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NANOPARTICLE-ASSISTED IMMUNOASSAYS FOR POINT-OF-CARE TESTING

– With Specific Interest in Minimally Interference-Prone Assays for Cardiac Troponin I

Heidi Hyytiä

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The way to get started is to quit talking and begin doing.

- Walt Disney -

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications, referred to in the text by their Roman numerals (**I–IV**):

I Heidi Hyytiä, Noora Ristiniemi, Päivi Laitinen, Timo Lövgren and Kim Pettersson (2014). Extension of dynamic range of sensitive nanoparticle-based immunoassays. *Anal Biochem* **446**:82–86.

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ABBREVIATIONS

ACS	acute coronary syndrome
BGG	bovine gamma globulin
BSA	bovine serum albumin
CA	cancer antigen
CEA	carcinoembryonic antigen
cFab	chimeric fragment antigen binding
CDR	complementarity determining region
CI	confidence interval
C_H	constant heavy domain of antibody
CK-MB	creatine kinase muscle-brain fraction
C_L	constant light domain of antibody
CRP	C-reactive protein
cTn	cardiac troponin
cTnI	cardiac troponin I
cTnT	cardiac troponin T
CV	coefficient of variation
ED	emergency department
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide
Fab	fragment antigen binding
Fc	fragment crystallizable
FDP	fibrin degradation product
FEU	fibrinogen equivalent units
FSH	follicle-stimulating hormone
HAAA	human anti-animal antibody
HACA	human anti-chimeric antibody
HAMA	human anti-mouse antibody
HBR	heterophilic blocking reagent
HBT	heterophilic blocking tube
hCG	human chorionic gonadotropin
IgG	immunoglobulin class G
IgM	immunoglobulin class M
IIR	immunoglobulin inhibiting reagent
IU	international unit
LoB	limit of blank
LoD	limit of detection
LoQ	limit of quantitation
Mab	monoclonal antibody
MES	2-(*N*-morpholino)ethanesulfonic acid
MI	myocardial infarction
mIgG	murine/mouse immunoglobulin G
NHS	N-hydroxysuccinimide
PEG	polyethylene glycol
POCT	point-of-care testing

RF	rheumatoid factor
scFv	single-chain variable fragment
skTnI	skeletal troponin I
skTnT	skeletal troponin T
sulfo-NHS	N-hydroxysulfosuccinimide
TAT	turn-around time
TnC	troponin C
TnI	troponin I
TnT	troponin T
TSH	thyroid-stimulating hormone
V_H	variable heavy domain of antibody
V_L	variable light domain of antibody
VTE	venous thromboembolism

ABSTRACT

Cardiac troponins (cTn) I and T are the current golden standard biochemical markers in the diagnosis and risk stratification of patients with suspected acute coronary syndrome. During the past few years, novel assays capable of detecting cTn-concentrations in >50% of apparently healthy individuals have become readily available. With the emerging of these high sensitivity cTn assays, reductions in the assay specificity have caused elevations in the measured cTn levels that do not correlate with the clinical picture of the patient. The increased assay sensitivity may reveal that various analytical interference mechanisms exist.

This doctoral thesis focused on developing nanoparticle-assisted immunometric assays that could possibly be applied to an automated point-of-care system. The main objective was to develop minimally interference-prone assays for cTnI by employing recombinant antibody fragments. Fast 5- and 15-minute assays for cTnI and D-dimer, a degradation product of fibrin, based on intrinsically fluorescent nanoparticles were introduced, thus highlighting the versatility of nanoparticles as universally applicable labels. The utilization of antibody fragments in different versions of the developed cTnI-assay enabled decreases in the used antibody amounts without sacrificing assay sensitivity. In addition, the utilization of recombinant antibody fragments was shown to significantly decrease the measured cTnI concentrations in an apparently healthy population, as well as in samples containing known amounts of potentially interfering factors: triglycerides, bilirubin, rheumatoid factors, or human anti-mouse antibodies. When determining the specificity of four commercially available antibodies for cTnI, two out of the four cross-reacted with skeletal troponin I, but caused cross-reactivity issues in patient samples only when paired together.

In conclusion, the results of this thesis emphasize the importance of careful antibody selection when developing cTnI assays. The results with different recombinant antibody fragments suggest that the utilization of antibody fragments should strongly be encouraged in the immunoassay field, especially with analytes such as cTnI that require highly sensitive assay approaches.

TIIVISTELMÄ

Sydänperäiset troponiinit (cTn, *engl*. cardiac troponin) I ja T ovat tällä hetkellä suositusten mukaiset biomerkkiaineet akuutin sepelvaltimotautikohtauksen diagnosoinnissa ja riskinarvioinnissa. Viime vuosina markkinoille onkin tullut erittäin herkkiä cTn-määrityksiä, joilla on mahdollista mitata verenkierrosta cTn-pitoisuuksia jopa yli 50 prosentilla terveistä henkilöistä. Näiden herkkien cTnI-määritysten käyttöönotto on kuitenkin johtanut määritysten spesifisyyden laskuun, jolloin potilaan kliininen kuva ei aina tue mitattua biomerkkiaineen määritysrajan ylittävää pitoisuutta. Spesifisyyden lasku saattaa johtua erilaisista analyyttisistä häiriötekijöistä.

Väitöskirjatyön tarkoituksena oli kehittää nanopartikkelileimoihin perustuvia immunometrisiä määrityksiä, joita voitaisiin käyttää automatisoidussa vieritestaussysteemissä. Tärkeimpänä tavoitteena oli kehittää rekombinanttisia vasta-ainefragmentteja hyväksi käyttäen cTnI-immunomääritys, joka ei olisi herkkä erilaisille analyyttisille häiriöille. Fluoresoivien nanopartikkelien yleiskäyttöisyys leimateknologiana todistettiin kehittämällä cTnI:lle ja fibriinin hajoamistuotteelle D-dimeerille nopeita määrityksiä, joiden valmistumisaika on 5 tai 15 minuuttia. Vasta-ainefragmenttien käytön avulla voitiin vähentää käytettyjen vasta-aineiden määriä cTnI-määrityksessä sen herkkyyttä alentamatta. Lisäksi rekombinanttisten vasta-ainefragmenttien käyttö alensi merkittävästi mitattuja cTnI-arvoja terveiltä ihmisiltä peräisin olevissa näytteissä, mutta myös näytteissä, joissa oli tunnettu määrä mahdollisia häiriötekijöitä: triglyseridejä, bilirubiinia, reumatekijöitä tai ihmisen anti-hiiri vasta-aineita. Kaupallisten cTnI vasta-aineiden spesifisyyttä tutkittaessa havaittiin, että kaksi neljästä tutkitusta vasta-aineesta sitoutui cTnI:n lisäksi myös luusto-lihasperäiseen troponiini I:hin, mutta ristireaktio aiheutti virheellisiä tuloksia vain silloin, kun näitä kahta vasta-ainetta käytettiin parina samassa määrityksessä.

Väitöskirjatyön perusteella huolellinen vasta-aineiden valinta on äärimmäisen tärkeää cTnI-määritysten kehityksessä, jotta voidaan taata määritysten cTnI-spesifisyys. Lisäksi tulokset rekombinanttivasta-ainefragmenteilla osoittavat, että niiden käyttöä tulisi vahvasti lisätä erilaisissa kaupallisissa immunomäärityksissä – etenkin sellaisilla analyyteillä, joilla pyritään kehittämään hyvin herkkiä määrityksiä.

1 INTRODUCTION

The history of immunoassays goes back several decades to the late 1950s, when radioimmunoassay for insulin was first introduced (Yalow and Berson, 1959). Since then, many advances and changes have been introduced, the most influential being the development of hybridoma technology in the 1970s (Köhler and Milstein, 1975). Hybridoma technology provides a continual source of monoclonal antibodies (Mabs) that have specificity to a certain epitope. This has ensured their predominant position as immunoassay reagents, as well as their utilization as a growing technology in cancer therapies (Ribatti, 2014). Not long after the introduction of hybridoma technology, in the early 1980s, the utilization of recombinant DNA techniques resulted in the production of recombinant antibodies nearly identical to the ones obtained from hybridomas (Boss *et al.*, 1984). During the last 20 years, automated plate-reading systems and the introduction of personal computers for data analysis have brought immunoassay technologies to everyday clinical use.

Immunoassay interferences have been acknowledged from the beginning: already in 1956, Berson *et al.* noticed that labeled insulin was bound unexpectedly in the sera of patients receiving insulin treatment. Since then, it has been noted that whenever immunoassays are used in clinical laboratories, they are vulnerable to different analytical and preanalytical interferences leading to either false positive or negative results (Bolstad *et al.*, 2013; Tate and Ward, 2004). One source of these interferences is the antibody parts originating in the host mammal, e.g., mouse (Kricka, 1999). Also, the current desire to develop increasingly sensitive assays for certain analytes, such as cardiac troponins (cTn), may render the assays increasingly susceptible to low-affinity interferences, causing decreasing assay specificities and difficulties in the diagnostic process (Zaidi and Cowell, 2010; Robier *et al.*, 2014). Some of these matrix-related interferences could be avoided by employing antibody fragments that can be optimized by eliminating the components most prone to different interferences (Hudson, 1998).

One of the trends in the immunoassay field during the past few years has been the introduction of fast and simple point-of-care testing (POCT), described as easy to use rapid assays that do not need to be performed by trained laboratory personnel in a central laboratory environment. The motivation behind this POCT trend has been the possibility to triage patients as early as possible along with the interest in the promotion of healthcare in the developing countries. In 2012, the global *in vitro* diagnostics market was worth $72 billion, of which the immunoassay market, excluding POCT, covered $18.2 billion. Although as a whole the immunoassay market is showing signs of maturing, POCT has been the fastest growing sector of *in vitro* diagnostics with an annual sales of $21.5 billion in 2012. (Huckle, 2013.)

In this thesis, highly fluorescent nanoparticles were used to develop immunoassays possibly applicable to POCT purposes. Special interest was focused

on cardiac troponin I (cTnI) assays, in which recombinant antibody fragments were used to develop rapid and minimally interference-prone assays. Also, the versatility of nanoparticles as universal labels for POCT applications as well as the specificity of commercial cTnI antibodies was studied. The following literature review will offer an overview of immunoassay interferences, and different approaches used to avoid and eliminate them. The focus will be on, though not restricted to, immunometric assays.

2 LITERATURE REVIEW

2.1 Cardiac troponin I

Acute coronary syndrome (ACS) that includes unstable angina and myocardial infarction (MI) is one of the leading causes of death worldwide. Approximately 15–20 million people present to the emergency department (ED) yearly with symptoms indicative of ACS (Mueller, 2014). The current recommendation for diagnosing MI includes detecting the rise and/or fall of the cardiac isoforms of troponins I (cTnI) or T (cTnT), along with an analysis of electrocardiographic abnormalities and patient symptoms (Thygesen *et al.*, 2012).

The troponin complex (TnI-TnT-TnC) functions as a regulator of muscle contraction and is found in the filaments of both cardiac and skeletal striated muscles (Farah and Reinach, 1995). TnI is a 21–24 kDa protein (Perry, 1999) existing as three genetic isoforms: cardiac-specific cTnI and two different subforms (fast and slow-twitch) of skeletal muscle specific troponin I (skTnI) (Hastings, 1997).

The prevailing view is that cTnI is only released from cardiac muscle after irreversible damage to the heart, although some suggestions of cTnI release due to reversible injury have also been made (Bergmann *et al.*, 2009; Hickman *et al.*, 2010). After cell membrane disruption, the free cytosolic pool of cTnI (2–6%) is released in the circulation within hours after the myocardial damage (Hickman *et al.*, 2010). This initial cTnI release is then followed by the breakage of the myofibrils causing the release of the complexed cTnI during the following 5–7 days (Wu and Christenson, 2013). In MI, cTnI has mainly been found as a part of binary (cTnI-TnC) or ternary complexes (cTnI-cTnT-cTnC) and only in small amounts as free cTnI (Katrukha *et al.*, 1997; Giuliani *et al.*, 1999; Bates *et al.*, 2010).

