areas under the receiver operating characteristic curves (AUROC) based on the measurements on the day before initiation of antibiotic therapy was 0.78 (0.68, 0.89) for calprotectin (cut-off value: 1.8 mg/L), 0.71 (0.68, 0.89) for CRP (cut-off value 130 mg/L), 0.50 (0.34, 0.66) for procalcitonin (cut-off value 0.78 µg/L) and 0.44 x 10^9/L (0.33, 0.56) for white blood cell count (cut-off value 8.5 x 10^9/L). These results leave calprotectin as the superior marker to predict a bacterial infection, 24 hours prior to confirmation of an infection.

At the day of initiation of antibiotic therapy, the AUROC were for calprotectin 0.76 (0.65-0.86) with cut-off value 3.4 mg/L, for CRP 0.69 (0.60-0.81) with cut-off value 133 mg/L, for procalcitonin 0.63 (0.49-0.77) with cut-off value 0.66 µg/L, and for white blood cell count 0.54 (0.43-0.65) with cut-off value 10.7 x 10^9/L. Also, here calprotectin was superior to the other marker for bacterial infection.

The data were collected prospectively, and the treating clinicians, laboratory staff and infectious disease specialist were blinded to the calprotectin results, which enabled an unbiased assessment of calprotectin as a predictor of bacterial infections. This was considered as a strength to this study. One of the limitations of the study was the exclusion of patients with renal dysfunction on admission to ICU. Hence, an extrapolation of the predictive value for calprotectin cannot be applied to patients with acute renal injury or chronic renal dysfunction at admission. In addition, we used a consensus classification to define infections, which entails a risk of misclassifications by including patients with lower likelihood of infections.

Calprotectin concentration in patients with neutropenia or leukopenia:

The calprotectin concentration and neutrophil counts were measured in 52 patient samples for comparison of calprotectin levels in patients with neutropenia/leukopenia with patients with normal and increased numbers of neutrophils. The results were sorted into three groups dependent of the observed neutrophil counts. The groups were defined as; low neutrophil counts (below normal range), normal neutrophil counts (1.3-5.4 x 10^9) and high neutrophil counts (above normal range). For each group the mean, 95% confidence interval and standard deviations were calculated for evaluation, see **table 2** and **figure 6**. The 95% confidence interval from all three groups were overlapping.

Table 2. Calprotectin vs neutrophil counts. Calculated means, 95% confidence intervals and standard deviations in the three groups of different levels of neutrophil counts.

	N	Mean	95% CI	SD
High neutrophils number	9	5.55	2.514 to 8.591	10.46
Low neutrophils number	14	0.85	-1.588 to 3.285	1.25
Normal neutrophils number	30	1.84	0.173 to 3.501	2.16

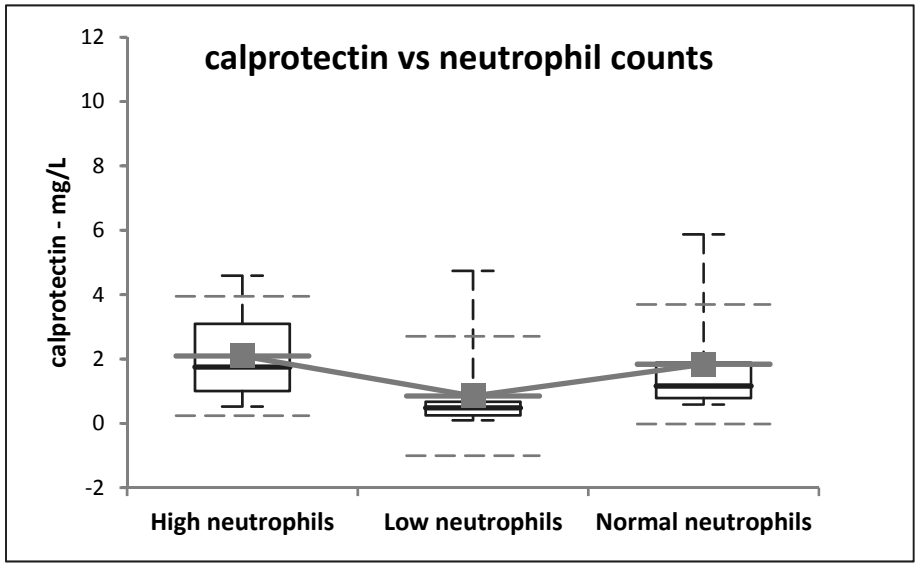

Figure 6. **Calprotectin concentrations in patients with normal, low and high numbers of neutrophils**. Calprotectin concentrations and the numbers of neutrophils were measured in 52 patients. The results were sorted into three groups dependent of neutrophil counts; within the normal range (n=30), below the normal range (n=14) and above normal range (n=9). The results for each group were plotted as a box plot with 95% confidence interval, ±1 STD.