2.1.1 Immunoassays for cardiac troponin I

The first report of cTnI as a marker for MI was published only in 1987 (Cummins *et al.*), and the first commercial assays were retailed by Sanofi Pasteur (Larue *et al.*, 1993) and Dade Behring (Adams *et al.*, 1993) in 1996. During the past 30 years, cTnI and cTnT have become the golden standard biochemical markers for diagnosing MI, due to the first Universal Definition of MI that encouraged the assay manufacturers to reduce the total imprecision (coefficient of variation, CV) of the cTn assays to be ≤10% at the MI decision level of (99th percentile) (Alpert *et al.*, 2000; Thygesen *et al.*, 2012).

To enable the diagnosis of ACS as early as possible, a new generation of high-sensitivity assays enabling the measurement of cTns in at least 50% (ideally 95%) of the reference population with a total imprecision (CV) of ≤10% have emerged the market during the past few years (Apple *et al.*, 2012a). These high sensitivity

assays have facilitated superior performance in rapid ACS diagnosis (Keller *et al.*, 2009; Reichlin *et al.*, 2009) as well as in patient risk stratification (Giannitsis *et al.*, 2010a; Lindahl *et al.*, 2010).

2.1.2 Point-of-care testing

Throughout the years, POCT has been defined in various ways. One of the most recent ones is: testing conducted outside the laboratory setting, near the patient, and by a person whose primary training is not in the clinical laboratory sciences (Nichols, 2013). Whatever the definition, none of them prefer a particular technology or a method over another. Thus, the testing should be performed near the patient, and facilitate a short turn-around time (TAT), i.e., decreased time from the sampling to obtaining the result (Drain *et al.*, 2014). This decrease in assay TAT is considered to be the biggest advantage POCT has to offer; the time savings are obtained by using whole blood instead of plasma or serum and eliminating blood transportation (Drenck, 2001). Resources being limited, experts in the Western world have come to the realization that healthcare budgets cannot grow at the rate they have been doing during the past decades. Thus, governments are actively seeking ways to lower healthcare costs (St John and Price, 2013). POCT has been subject to animated discussion during the past decades, and the evidence for its cost efficiency seems still to be limited and under debate (Fermann and Suyama, 2002; St John and Price, 2013).

It has been shown that in the Western world a substantial proportion of healthcare resources targeted to EDs are consumed by patients presenting with chest pains (Goodacre *et al.*, 2005). Since the majority of these patients are not diagnosed with ACS or another life threatening condition (Lindsell *et al.*, 2006), an effective and rapid diagnosis would benefit all parties involved. POCT is expected to assist in meeting the ED guidelines for cardiac markers: TAT from blood collection to the result should be a maximum of 30 minutes, and the implementation of POCT should occur if the results cannot be obtained in under 60 minutes (Storrow *et al.*, 2007).

A recent study by Blick (2014) reports an average TAT of 13.1 min (±5.9 min) with a cTnI POCT assay, when the TAT for a central laboratory cTnI assay was 48.2 min (±9.0 min). When savings in the TAT were projected into annual bed-savings, a total of $738,000 was accumulated. Apple *et al.* (2006) have reported that by implementing POCT for the measurement of cTnI, the time a patient spent in the hospital was decreased by 8% and the overall costs were reduced by 25%.

There are also challenges in POCT, the implementation of which requires resources to acquire the devices, as well as to train the staff (Drenck, 2001). The ED staff in the study conducted by Blick (2014) had the advantage of knowing the POCT device from previous use for other analytes, thus no extra costs were needed for the training of the staff. Whether the minimization of assay TAT offers added value in the actual ACS diagnosis remains a matter of debate. This is due to the low number of studies that address the issue of whether POCT has actual

clinical impact. It has been shown that, theoretically, the implementation of POCT and redesign of the current pathways can limit the length of ED admissions and increase the patient flow via reducing the laboratory TAT time (Storrow *et al.*, 2008). According to the experts, one of the biggest shortcomings in the studies involving the POCT of cTns is that, currently, there are no studies that would have clearly demonstrated that saving 60 minutes in the diagnosis of MI reduces the risk of future myocardial events (Bingisser *et al.*, 2012).

2.2 Lanthanide-doped polystyrene nanoparticles

The European Committee for Standardization has defined nanomaterials as particles having external dimensions on the nanoscale, i.e., the maximum size of nanoparticles should be in the order of 100 nm (Lövestam *et al.*, 2010). A number of different materials can be used to obtain nanosized particles (Grillo *et al.*, 2014). Since lanthanide-doped polystyrene particles are used as the labels in this thesis, the following section shortly describes lanthanide chelate technology and its use with nanosized polystyrene particles.

2.2.1 Lanthanide chelate technology

Labels constructed to include chelated lanthanide ions (Eu^{3+}, Tb^{3+}, Dy^{3+}, Sm^{3+}) can be excited with certain wavelengths of light, and after a series of energy transfers a luminescence signal from the lanthanide ion can be measured. Unlike traditional labels, lanthanide luminescence has relatively long decay times, between µs and ms range, enabling its use in time-resolved mode and resulting in minimal interference from background fluorescence. Lanthanide labels also have a large Stokes shift, which means there is no overlap between the excitation and emission spectra. Therefore, self-quenching is not a problem, as is commonly the case with traditional labels. (Hagan and Zuchner, 2011) The introduction of lanthanide chelate technology in the 1980s (Soini and Kojola, 1983; Mukkala *et al.*, 1989) and its commercialization have enabled the development of wide range of immunoassays and DNA hybridization assays (Diamandis and Christopoulos, 1990).

2.2.2 Production of lanthanide-doped polystyrene nanoparticles

Attaching several chelates into a single biomolecule has been reported to assist in the development of sensitive assays (Diamandis *et al.*, 1989), but it can ultimately cause increases in the assay nonspecific binding (Laukkanen *et al.*, 1995). Since only a number of individual chelates can be attached to a single protein, the lanthanide chelates have been incorporated into a shell protecting the labels.

The polymerization of styrene monomers is a well-recognized method for achieving nanoscale particles (Piskin *et al.*, 1994). One way of manufacturing nanoparticles doped with lanthanide chelates is their preparation in aqueous solutions. As the chelates are hydrophobic, they are forced into a less hydrophilic environment in the polymer capsules (Huhtinen *et al.*, 2005). Over 30,000 of these

different chelates can effectively be incorporated into a single nanoscale shell to produce a particulate label, a so-called nanoparticle, that does not affect the individual fluorescence properties of the chelates, and even a single particle can be detected in a solution (Härmä *et al.*, 2001; Soukka *et al.*, 2001b). The composition of the final particle solution can be modified by using different amounts of co-polymers in the polymerization reaction. These co-polymers include acrylic or methacrylic acid, which produce carboxyl groups on the particle surface (Shirahama and Suzawa, 1984). By the addition of these co-polymers the colloidal stability and functionality of the particles can be altered (Lück *et al.*, 1998). In the end, the particles are separated from the excess of the label (Matsuya *et al.*, 2003). Approaches with simultaneous polymerization and chelate encapsulation have also been introduced (Chen *et al.*, 1999; Tamaki and Shimomura, 2002). To prevent the chelate from leaking out of the polystyrene particle, Hakala *et al.* (2006) have described a method for covalently linking the chelate into the particle shell. Commercially available lanthanide-doped polystyrene particles with modified surfaces are also readily available.

2.2.3 Functionalization of polystyrene nanoparticles

The simplest way of attaching the target molecule to the particles is physical adsorption (Dezelić *et al.*, 1971), which is a reversible process. The adsorbed molecule can partially desorb due to pH changes (Ortega-Vinuesa and Hidalgo-Alvarez, 1995), detergent addition, or displacement by another protein (Bale *et al.*, 1989).

The functional groups formed during the polymerization reaction are conveyed onto the particle surface to enable an efficient covalent conjugation of biomolecules with the particles (Bale Oenick *et al.*, 1990). Therefore, specific coupling schemes are available for different functional groups (Brinkley, 1992; Holmes and Lantz, 2001). The utilization of polystyrene lanthanide-doped nanoparticles have been demonstrated in a number of different bioaffinity assays for protein (Soukka *et al.*, 2001b), nucleic acids (Huhtinen *et al.*, 2004), and virus (Valanne *et al.*, 2005) diagnostics. Traditionally, the bioconjugation of widely used carboxyl modified particles is performed by using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) -chemistry. EDC activates the carboxylic groups on the particle surface and enables their reaction with primary amine groups on the binder molecules (**Figure 1**). The incorporation of N-hydroxysuccinimide (NHS) or N-hydroxysulfosuccinimide (sulfo-NHS) yields a more stable intermediate than EDC alone (Staros *et al.*, 1986; Griffin *et al.*, 1994).

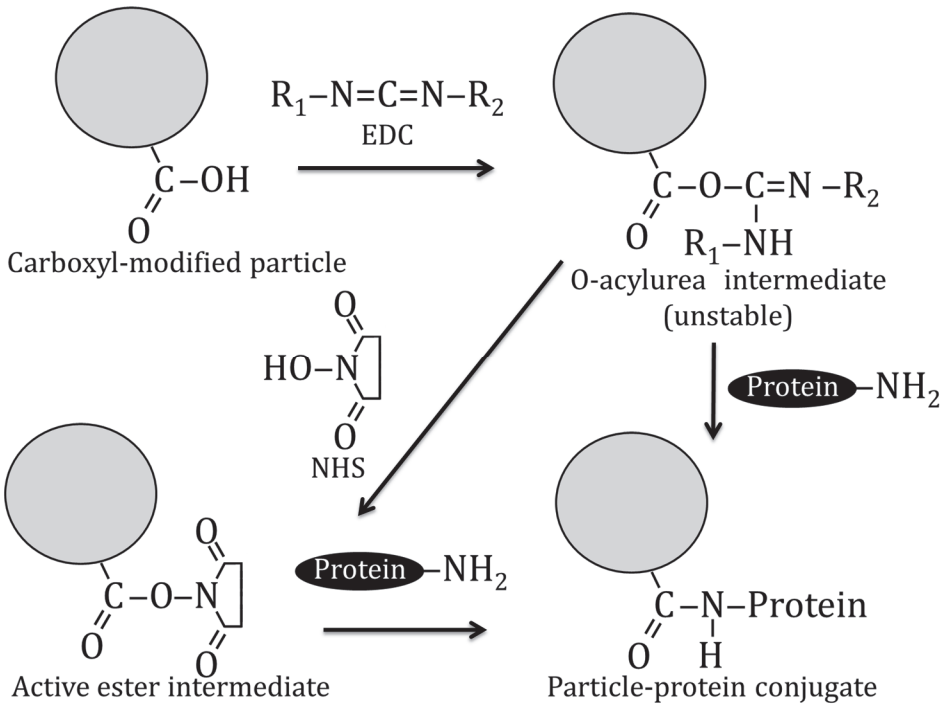

Figure 1. Schematic presentation of the activation chemistry of carboxyl-modified particles. EDC activation of the COOH-group yields an unstable intermediate that can directly be used to couple the desired protein or other primary amine-containing molecule to the particle. Some proteins are inactivated from direct exposure to EDC, and the addition of NHS or sulfo-NHS forms a more stable intermediate, thus enabling washing steps and the removal of the unreactive EDC prior to the coupling of the binder molecules. (Adapted from Griffin *et al.*, 1994).

2.2.4 Immunometric assays utilizing polystyrene nanoparticles

Soukka *et al.* (2001a) have demonstrated that by optimizing the amount of activation reagents and the protein amount in the coating reaction, up to 200 active binding sites can be created onto a single 107 nm (diameter) particle. This causes an increase in the binding area of the label and enhances the association rate of the detection antibody used (Soukka *et al.*, 2001a). This avidity-based enhancement of assay signal assists in the development of highly sensitive assays with simple test designs (Härmä *et al.*, 2001; Soukka *et al.*, 2001b). Thus, lanthanide-doped polystyrene particles have enabled the development of sensitive assays for different protein and viral analytes (Soukka *et al.*, 2003; Valanne *et al.*, 2005; Järvenpää *et al.*, 2012).

2.3 Immunoassay interferences

The concept of immunoassay interference is almost as old as immunoassay technology itself. The first substantial reports of immunoassay interference date back to the 1970s and 1980s (Sgouris, 1973; Hunter and Budd, 1980).

Nevertheless, immunoassay interferences are frequently reported with new assays, as well as with assays that are widely used in the clinical field (Xu *et al.*, 2013; Lippi *et al.*, 2014). Over the years, false immunoassay results have led to detrimental misdiagnosis and unnecessary treatments of patients (Rotmensch and Cole, 2000; Ballieux *et al.*, 2008). The effects of immunoassay interference depend on the interference type, as well as the method and analyte used. They cause either false positive or false negative results, but these can be observed with one method and be corrected with an assay from a different manufacturer (Janssen *et al.*, 2014; Robier *et al.*, 2014).

2.3.1 Interference mechanisms of circulating human antibodies

Antibodies used in different immunoassays share a common basic structure. An immunoglobulin class G (IgG) monoclonal antibody (Mab) consists of a light chain and a heavy chain. In the light chain, there are constant (C_L) and variable domains (V_L), whereas the larger heavy chain consists of a variable domain (V_H) and three different constant domains (C_H1, C_H2 and C_H3). Domains present in the so-called fragment antigen binding (Fab) -region consist of V_L, C_L, V_H, and C_H1. Fragment crystallizable (Fc) -region contains the remaining constant heavy domains C_H2 and C_H3. (Lipman *et al.*, 2005) The basic structure of an IgG class antibody can be seen in **Figure 2.**

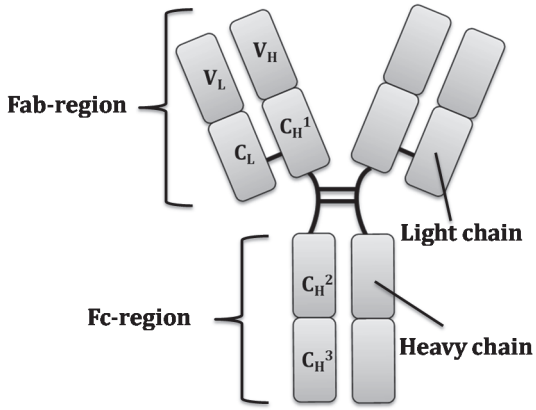

Figure 2. The basic structure of an IgG class antibody, commonly used in immunometric assays

A false positive immunoassay response is obtained when a circulating interfering human antibody bridges the capture and detection antibodies without the presence of an analyte. Negative interference, however, can be detected when the interfering antibody blocks the binding sites of the assay antibodies (one or all), so that no binding of the analyte can occur (**Figure 3A**). (Kricka, 1999)

The most common types of human antibodies binding different parts of the assay antibodies are presented in **Figure 3B**. Human anti-isotype antibodies bind to the Fc-region of, for example, murine IgG antibodies (mIgG) (Csako *et al.*, 1988; Vaidya and Beatty, 1992; Bolstad *et al.*, 2011). Furthermore, interfering circulating antibodies can be directed against the idiotype of the antibody (Goodman *et al.*, 1985; Kuus-Reichel *et al.*, 1994), or against the anti-idiotype antibody, hence called anti-anti idiotype antibodies (Frödin *et al.*, 1992; Reinsberg, 1995).

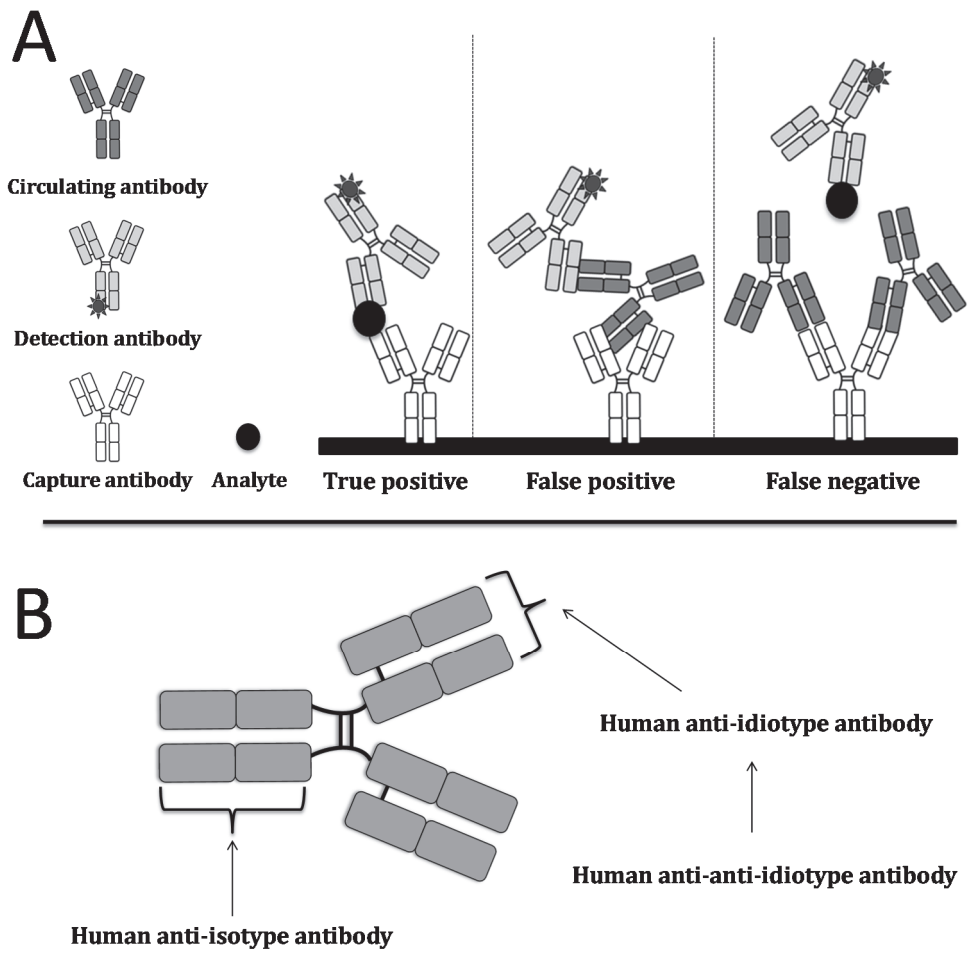

Figure 3. A) Examples of immunometric assays for a true positive, a false positive and a false negative immunoassay result. B) Examples of specificities of different possibly interfering heterophilic and similar antibodies.

2.3.2 Heterophilic-type interference

During the past 40 years, different immunoassays have been reported to suffer from interferences caused by circulating antibodies that have affinity to assay

antibodies. Heterophilic and similar antibodies can express immunoglobulin classes G, A, M, and somewhat infrequently E (Kricka, 1999). One very prominent field affected by heterophilic-type interference has been the assays for different tumor markers, including human chorionic gonadotropin (hCG) (Rotmensch and Cole, 2000; Gallagher *et al.*, 2010), prostate-specific antigen (PSA)(Fritz *et al.*, 2009), cancer antigen 125 (CA-125) (Bertholf *et al.*, 2002), carcinoembryonic antigen (CEA) (Bjerner *et al.*, 2002), and calcitonin (Papapetrou *et al.*, 2006). Different categorizations of heterophilic-type interference can be made. In this thesis, as suggested by Kaplan and Levinson, the term human anti-animal antibodies (HAAAs) and their subclasses, including human anti-mouse antibodies (HAMAs), have been reserved for high-affinity human antibodies that are produced against specific immunogens in response to a known exposure of antibodies of foreign origin through diagnostic or therapeutic purposes. Contrary to this, heterophilic antibodies are usually multispecific, weak antibodies that are produced against poorly defined antigens without any history of specific medical treatment with animal antibodies. (Kaplan and Levinson, 1999) However, the terms HAAA and heterophilic antibodies are often used synonymously in the literature.

2.3.2.1 Human anti-animal, anti-chimeric and anti-human antibodies

During the past years, therapeutic antibodies have increasingly been introduced, thus resulting in reports of specific HAAAs (Hwang and Foote, 2005) and the introduction of engineered antibodies (Presta, 2006). The most commonly described HAAAs are HAMAs, since antibodies of murine origin are most commonly used in commercial immunoassays (Kricka, 1999). Anti-isotype HAMAs seem to be more common than other types of HAMA: Lind *et al.* (1991) reported that after an administration of a medical murine Mab, 29 out of the 141 study participants showed a positive HAMA response: 80% being anti-isotypic and the remaining 20% anti-idiotypic. The reported HAMA concentrations in the circulation vary hugely, and can be as high as on the g/L scale (Moseley *et al.*, 1988). The duration of the response can be maintained for years after the exposure to foreign Igs (Baum *et al.*, 1994). In addition to HAMA, specific HAAA responses have been observed against other animals serving as hosts for immunotherapy. The existence of both human anti-rabbit (Hiemstra *et al.*, 1988) and anti-horse antibodies have been reported (Harkiss, 1984).

When the Fc-region of the Mab is substituted with the corresponding human sequences, the human antibody response is often reduced (Hwang and Foote, 2005). Nevertheless, significant human anti-chimeric antibody (HACA) responses have also been reported (Colnot *et al.*, 2000; Afif *et al.*, 2010). During the recent years, humanized antibodies with the only non-human parts remaining being the analyte-recognizing ones have also emerged. With these humanized antibodies, the immunogenicity cannot be predicted. A humanized Mab A33 that is used in the treatment of colon cancer has showed a human anti-human antibody response in 66% of the treated patients (Ritter *et al.*, 2001).

The production of HAAAs is a complex process, since not all antibodies of animal origin are reported to cause corresponding HAAAs, or interferences in immunoassays. Patients injected with Arcitumomab, a murine Fab-fragment used in the diagnostic imaging of colorectal cancer, do not form HAAA-responses against the drug (Wegener *et al.*, 2000). Furthermore, not all HAAA-responses cause interferences in immunoassays. Hemophiliacs treated with porcine factor VIII, form HAAAs against the medication, but no immunoassay interferences from these HAAAs have been reported (Morrison *et al.*, 1993).

2.3.2.2 Heterophilic antibodies

Although heterophilic antibodies are generally considered to be low-affinity multispecific antibodies, also high-affinity antibodies especially against immunoassay antibody sub-class IgG1 have been found (Bjerner *et al.*, 2005; Bolstad *et al.*, 2011). Heterophilic antibody interference seems to be caused mainly by human immunoglobulin class M (IgM) antibodies (Bjerner *et al.*, 2005). Due to the polyvalence of IgM class antibodies, the interference from IgM class antibodies is often considered more effective than the interference from IgG class antibodies. Despite the current view that heterophilic antibodies are formed without a known exposure to animal antibodies, they are more common in people that are routinely handling animals (Davies, 2013), and their production is often considered as an antigen driven process (Check *et al.*, 1995). Sources proposed for the induction of heterophilic antibodies include exposure to animals (Berglund and Holmberg, 1989) and animal-based products (Dahlmann and Bidlingmaier, 1989), blood transfusion (Davidsohn and Lee, 1966), autoimmune diseases (Falchuk and Isselbacher, 1976), and maternal transfer (Larsson *et al.*, 1981).