Conclusions

Paper I:

The method for extraction and purification of calprotectin from human granulocytes manage to purify 1.5 to 2.5 mg per buffy coat. This yield is sufficient to use in a production-line, supplying antigens for affinity purification of antibodies and for calibrator and control production.

Paper II:

The designed assay meets the defined criteria for limit of quantitation, antigen excess, linearity, precision, interference and calibration curve stability. In addition, the design is robust with low lot to lot variation for the antibodies. The method is commutable with another commercially available method for quantitative measurement of serum/plasma calprotectin. The assay is therefore ready to use for clinical research purposes. For commercial use/CE labelling a more extended testing program must be performed.

Paper III:

The four most influencing factors on sensitivity and antigen excess for this PETIA was conductivity and pH of conjugate buffer, coating grade and conductivity of dialysis buffer II.

Paper IV:

The reference interval in serum for healthy elderly subject, both females and males were 0.3 - 2.6 mg/L. There was no difference between females and males when calculated separately.

Paper V:

Calprotectin was an early and specific marker for bacterial infections. Our findings suggest that calprotectin may be more robust than conventional markers such as CRP, PCT and WBC.

General Discussion and Future Work

Methods for measuring calprotectin in serum or plasma has been available for a while. As far as we know there are no PETIAs, but several ELISAs available. A PETIA can be set up on the main clinical chemistry analysers and will therefore be available 24 hours a day. The actual time from blood sampling to provided answer is of course dependent of the clinic's routines, but these analysers have random access capacity providing rapid test results. In theory, answers can be provided within 30 minutes from blood sampling if serum or plasma samples are centrifuged and analysed directly from withdrawal.

The calprotectin PETIA developed in this project are technically able to measure calprotectin levels in all natural, blood samples, in the range from 0.3 to 56 mg/L, which should cover most relevant levels for clinical use.

Prior a potential launch to the market an assay must be developed according to the IVD directive to meet the CE mark criteria for the European market. For other markets, like US have similar requirements, but may differ in some ways. In common for all markets, thoroughly testing and validations are required in order to be qualified for registration.

The clinical usefulness of calprotectin measurements in specific patient groups remain to be evaluated. However, a strong correlation between calprotectin levels and infection is seen in paper V. If calprotectin proves to be clinically relevant for diagnosis of sepsis, it is valuable to get access to the results as soon as possible after admission to ICU, and therefore we believe a calprotectin PETIA is convenient.

There is a shift in calprotectin values between plasma and serum. This forces the establishment of a reference interval for all matrices in use for clinical evaluations. The reason for the shift is not well known, but a hypothesis is that coagulant agents activate the neutrophils in the sample enabling excretion of calprotectin from the granulocytes into the serum during the coagulation process prior to centrifugation. In the literature there are different statements of how the calprotectin molecule is configured from the subunits S100A8 and S100A9. Better understanding of the configuration of this molecule is demanded to fully understand its function.

Norsk Sammenfatning

Utviklingen av antibiotikaresistente bakterier er en økende trussel for menneskers helse. Små infeksjoner som ikke kan behandles kan utvikle seg til blodforgiftning og bli fatale. En foreslått strategi for å bremse utviklingen av antibiotikaresistente bakterier er å redusere bruken av antibiotika. En del av denne strategien vil være å utvikle metoder som kan påvise infeksjoner raskt slik at behandlingen kan begynne tidlig før infeksjonen har utviklet seg. Ved å starte behandlingen tidlig vil mindre antibiotika være nødvendig. I dag blir Procalcitonin og CRP brukt for å påvise infeksjoner. Det tar gjerne ett par dager fra infeksjonen starter til CRP verdiene er høye nok til at pasienten får behandling. Fordi calprotectin-konsentrasjonen i blodet øker raskere enn procalcitonin og CRP konsentrasjonen ved en infeksjon og fordi calprotectin kan skille bedre mellom bakterielle og virale infeksjoner, er det interessant å kunne måle calprotectin ved mistanke om infeksjon. Vi utviklet derfor en metode for å måle calprotectin konsentrasjonen i blodet, som kan anvendes med eksisterende rutine-instrument på sykehuslaboratorier i hele verden. Testen tar 10 minutter fra start til resultatet foreligger. I prosjektet måtte vi først utvikle metoder for å rense antigener og antistoffer, som er avgjørende råvarer i produksjonen av metoden. Deretter utviklet vi selve metoden og testet den klinisk. Resultatene fra den kliniske testingen viser at calprotectin er en lovende markør for tidlig påvisning av alvorlige infeksjoner og blodforgiftning.

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