In many cases heterophilic-type antibody interference can be traced to be against antibodies from a specific origin. Heterophilic human anti-bovine antibodies (Andersen *et al.*, 2004), anti-sheep antibodies (Monchamp *et al.*, 2007), anti-rabbit antibodies (Butch, 2000), and anti-goat antibodies (Cavalier *et al.*, 2009) have all been reported to cause immunoassay interferences. Although these types of immunoassay interferences are nowadays often caught before causing detrimental effects, reports of overlooking these interferences have been published throughout the years. In a report from Berglund and Holmberg (1989), a case was presented, where heterophilic antibodies specific to rabbit antibodies caused falsely elevated levels of follicle-stimulating hormone (FSH) in a patient presenting with infertility and amenorrhea. Due to the false levels of FSH, a series of unnecessary procedures including an ovarian biopsy were done.

IgGs from different mammals possess sequence homology. Therefore, heterophilic and similar antibodies are known to cross-react with IgGs from different animal species. Thompson *et al.* (1986) showed that the administration of non-immune sera from different animals could effectively eliminate heterophilic interference against creatine kinase muscle-brain fraction (CK-MB) assay antibodies. The addition of mouse serum abolished the interference in 100% of the samples, when

the additions of sheep, cow, guinea pig, rat and rabbit serum abolished the interference in 78%, 78%, 69%, 70% and 25% of the samples, respectively. Sampson *et al.* (1994), however, reported that heterophilic interference in two male samples could equally be blocked by using mouse, sheep, or goat serum, as well as by mIgG1, mIgG2a and rat IgG.

Heterophilic-type interference can also be caused by antibodies produced against micro-organisms. Covinsky *et al.* (2000) have reported on a 56-year-old male suffering from *Escherichia coli* septisemia, who presented with falsely elevated assay values for cTnI, thyrotropin, hCG, α-fetoprotein, and CA-125. Sample incubation with mouse Mabs or *E.coli* removed the interference and corrected the assay results. Thus, an extremely restricted IgM antibody response against *E.coli* was causing the spuriously elevated assay values.

2.3.2.3 Rheumatoid factors

Rheumatoid factors (RF) are autoantibodies (usually IgM) directed against a patient's own IgG and IgA antibodies. About 70% of the people affected with rheumatoid arthritis are found to have circulating RFs (Wolfe *et al.*, 1991), but RFs can also be found in approximately 5% of the healthy population (Nielsen *et al.*, 2012). Originally, RFs were defined by their ability to bind to antigenic determinants in the antibody Fc-region (Carson *et al.*, 1987), but they can be targeted to other regions as well (Selby, 1999). The presence of RFs has been connected to falsely elevated analyte values in numerous assays, including thyroid testing (Martel *et al.*, 2000), tumor marker assays (Berth *et al.*, 2006), and cTns (Fitzmaurice *et al.*, 1998) most probably due to cross-reactivity of the Fc-region in different animal species.

2.3.2.4 Incidence of heterophilic-type interference

The incidence of heterophilic-type interference is highly dependent on the immunoassay type and analyte, and varies greatly between different studies. In a study by Andersen *et al.* (2004), it was observed that human heterophilic antibodies with specificity to bovine antibodies were present in 99% of the donor serum samples and caused a false positive interference rate of 81% in an immunoassay for endometrial protein PP14. A recent study by Koshida *et al.* (2010) indicated the existence of heterophilic antibodies with anti-mouse specificity in >10% of the samples tested. An older study by Boscato and Stuart (1986) observed an approximately 15% interference rate in non-blocked immunoassays with roughly 40% of the serum samples containing significant amounts of heterophilic type antibodies.

The extent of the immunoassay interference incidence was clearly highlighted a decade ago, when Marks *et al.* (2002) measured the concentrations of 74 different analytes in samples obtained from 10 different individuals with diseases associated with RFs. The study was conducted in 66 clinical laboratories in 7 countries. Examples of the different analytes affected by heterophilic interference

can be seen in **Table 1**. Of the 3445 results obtained, approximately 9% were found to be falsely positive. Of those false positives, 21% (1.8% of all results) were considered as potentially leading to incorrect clinical interpretation.

Table 1. An example of false positive results with immunoassays in a multicenter study of 74 analytes. (Adapted from Marks *et al.*, 2002).

Analyte	Assay systems tested (n)	Heterophilic false positives/number of analyses performed[a]	Etiologically uncertain false positives/number of analyses performed[b]
Cortisol	8	0/85	17/85 (20%)
CA-19-9	10	1/84 (1.2%)	9/84 (11%)
Estradiol	7	3/59 (5.1%)	34/59 (58%)
Myoglobin	7	28/59 (48%)	0/59
TSH	14	10/249 (4.0%)	4/249 (1.6%)
cTnI	9	18/156 (12%)	9/156 (5.8%)

[a] Samples that were reduced to reference ranges by pre-treatment with blocking reagent, and samples that were originally within reference range, but were reduced >30 by pre-treatment with blocking reagent.
[b] Samples that were not reduced to reference ranges by pre-treatment with blocking reagent.

2.3.3 Autoantibodies against the analyte

Autoantibodies are produced against autoantigens when the body's self-tolerance is failing. Autoantibodies are produced in many autoimmune diseases, e.g, rheumatoid arthritis, but can also be found in apparently healthy individuals (Nielsen *et al.*, 2012). Autoantibodies against the assay analyte can cause either false negative or false positive signals in immunoassays. In thyroid testing, the prevalence of autoantibodies has been estimated to be between 0% and 25 %, and their occurrence has been connected to interferences in thyroid function tests (Despres and Grant, 1998). Falsely decreased values of insulin can be measured, if the insulin autoantibodies mask the binding sites of the assay antibodies (Kim *et al.*, 2011). Autoantibodies have been detected against various other analytes as well, including PSA (Zisman *et al.*, 1995; McNeel *et al.*, 2000) and calcitonin (Dorizzi *et al.*, 1991), but their impact on the measurement of the analytes is unknown.

A number of studies have highlighted the effects of anti-analyte autoantibodies against cTns. These autoantibodies can cause false negative results by inhibiting the cTn detection of the assay antibodies (Eriksson *et al.*, 2005b), or false positive results by stabilizing the antigen (Plebani *et al.*, 2002). The estimates of cTn autoantibody prevalence in an apparently healthy population vary extensively from 0% (Shmilovich *et al.*, 2007) to as high as 20% (Lappé *et al.*, 2011). This huge variation is possibly due to the differences in the detection methods, assay sensitivities, and the number of study subjects.

2.3.4 Other proteins

Various other proteins can also cause interferences in immunoassays. When an antigen binds to a solid-phase antibody, or an intact Mab is bound to a plastic surface, an immune complex is formed that may activate the complement system (Carlander and Larsson, 2001; Nilsson *et al.*, 1993). Complement factor C4b binds to the Fab-region of the antibody (Campbell *et al.*, 1980), which may cause the antibody binding sites to be blocked by the complement. It has also been reported that antibody subclasses show different susceptibilities to complement-derived interference so that subclass IgG2 antibodies are more prone to complement-derived interference than subclass IgG1 antibodies (Börmer, 1989).

Lysozyme binds proteins with low isoelectric points, thus possibly bridging the assay antibodies (Selby, 1999). Different hormone-binding proteins can alter the measured analyte concentration by blocking the analyte or by removing the analyte from the assay antibodies (Tate and Ward, 2004). Albumin may cause interference as a result of a high blood concentration, or the ability to bind or release vast quantities of ligands (Selby, 1999).

Paraproteins, which are antibodies that do not fight against infection, are reported to cause falsely decreased immunoassay values for vancomycin (LeGatt *et al.*, 2012) and thyroid-stimulating hormone (TSH) (Loh *et al.*, 2012). Macrohormones, which are hormones conjugated with immunoglobulins, can also cause assay interferences, usually by artefactually elevating assay results. The most commonly encountered macrohormone is macroprolactin, which may cause false diagnoses of hyperprolactinaemia (Fahie-Wilson and Smith, 2013), but reports of macrohormone-induced elevations of assay results have also been documented with TSH (Loh *et al.*, 2012) and B-type natriuretic peptide (Janssen *et al.*, 2014).

2.3.5 Hemolysis, icterus and lipemia

Unlike assays employing spectral and chemical techniques, the majority of immunoassays are not affected by hemolysis, icterus and lipemia (Tate and Ward, 2004). Nevertheless, certain assays and analytes have been found to be affected by them (Ji and Meng, 2011). Hemolysis has been associated with decreased cTnT values, possibly due to the degradation of cTnT by the released proteases from hemolyzed blood cells (Sodi *et al.*, 2006; Ji and Meng, 2011). Icterus, i.e., elevated levels of bilirubin, has been linked to statistically significant decreases in a specific cTnI assay without understanding the mechanism (ver Elst *et al.*, 1999; Dasgupta *et al.*, 2001). High level of sample triglycerides and/or cholesterol, known as lipemia, however, has caused negative interference in a competitive testosterone immunoassay (Owen *et al.*, 2010).

2.3.6 Cross-reactivity

Cross-reactivity occurs, when endogenous molecules with similar or identical epitopes to the analyte being measured are present in the circulation (Kroll and Elin, 1994). Therefore, cross-reactivity requires a stereochemically permissive environment at the antigen-antibody interface (Chitarra *et al.*, 1993). Cross-reactivity can cause either false positive or false negative results depending on the epitopes present in the cross-reactant (Sturgeon and Viljoen, 2011). Modern digoxigen assays show detrimental cross-reactivity with immunoreactive factors present in renal failure, liver disease, and hypertension (Dasgupta, 2006). Early human hCG assays cross-reacted with luteinizing hormone (Thomas and Segers, 1985), but the development of new monoclonal antibodies has led to very specific hCG assays with which the cross-reactivity issues can be avoided. It has been recommended that, to ensure a clinically useful assessment of cross-reactivity, cross-reactivity should be calculated as the apparent percentual change in the measured endogenous analyte concentration when the cross-reactant is present in concentrations observed in healthy and diseased populations (Davies, 2013).

2.3.7 Pharmaceutical preparations

Some drugs and herbal remedies react as cross-contaminants. When measuring steroids, cortisol for example, is often problematic. Berthod and Rey (1988) reported cross-reactivities between 340%–810% for different hydrocortisone derivatives in an assay for cortisol, whereas immunoassays for cyclosporin A, a drug used as an anti-rejection drug for organ transplantation, have shown cross-reactivities up to 174% for the metabolites of the parent drug (Steimer, 1999).

Herbal remedies, especially used in the Eastern medicine, have recently raised concerns as interfering factors (Dasgupta and Bernard, 2006). A certain Chinese medication prepared from toad venom (Chan Su) containing bufadienolides that are components structurally similar to digoxin, have been linked to both false elevations and decreases of measured digoxin levels (Dasgupta *et al.*, 2000).

2.3.8 Sample processing

Blood collection and the sample type (serum vs. plasma) may affect the measured results in some assay systems and analytes (Evans *et al.*, 2001; Krintus *et al.*, 2014). False elevations of cTnI values have been observed in Abbott AxSym® assay system due to fibrin present in serum samples (McClennen *et al.*, 2003; Kazmierczak *et al.*, 2005). Chang *et al.* (2003) have reported that when serum separator tubes were used instead of plain tubes, increased assay values for C-reactive protein (CRP) were measured. The proposed reason for the observation was that the gel used in the tubes adsorbed macromolecules that formed complexes with CRP, and the release of CRP from these complexes enhanced the antigen-antibody reaction causing increased CRP concentrations to be measured.

Inappropriate sample handling and storage can also affect the assay results. Jane Ellis *et al.* (2003) have reported that the hormone adrenocorticotrophin was stable only for 18 hours in plasma (at +4°C), compared to the stability of over 120 hours for 18 other hormones. Additionally, falsely elevated values due to inappropriate sampling procedures are an inherent risk that should not be disregarded. If a sample with very high analyte concentration is followed by a sample with low analyte concentration, a carryover of the analyte may occur (Armbruster and Alexander, 2006).

2.3.9 High-dose hook effect

In assay systems where wide dynamic ranges are required (e.g., hCG, PSA) a saturation of the solid-phase and detection antibodies with the circulating antigen can occur. This phenomenon called high dose hook effect, although not interference as such, can lead to a possible misdiagnosis of a condition (Sturgeon and McAllister, 1998). A diagrammatic representation of a high-dose hook can be seen in **Figure 4**. High-dose hooks are mostly seen in one-step immunometric assays, where the simultaneous addition of capture and detection antibodies allows them to compete for the binding of the analyte. Thus, an excess of the analyte can minimize the number of analytes bound simultaneously to the capture and detection antibodies (Tate and Ward, 2004). The high-dose hook effect can be reduced with basic assay design: the possible steps include increasing the amount of assay antibodies, diluting the sample, and adding an extra wash step before the addition of detection antibodies (Selby, 1999; Demers and Spencer, 2003; Cole *et al.*, 2001).

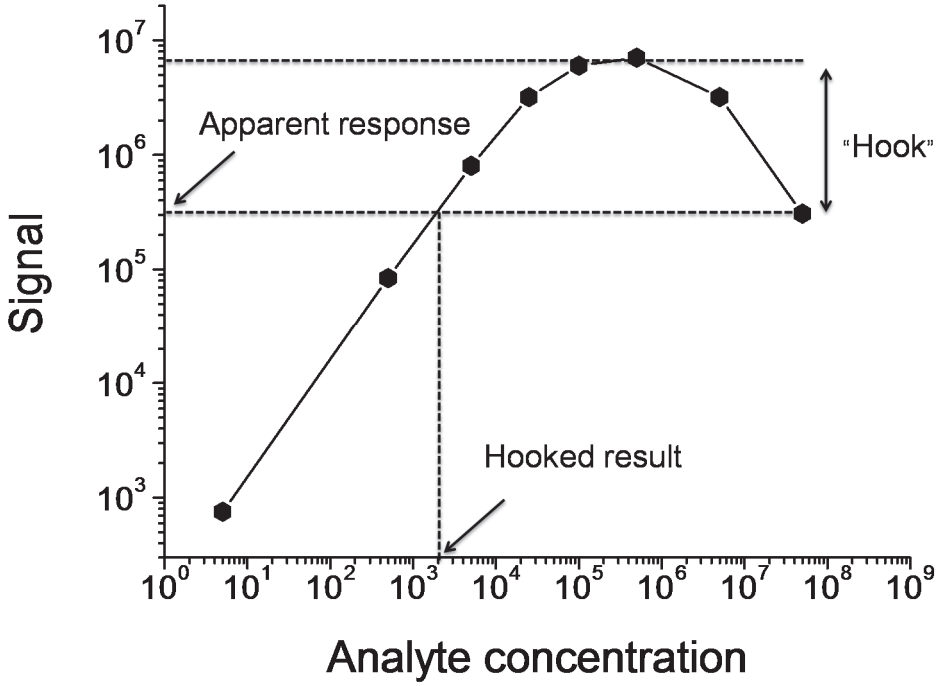

Figure 4. An example of a possible high-dose hook effect. High analyte concentration saturates the assay antibodies, thus resulting in a falsely low assay signal and an invalid estimation of the analyte value.

2.4 Heterophilic-type interference: prevention and elimination

2.4.1 Immunosuppressant therapy

As noted earlier, the formation of HAAAs is a complicated process, and not all HAAAs cause assay interferences. There are few studies, where the sole administration of a therapeutic drug is compared with a situation where it is administrated simultaneously with an immunosuppressant drug, but the administration of an immunosuppressant does not always decrease the formation of anti-antibody response (Sandborn *et al.*, 2001). When a group of breast cancer patients were treated only with a radiolabeled chimeric Mab, they all developed a specific HACA-response, but when cyclosporin was simultaneously dispensed, no HACA-responses were observed (Richman *et al.*, 1995). In another study, when infliximab, a chimeric monoclonal IgG against tumor necrosis factor alpha was repeatedly administered, antibodies against the drug were formed in 75% of the patients if no immunosuppression therapy was included. A simultaneous administration of an immunosuppressant drug decreased the incidence of HACA-response to 43%. (Baert *et al.*, 2003)

2.4.2 Immunoassay design

Immunoassay design has a major effect on the probability of possible interferences caused by the sample matrix. Currently, most immunometric assays are designed to use subclass IgG1 Mabs originating in the mouse (Bolstad *et al.*, 2013). One successful approach to the removal of assay interferences is the utilization of capture and detection antibodies of differing animal origins (Hennig *et al.*, 2000; Carlander and Larsson, 2001).

Mabs possessing the highly immunogenic Fc-region are prone to different interferences, and since there is no single blocking reagent that could remove all interferences from heterophilic and similar antibodies (Dasgupta *et al.*, 1999), the removal of the Fc-region of the antibody is considered to be one of the most effective ways of removing many interferences (Csako *et al.*, 1988). Thus many companies have started to employ antibody fragments in their commercial assays (Bolstad *et al.*, 2013).

2.4.2.1 Enzymatically cleaved fragments

The enzymatic cleavage of Mabs can be done with different enzymes. Traditionally, the non-specific thiol protease papain, is used to obtain Fab-fragments, whereas F(ab')$_2$-fragments are obtained by using pepsin (**Figure 5**). (Liddell, 2013) The production of F(ab')$_2$-fragments with pepsin has proven more difficult than the production of Fab-fragments: different antibody species and subclasses are variably susceptible to pepsin digestion. Especially IgG subclass 2b is prone to complete degradation, so an optimization of the degradation process is crucial for each antibody (Wilson *et al.*, 2002). Because of the differences and difficulties in pepsin and papain digestions of different IgG subclasses, also enzymes bromelain and ficin are widely used for antibody fragmentation due to a rapid fragmentation process and the stability of the obtained fragments (Mariani *et al.*, 1991).

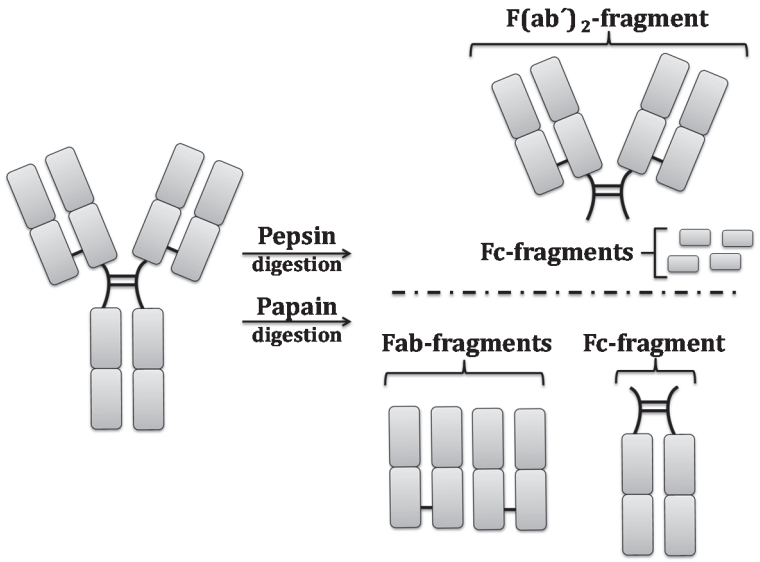

Figure 5. Enzymatic papain and pepsin digestions of an intact Mab to obtain Fab- and F(ab')$_2$-fragments**.**

Vaidya & Beatty (1992) reported that out of about 2600 samples tested, 81 were observed to contain heterophilic antibodies, and out of those samples 22 % (n=18) gave unexpectedly high signals in their assays for CK-MB. They reported that the combinatorial use of F(ab')$_2$-fragments and mIgG eliminated all heterophilic interferences in the assay. A more recent study by Väisänen *et al.* (2006) reported that in an assay for free human kallikrein 2, the employment of F(ab')$_2$-fragments instead of intact Mabs resulted in a 10–100-fold decrease in false positive signals, thus practically eliminating assay interference. In a study by Bjerner *et al.* (2002) an introduction of an F(ab')$_2$-fragment reduced the immunoassay interference from 4% to 0.1%. Enzymatically produced Fab-fragments have less frequently than F(ab')$_2$-fragments been applied in the elimination of assay interferences, but already in 1979 it was observed that when enzymatically cleaved Fab-fragments were applied into an assay for insulin, a total elimination of RF-interference occurred (Kato *et al.*, 1979).

2.4.2.2 Recombinant antibodies and antibody fragments

The proteolytic fragmentation of intact Mabs is a labor-intensive and expensive process, so recombinant antibody fragments expressed in *E.coli* have been available for nearly 20 years (Hayden *et al.*, 1997). Furthermore, the introduction of different recombinant techniques in the 1980s and 1990s has also enabled the production of antibody fragments of fully human origin (Jespers *et al.*, 1994). These well-established recombinant DNA techniques can be harnessed to incorporating specific reactive groups into the fragments, thus facilitating easy purification and site-directed labeling of the fragments, along with enabling the

continual and homogenous production of the fragments (Lyons *et al.*, 1990; Knappik and Plückthun, 1994; Lindner *et al.*, 1997). The use of site-specific labeling should enable the formation of highly oriented antibody surfaces and the development of increasingly sensitive immunoassays (Peluso *et al.*, 2003). It has been reported that recombinantly produced antibody fragments have equal affinities to corresponding Mabs (Altshuler *et al.*, 2012). Also, the production of recombinant fragments in *E.coli* and the biotinylation of the fragment as part of the purification process do not alter the stability of the produced fragments (Eriksson *et al.*, 2000; Ylikotila *et al.*, 2006).

The first report for an immunometric assay for PSA completely based on recombinant Fab-fragments was published in the year 2000 (Eriksson *et al.*, 2000). Since then, a number of assays based on recombinant Fab-fragments for different analytes, e.g., cTnI (Ylikotila *et al.*, 2006) and lectins (Kelly *et al.*, 2005) have been published. As for commercial immunoassays, the 4th generation cTnT assay from Roche (Mannheim, Germany) utilized two murine Fab-fragments in a sandwich format, and the new 5th generation high sensitivity modification of the assay utilizes a chimeric version of the detection antibody (Giannitsis *et al.*, 2010b).

Although the first assay utilizing single-chain variable fragments (scFv) consisting of the variable domains (V_H, V_L) connected by a flexible linker, was described already in 1997 (Kerschbaumer *et al.*, 1997), small scFvs have traditionally been considered to be less stable than bigger antibody fragments (Raag and Whitlow, 1995). Some scFvs might remain stable for less than six months in +4°C (Hu *et al.*, 2007), causing their use in commercial assays to be somewhat limited. It has been shown that the constant parts present in Fab-fragments significantly increase antibody stability (Shimba *et al.*, 1995). Antibody fragments are likely to increase their popularity especially in immunoassays detecting untraditional analytes: not because of a desire to avoid immunoassay interferences, but because traditional animal-based immunization methods cannot be applied to certain analytes, e.g., toxic components and small haptens (Zhang *et al.*, 2014; Alvarenga *et al*., 2014; Oyama *et al.*, 2013). Large antibody libraries can easily be used to obtain binders for any kinds of analytes, making animal immunization redundant (Nissim *et al.*, 1994; Brockmann *et al.*, 2011). Despite their possible challenges in long-term stabilities, scFv-fragments have successfully been employed in a variety of different immunoassays (Warren *et al.*, 2005; Wang *et al.*, 2008; Brockmann *et al.*, 2010).

Small scFv-fragments contain less material from the original host species than larger recombinant antibody fragments or enzymatically fragmented antibodies and are, therefore, less prone to heterophilic interferences. Warren *et al.* (2005) reported that out of 383 samples that produced a falsely elevated result in their F(ab')$_2$-assay, 374 were corrected when the used F(ab')$_2$-fragment was replaced with the corresponding scFv-fragment.

Chimeric antibodies and antibody fragments are antibodies in which sequences of two different origins have been combined. Usually chimeric antibodies are considered to be of non-human origin, e.g., murine antibodies that are modified to contain human sequences. For example, the constant domains of a mouse antibody can be replaced with the corresponding human sequences, thus potentially minimizing the immunoreactive properties of the mouse parts (Liu *et al.*, 1987). Chimeric antibodies can easily be produced and secreted in *E.coli* as fully active constructs (Better *et al.*, 1988). When intact Mab and chimeric human/mouse versions of the same tracer antibody was tested in an immunoassay for CEA, it was noted that when no assay blockers were used, 146 (29.0%) out of the 503 serum samples tested produced a falsely elevated result with the Mab-version, whereas only 14 (2.8%) did so with the chimeric system (Kuroki *et al*., 1995).

Humanized antibodies are distinct from chimeric antibodies. They are antibodies of non-human origin, whose protein sequences have been modified to increase their similarity to antibody variants produced naturally in humans. The most common way of obtaining humanized antibodies is called complementarity determining region (CDR) grafting. When performing the humanization of a mouse antibody by CDR grafting, the mouse CDRs responsible for the antibody binding are inserted into a human framework with different molecular techniques. The binding affinity of the humanized antibodies may suffer, however, since the human framework residues may be involved in the actual binding reaction. (Safdari *et al.*, 2013) Nowadays, humanized antibodies are used for treating different human diseases, but no studies of their use in reducing matrix-related interferences in immunoassays have yet been published. A schematic presentation of murine, human, chimeric, and humanized Fab-fragments is presented in **Figure 6**.

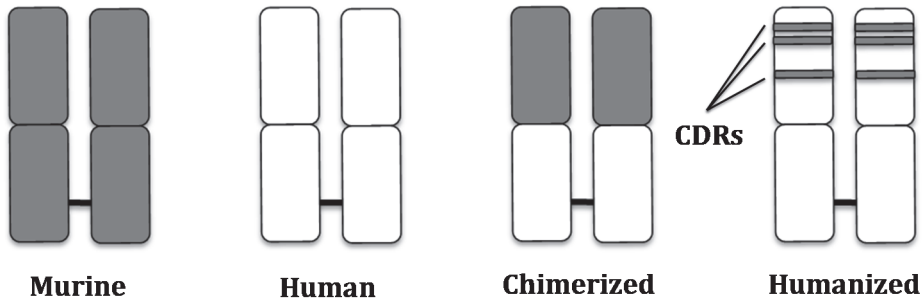

Figure 6. An example of a murine, human, chimeric, and humanized versions of an Fab-fragment.

2.4.3 Physical and chemical techniques

Immunoassay interference can be removed by applying a number of different chemical and physical techniques. These techniques are usually time consuming,

tedious, and often constrained to a specific group of interferences. Different approaches have been tested on samples where either falsely negative or falsely positive results have been obtained. It must be noted though, that all of the physical and chemical approaches are highly specific to each analyte and interference type, and cannot be universally applied.

2.4.3.1 Precipitation

The precipitation of interfering proteins can be performed by using polyethylene glycols (PEGs) or ammonium sulfate, which precipitate proteins by lowering their solubility in aqueous solutions, e.g., plasma and serum (Bolstad *et al.*, 2013). PEG precipitation has been used to remove proteins, e.g., prolactin macrocomplexes and thyroid autoantibodies from blood samples (Fahie-Wilson and Smith, 2013; Despres and Grant, 1998). The precipitation with PEGs and ammonium sulfate has been shown to clear near all Igs from the sample, but it can also abolish and irreversibly denature clinically important proteins (Bolstad *et al.*, 2013). All sample proteins can also be precipitated with ethanol (Levine *et al.*, 1990) as well as with trichloroacetic acid, which has been used in eliminating heterophilic interference in digoxin assays (Liendo *et al.*, 1996).

2.4.3.2 Affinity extraction

Different affinity-based extraction methods include the application of ion exchange, and protein G, A and L –based approaches (Turpeinen *et al.*, 1990; de Jager *et al.*, 2005). Of the affinity-based methods, approaches using proteins G and A are the most universally applicable. Protein G binds to the Fc-region of all four IgG subclasses, when protein A is known to bind to only three of the four subclasses. A case study by Lippi *et al*. (2013a) demonstrated that by applying affinity extraction with protein A, a false positive troponin level caused by heterophilic antibodies could be decreased from 7.9 µg/L to <0.2 µg/L. In another study, the immunodepletion of sample IgGs with protein G affinity chromatography decreased the measured falsely elevated TSH value from 25.2 mIU to 2.7 mIU (Ross *et al.*, 2008). Protein-L has higher affinity against IgM than against either IgA or IgG, especially through kappa light chain interactions (Åkerström and Björck, 1989). In a study by de Jager *et al.* (2005), total IgM, IgG, and IgA levels were cut by 60%, while RF-associated IgM levels were cut by 89% after an incubation of 2 h with protein L.

2.4.3.3 Other approaches

Numerous other approaches have also been tested to remove sources of possible interference. One of these is size-exclusion (Liendo *et al.*, 1996), which usually requires relatively high sample volumes. The use of size-exclusion is also restricted because the analyte is usually highly diluted during the process (Bolstad *et al.*, 2013). Heating a sample at 90°C (Primus *et al.*, 1988), and sample treatment with dithioerythritol (Müller *et al.*, 1985) can only be used with analytes that can endure high temperatures and antibody-denaturing conditions.

2.4.4 Blocking agents

The most commonly utilized way of decreasing assay interferences is to use specific blocking agents as part of the assay buffers. There are several specific requirements the employed blockers should meet. The blocker substance should: 1) inhibit the nonspecific binding of the assay components to the assay surface; 2) inhibit non-specific protein-protein interactions; 3) obtain no cross-reactivity with the assay components (antibodies, analyte); 4) not inhibit specific reactions in the assay and 5) to exhibit very little lot-to-lot variation.

Basic protein blockers including bovine serum albumin (BSA), casein, and gelatin can be used to decrease undesirable protein-protein interactions (Wakayama *et al.*, 2008; Kenna *et al.*, 1985). An addition of excess nonspecific Igs, e.g., bovine gamma globulin (BGG) has been proven to decrease the interactions between assay antibodies and interfering factors (Hunter and Budd, 1980; Frengen *et al.*, 1994). To minimize interferences from heterophilic and similar antibodies with a specificity to assay antibodies, polyclonal IgGs from the antibody source species should be applied directly or as a part of serum preparation (Prince *et al.*, 1973; Boscato and Stuart, 1986; Boerman *et al.*, 1990). As the most commonly used assay antibodies are of murine origin, the addition of free polyclonal mIgGs in native or denatured form has been used to decrease interferences associated with heterophilic-type interferences specific to mouse antibodies (Boscato and Stuart, 1988; Primus *et al.*, 1988). The effects of these polyclonal mIgG preparations originating in a number of different animals are based on the fact that they contain all possibly cross-reactive allotypic and idiotypic epitopes of mIgG (Vaidya and Beatty, 1992; Reinsberg, 1996).

Denatured antibodies have proven to be more potent as blockers than native antibodies (Bjerner *et al.*, 2002). One plausible reason for this is that aggregated antibodies are more prone to form stable and intricate complexes with the interfering antibodies. When aggregated, several potent parts are in close proximity, thus providing a more alluring target for the interfering antibodies to bind to (Bolstad *et al.*, 2013).

Several different specific blocking reagents with distinct specificities and structures are commercially available (**Table 2**). The advantage of specific antibodies is that they are high-affinity antibodies (10^9 L/mol vs. 10^6 L/mol unspecific blockers), allowing them to be used in low concentrations (Kricka, 1999).

Table 2. Examples of commercially available specific blocking reagents.

Trade name	Consistency	Producer	Reference
Heterophilic Blocking Reagent (HBR)	Monoclonal mouse anti-human IgM	Scantibodies, (Santee CA)	(Kuroki *et al.*, 1995; Sosolik *et al.*, 1997)
MAB33 (MAK33)	Monoclonal $mIgG_1$	Roche Molecular Biochemicals	(Mössner *et al.*, 1990; Bjerner *et al.*, 2002)
Poly MAB33 (& other MAB33 family of	Polymeric monoclonal $mIgG_1$/Fab	Roche Molecular Biochemicals	(Kricka, 1999)
Immunoglobulin Inhibiting Reagent (IIR)	IgG and IgM (from multiple species) with high affinity to HAAA	Bioreclamation Inc, (East Meadow, NY)	(Nicholson *et al.*, 1996; Reinsberg, 1998)
Super Chemiblock	Igs with high affinity to RF/HAMA	Millipore, (Danvers, MA)	(Waterboer *et al.*, 2006; van Gageldonk *et al.*, 2011)
Heteroblock	Mixture of active and passive blocking reagents	Omega Biologicals Inc. (Bozeman, MT)	(Hueber *et al.*, 2007)
Heterophilic Blocking Tube (HBT)	Lyophilized binders against heterophilic antibodies (500 µl sample /tube)	Scantibodies	(Butler and Cole, 2001)

In the original study on MAK33, Mössner *et al.* (1990) reported that when 10,000 sera samples were analyzed in a 1-step assay for CEA, 72 samples were noticed to contain heterophilic interferences. Out of the 72 samples, 7 could not be corrected with the addition of non-immune mIgG, but showed normal values with the addition of MAK33. In a study by Bjerner *et al.* (2002) immunoassay interference was observed to be 4%, but when heat-treated MAK33 was applied, the interference incidence dropped to 0.86%.

Both IIR and HBR have shown to be specific blocking agents in HAMA interferences observed in patients that have had cancer treatments with therapeutic antibodies (Nicholson *et al.*, 1996; Sosolik *et al.*, 1997). In a study by Reinsberg, IIR blocked HAMA interferences in more cases than MAK33, but not in as many cases as polyclonal non-immune mIgG. The proposed reason for this was that HAMA is usually directed against the allotypic and cross-reactive idiotypic antibody determinants, thus preventing the blocking effect of reagents such as MAK33 designed to block unspecific heterophilic interferences. (Reinsberg, 1998) However, both IIR and MAK33 have also proven their utility in blocking other than specific HAAA interferences. In an assay for CEA, the addition of HBR decreased heterophilic-type interference from 29% to 4.8%, when the addition of normal mouse sera to 1% of final assay volume decreased the interference only to

6.2% (Kuroki *et al.*, 1995). In another study, the addition of IIR decreased the interference in serum samples from 28% to 0% and 12% to 0% in rheumatoid arthritis and osteoarthritis patients, respectively (DeForge *et al.*, 2010). An alternative to HBR is the use of Heterophilic Blocking Tubes (HBTs). It has been reported that in 11 samples that were observed to give falsely elevated hCG values and were treated with HBTs, 9 were fully blocked and even the remaining 2 were observed to have >50% reduced analyte values (Butler and Cole, 2001).

It has been demonstrated that the addition of 3 µg/mL of HeteroBlock in serum diluent yielded effects comparable to immunoglobulin depletion by protein L-sepharose precipitation (Hueber *et al.*, 2007). The use of Super Chemiblok has mostly been restricted to multiplex bead-based fluorescent immunoassays (Luminex technology), where it has been noted that the incubation of sample serum with Super Chemiblok significantly decreases the unspecific human antibody binding to the assay beads (Waterboer *et al.*, 2006).

All the available blockers serve an important purpose, but their applicability is highly case-specific. Hence, the addition of different types of blockers can be used to identify the type of interference in a specific sample. Often, a combination of different blocking approaches is needed to obtain the desired result: de Jager *et al.* (2005) observed a reduced non-specific binding and more accurate recovery rates of cytokines, when using the protein L-sepharose in a combination with 10% rodent serum as a blocker. This combination treatment resulted in 89% depletion of plasma IgM RFs.

When a false positive result is obtained that does not correlate with the clinical picture of the patient, the recommended procedures include linearity studies, recovery experiments, and sample treatment with HBTs, or an addition of blocking reagents with re-measuring the sample with an alternate assay (Sturgeon and Viljoen, 2011; Ismail, 2007). As the rate of assay interference varies greatly between different assay types and analytes, the comparison of a set of different blockers is often impossible (Marks, 2002). The fact that commercial assays contain certain blockers, cuts back the interference problem but does not totally abolish it: when 21,000 samples were screened with routine testing for thyroid function, 7 patients (0.03%) showed falsely increased analyte values (Ward *et al.*, 1997). It has also been reported that not all blocking agents reduce interferences as expected, and sometimes they can even increase falsely elevated analyte values with certain assays (Koshida *et al.*, 2010; van Gageldonk *et al.*, 2011). Different screening techniques have also been tested, but their use is not currently warranted (Emerson *et al.*, 2003). It has been proposed that in samples with a high likelihood of false positives (e.g., tumor markers) it would be more cost-efficient to add a blocker to all tests rather than re-measure all positive results (Bjerner *et al.*, 2012). As different types of immunoassay interferences are regularly encountered in the clinical laboratories, different and novel approaches eliminating them should be thoroughly studied. It is also important to acknowledge the possible presence of these interferences and encourage communication between clinical laboratories and physicians.

3 AIMS OF THE STUDY

The overall aim of this thesis was to develop sensitive, fast, and minimally interference-prone immunoassays for cTnI that could be utilized in POCT settings. For this purpose intrinsically fluorescent Eu-doped nanoparticles were employed as labels, recombinant antibody fragments were utilized when possible, and the specificity of different cTnI-specific antibodies was studied.

More specifically, the aims were:

I To illustrate that intrinsically fluorescent nanoparticles can be universally employed in immunoassays designed for POCT applications, whether the primary assay requirement is sensitivity or a wide dynamic range around a predetermined analyte concentration.

II To study the effects that different antibody molecular forms have on matrix-related interferences in a cTnI research immunoassay.

III To present a novel heterogeneous research immunoassay for cTnI and to emphasize the effects the removal of antibody Fc-region and chimerization of Fab-fragment have on matrix-related interferences in samples containing known amounts of possibly interfering substances.

IV To evaluate the skTnI cross-reactivity of four different cTnI antibodies in four different versions of a cTnI research assay possessing identical epitope specificity determined by peptide mapping.

4 SUMMARY OF MATERIALS AND METHODS

A summary of materials and methods is described here. More detailed information can be found in the original publications (**I–IV**).

4.1 Samples

The summary of sample panels used in the study is described in **Table 3**. All samples were collected according to normal laboratory routines and were stored at -20°C for short-term preservation or at -70°C for long-term preservation. Informed consent was obtained from all participants and the study protocols were approved by the local ethics committees. All the study protocols were in accordance with the Helsinki declaration as revised in 2006.

Samples from Oulu University hospital (**I**, **III**, **IV**) were collected, and the reference analyte value was measured, as a part of routine analysis. Thereafter, the samples were frozen and shipped to the University of Turku without any identification labels or final diagnoses. Routine in-house plasma samples that were collected at the University of Turku, Department of Biotechnology (UTU/BT) were used for the optimization process of the immunoassays, as well as for assessing the matrix-related interferences of the different assay versions (**II–IV**). All the normal samples used in cTnI assay versions were tested for cardiac specific autoantibodies (Eriksson *et al.*, 2005a; Savukoski *et al.*, 2014).

Table 3. Description of the samples used in the studies. Samples obtained from one individual may have been used in several studies.

Collection place	Study population	Matrix	Number of samples	Publication and use
Oulu University Hospital	Left over	Citrate plasma	65	**I** D-Dimer method comparison
UTU/BT	Healthy volunteers	Heparin plasma /serum	32(**I**)/64(**III**) / 3(**IV**)	**II, III, IV** Assay interference studies/ recovery & reference population studies
Oulu University Hospital	Left over	Heparin plasma	265(**III**)/ 101(**IV**)	**III, IV** Method comparison
ProMedDx (Norton, MA, United States)	Triglyceride containing	Heparin plasma	12	**III** Assay interference studies
	Bilirubin containing	Heparin plasma	12	
	RF containing	Serum	12	
	HAMA containing	Serum	3	

4.2 Reagents

4.2.1 Antibodies

The antibodies used in the studies are listed in **Table 4**. According to the manufacturer, Mab-FDP14 recognizes the degraded forms of human fibrin and fibrinogen, but not intact fibrin and fibrinogen, and F(ab')$_2$-8D3 recognizes the D-dimer domain contained in the cross-linked degradation products of human fibrin. All the used commercial cTnI antibodies were claimed to be cTnI specific by their original manufacturers.

Table 4. Antibodies used in the studies.

Clone name	Specificity	Molecular form	Epitope (amino acid residues)	Manufacturer	Publication
19C7	cTnI	Mab	41–49	HyTest Ltd. (Turku, Finland)	**II–IV**
8I7	cTnI	Mab	169-178[a] (137–148)	International Point of Care (IPOC, Toronto, Canada)	**I–IV**
9707	cTnI	Mab/F(ab')$_2$/ Fab/cFab[b]	190–196	Medix Biochemica (Kauniainen, Finland)	**I–IV**
625	cTnI	Mab	169–178	HyTest Ltd.	**IV**
MF4	cTnI	Fab	190–196	HyTest Ltd.	**IV**
11N11	cTnI	cFab	160–179	UTU/BT	**III**
FDP14	D-dimer	Mab	-	Biokit (Barcelona, Spain)	**I**
8D3	D-dimer	F(ab')$_2$	-	Biokit	**I**

[a] (Vylegzhanina *et al.*, 2013)
[b] cFab, chimeric fragment antigen binding

The F(ab')$_2$-9707 used in the assays was produced from Mab-9707 by enzymatic digestion with bromelain (ID-Diluent 1; Diamed, Cressier, Switzerland) (Väisänen *et al.*, 2006). Recombinant Fab-9707 and Fab-MF4 were cloned from hybridoma cell lines of the corresponding Mabs at the Department of Biotechnology, University of Turku. The cFab-11N11 used was developed at the University of Turku Biotechnology department. Both cFab-11N11 and cFab-9707 were modified as mouse/human chimeric antibody fragments by replacing the constant parts of the light and heavy chains originating from mouse with the corresponding human antibody sequences. Unlike cFab-11N11, Fab/cFab-9707 and Fab-MF4 were designed to contain an unpaired cysteine residue in the C-terminal end of the heavy chain peptide to facilitate their site-specific biotinylation.

4.2.2 Calibrators

4.2.2.1 D-dimer

D-dimer is a degradation product of fibrin that is liberated into circulation in certain pathological conditions, e.g., venous thromboembolism (VTE), which can be classified as deep vein thrombosis and pulmonary embolism (Bounameaux *et al.*, 2010). D-dimer is a term used for several cross-linked fibrin degradation products (FDPs) of various sizes, all containing the D-dimer epitope. The sizes of the FDPs vary from 200 kDa of free D-dimer fragment to over 2300 kDa (Walker and Nesheim, 1999). Although present in VTE, D-dimer is not VTE specific; slightly elevated D-dimer levels can also be measured in several different illnesses and medical conditions, including infections, traumas, collagen and autoimmune diseases, atherosclerosis, and even in uncomplicated pregnancies in healthy women (Eichinger, 2005; Adam *et al.*, 2009). This increase in the baseline level of D-dimer may be due to a condition in which a formation of fibrin occurs, but no evident thrombus is formed (Brenner *et al.*, 1990). Nonetheless, the risk for thrombosis is increased in many cases (Heit *et al.*, 2001; Isma *et al.*, 2009). The widely used clinical cut-off level of D-dimer is 500 ng/mL fibrinogen equivalent units (FEU) (Bounameaux *et al.*, 2010).

The D-dimer calibration material was purchased from Biokit, and was obtained as partially purified D-dimer from human fibrin that was digested with human plasmin. The calibrator was diluted in a buffer containing 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4, and 10 g/L BSA (pH 7.2).

4.2.2.2 Troponin I

Human cTn (native, tissue derived, cTnI-cTnT-TnC complex) and native skTnI were purchased from HyTest Ltd. The skTnI was originally diluted with urea/Tris buffer, pH 7.5 (7 mol/L urea, 5 mmol/L ethylenediaminetetraacetic acid, 15 mmol/L mercapthoethanol, 20 mmol/L Tris). All TnI calibrators used in the assays were prepared by diluting them with Tris-based buffer (50 mmol/L Tris, 150 mmol/L NaCl, 0.5 g/L NaN_3) supplemented with 75 g/L BSA (pH 7.75).

4.2.3 Other reagents and assay buffers

All immunoassays were conducted in low-fluorescent streptavidin-coated 96-well microtitration wells (Kaivogen Oy, Turku, Finland). In this study, two immunoassay approaches, methodologically slightly different from each other, were studied. The preincubation of the sample and particles (**I**) was conducted on microtitration wells (Nunc, Roskilde, Denmark) coated with 5 g/L BSA. All solid-phase antibody dilutions were prepared to assay buffer (Kaivogen Buffer Solution Red, Kaivogen Oy) and microtitration wells were washed with Kaivogen wash solution (Kaivogen Oy). In study **I**, particles were diluted with particle buffer A, which consisted of assay buffer supplemented with 0.1 g/L native mIgG and 0.05 g/L denatured mIgG. In all the versions of the cTnI research assay (no

preincubation step, **II–IV**) the particles were diluted with particle buffer B containing: 37.5 mmol/L Tris, pH 7.75, 500 mmol/L NaCl, 0.4 g/L NaN_3, 0.6 g/L BGG, 25 g/L BSA, 50 g/L D-trehalose, 0.8 g/L native mIgG, 0.05 g/L denatured mIgG, 2 g/L casein, and 37.5 IU/mL heparin, modified from (von Lode *et al.*, 2003). In the assay based on chimeric antibody fragments (**III**), 0.15 mol/L biotinylated 5 kDa PEG was supplemented into the particle buffer B.

4.3 Preparation of assay reagents

4.3.1 Carboxyl-modified nanoparticles

Europium-fluorescent Fluoro-Max™ polysterene particles (Seradyn Inc, IN) were employed throughout this study. The particle size employed for publications **I**, **II,** and **IV** was 107 nm, whereas the size used for publication **III** was 100 nm. NHS- and EDC-chemistries at +23 °C and under vigorous shaking were used in all particle coatings.

The 107 nm particles (e.g., 1.5 x 10^{12} units) were washed with a phosphate buffer (10 mmol/L, pH 7.0) using microfiltration centrifugal devices (300 kDa cut-off, Pall, MA), and they were activated for 15 minutes with 10 mmol/L sulfo-NHS and 0.75 mmol/L EDC. The covalent coupling of detection antibodies (Mab-9707, **I;** Mab-817, **I, II,** and **IV**; Mab-FDP14, **I**; F(ab')$_2$-8D3, **I**; MAb-625, **IV**) was performed in 10 mmol/L phosphate buffer supplemented with 100 mmol/L NaCl (pH 8.0, 2h). The amounts of antibody used varied between 0.5 and 1.0 g/L. The blocking of the remaining active sites on the particles (10 g/L BSA, 15 minutes), as well as the final storage of the conjugated particles was done in a Tris-based buffer (e.g., 10 mmol/L Tris, 0.5 g/L NaN_3, pH 8.5). For particle storage, the buffer was supplemented with 2 g/L BSA.

The 100 nm particle coating (**III**) was executed in a 2-(*N*-morpholino)ethane-sulfonic acid (MES) buffer (20 mmol/L, pH 6.0). First, the particles (1.35 x 10^{12} units) were washed by using microfiltration centrifugal devices (300 kDa cut-off), after which they were activated for 15 minutes with 8 mmol/L sulfo-NHS and 1.5 mmol/L EDC. Then, cFab-11N11 (0.5 g/L) was let to adsorb to the activated particles for 30 minutes (20 mM MES, 100 mmol/l NaCl, pH 6.0.). After the 30-minute adsorption, the covalent coupling of the activated particles and cFab-11N11 was generated by increasing the reaction pH to 8.0 with 1M Na_2CO_3/$NaHCO_3$ -buffer (pH 9.3), and the reaction was continued for an additional 30-minute period. The blocking of the remaining active sites on the particles (10 g/L BSA, 30 minutes), as well as the final storage of the conjugated particles was done in Tris-based buffer (25 mM Tris, 150 mmol/L NaCl, 1 g/L NaN_3, pH 7.8). For particle storage, the buffer was supplemented with 2 g/L BSA.

Before the first instance of use, the particle solutions were mixed and sonicated. After that, the solutions were centrifuged to remove noncolloidal aggregates

(350 x g, 5 min). The particle concentrations were determined by diluting the particles with 0.1% (w/v) Triton X100 solution, and by comparing the time-resolved fluorescence of the preparation to a particle calibrator prepared from uncoated particle stock. The measurements were conducted with Victor™ 1420 multilabel counter (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland)

4.3.2 Labeling of solid-phase antibodies

Mab-FDP14 and F(ab')$_2$-8D3 (**I**) were biotinylated to free NH_2-groups with EZ-Link® NHS-Chromogenic-Biotin (Thermo Scientific, Waltham, MA, United States) for 60 minutes at +23 °C while protected from light. Mab-FDP14 (1.0 mg/mL) was biotinylated in 50 mmol/L Na_2CO_3/$NaHCO_3$ –buffer supplemented with 150 mmol/l NaCl (pH 9.6) with a 10-fold molar excess of the labeling reagent, whereas F(ab')$_2$-8D3 (1.0 mg/mL) was biotinylated in 100 mmol/L phosphate buffer (pH 7.2) supplemented with 150 mmol/L NaCl with a 5-fold molar excess of the labeling reagent.

Mab-19C7 (1.5 mg/mL, **II–IV**) and Mab-9707 (0.6 mg/mL, **I–II**) were biotinylated to free NH_2-groups with a 10-fold, Mab-8I7 (1.4 mg/mL, **I**) with 20-fold, and F(ab')$_2$-9707 (0.9 mg/mL, **II**) with a 30-fold molar excess of biotin isothiocyanate (University of Turku, Turku, Finland) (Eriksson *et al.*, 2003). Recombinant mouse antibody fragments Fab-9707 (**II**) and Fab-MF4 (**IV**) were biotinylated site-specifically to C-terminal free cysteine as a part of their production and purification process with EZ-Link® Maleimide-PEG$_2$-Biotin (Thermo Scientific) (Ylikotila *et al.*, 2006).

After the biotinylation reactions, all the antibodies were purified twice with NAP gel filtration colums (GE Healthcare Life Sciences, NY, United States) into 50 mmol/L Tris-HCl (pH 7.75), containing 150 mmol/L NaCl and 0.5 g/L NaN_3. The labeled antibodies were stabilized with 1 g/L BSA and stored at +4 °C.

4.4 Immunoassays

4.4.1 Immunoassay with preincubation step (I)

All the utilized immunoassays were performed as heterogeneous non-competitive immunoassay format. In publication **I**, an additional preincubation step was employed in order to simulate simple microfluidic chip processing, where the sample and detection antibodies are introduced prior to transferring them to the solid-phase surface. The assays utilized antibody pairs Mab-FDP14 – F(ab')$_2$-8D3 (D-dimer) and MAb-9707 – Mab-8I7 (cTnI) so that both antibodies in the pairs were tested as solid-phase and detection antibodies.

The preincubation of the particles (coated with Mab-FDP14, F(ab')$_2$-8D3, Mab-9707, or Mab-8I7) and sample was done so that 12 µl of sample or standard with 1.44 x 10^9 (D-dimer) or 1.8 x 10^8 (cTnI) antibody-coated particles were added in

48 µl of particle buffer A along with 0–25 µg/mL free solid-phase or detection antibodies to BSA-coated wells. After a preincubation of 30 seconds (+23 °C, shaking at 900 rpm), 50 µl of the solution was transferred to a fresh solid-phase surface.

The solid-phase surface was constructed on streptavidin-coated wells by incubating 120 ng of biotinylated solid-phase antibody Mab-FDP14, F(ab')$_2$-8D3, Mab-9707, or Mab-8I7 in 60 µL of assay buffer at +23°C with shaking at 900 rpm for 60 minutes. After the bio-incubation, the wells were washed with wash solution and the preincubated sample-particle solution was added into the wells.

The assay wells were incubated for either 5- or 15-minutes (+23 °C, shaking at 900 rpm) before washing with wash solution and a direct surface measurement of the long life-time fluorescence of the nanoparticles with Victor™ 1420 Multilabel Counter. The 15-minute assay incubation was employed only in method comparison. The basic principle of the developed two-site immunoassay with preincubation can be seen in **Figure 7.**

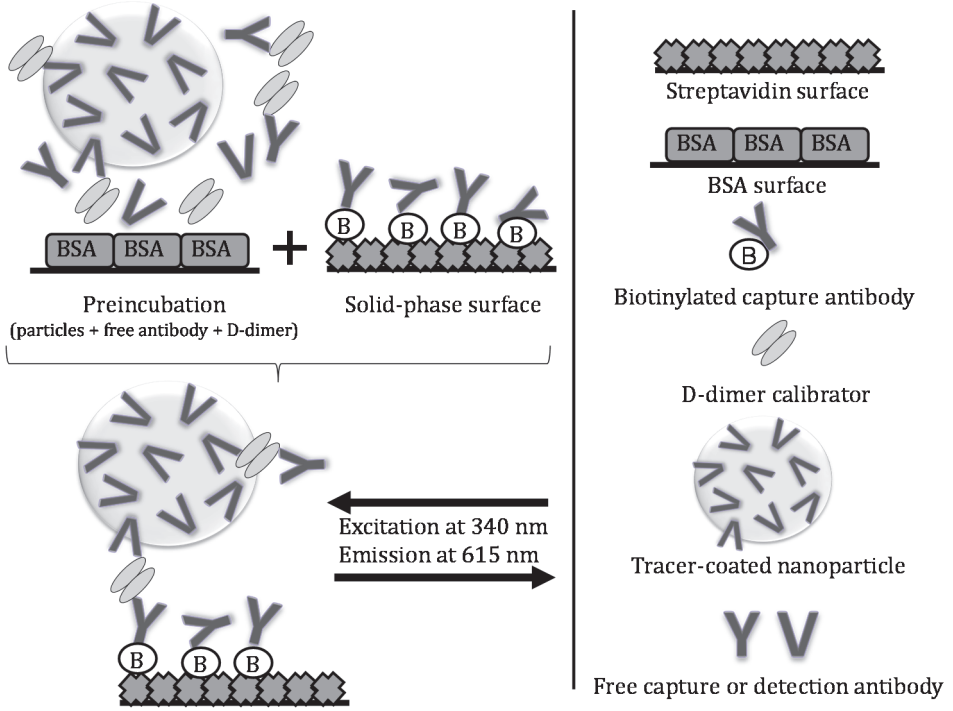

Figure 7. The basic principle of an immunoassay with a preincubation step used for immunoassay dynamic range extension (**I**). A D-dimer assay using Mab-FDP14 as the solid-phase and F(ab')$_2$-8D3 as the detection antibody attached to nanoparticles is used as an example. Adapted from (**I**).

4.4.2 Immunoassays for cardiac troponin I (II–IV)

All the different cTnI assay versions used in the original publications **II**–**IV** employed identical antibody configuration and assay epitopes as evidenced by peptide mapping. The assay epitopes were designed to employ an antibody configuration, where inhibiting effects of circulating autoantibodies mainly bound to the central part of cTnI (amino acid residues 30–110) would be minimal (Eriksson *et al.*, 2005b). The immunoassay configuration utilized in all assay versions is shown in **Figure 8**.

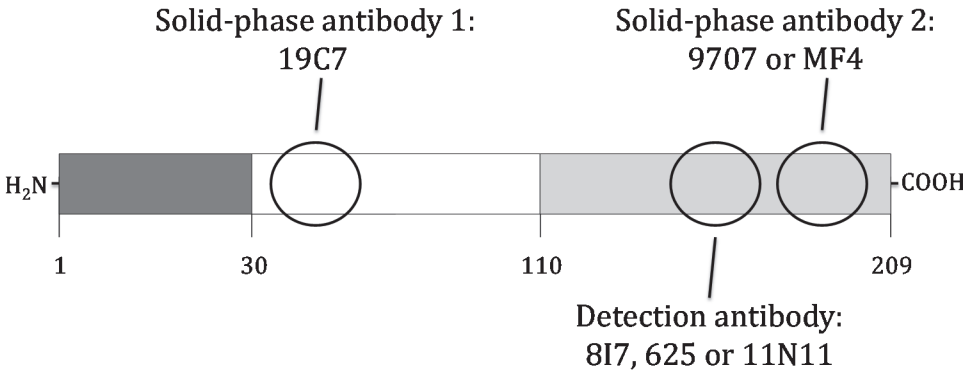

Figure 8. A schematic presentation of the antibody configuration and epitope specificities used in the different versions of a research assay for cTnI (**II–IV**). The bar represents the linear amino acid sequence of cTnI. The N-terminal region of cTnI (amino acid residues 1–29) is shown in dark grey, the central region in white and the C-terminal region (amino acid residues 111–209) in light grey. Circles indicate the approximate epitopes of the different antibodies used.

All the assays were performed so that fixed amounts of biotinylated solid-phase antibodies were incubated in 50 µL of assay buffer at +23 °C for 60 minutes in streptavidin-coated wells. After the incubation, the wells were washed with wash solution and 3.75 x 10^8 nanoparticle-bioconjugates in 40 µl particle buffer B along with 10 µL of calibrator solution or sample was applied to the solid-phase surface followed by a 15-minute incubation at +36 °C (650 rpm). After sample incubation, the wells were washed with wash solution and time-resolved fluorescence was measured from the assay wells as described in section 4.4.1.

The different versions of the cTnI assays developed during the study were assigned a version number by which they are later referred to. The specific amounts of the solid-phase antibodies, detection particles, and the assigned assay version numbers are listed in **Table 5.**

Table 5. Antibody and particle amounts of the different cTnI assay versions.

Assay version	**Solid-phase**	**Detection**
	Study **II** (Assays utilizing different molecular forms of 9707)	
1.	Mab-19C7 + Mab-9707 (100 + 100 ng/well)	Mab-8I7 ($3.75x10^8$ particles/well)
2.	Mab-19C7 + F(ab')$_2$-9707 (100 + 66 ng/well)*	
3.	Mab-19C7 + Fab-9707 (25 + 16.5 ng/well)	
4.	Mab-19C7 + cFab-9707 (25 + 16.5 ng/well)	
	Study **III** (Assay utilizing cFabs)	
5.	Mab-19C7 + cFab-9707 (25 + 16.5 ng/well)	cFab-11N11 ($3.75x10^8$ particles/well)
	Study **IV** (Assays utilizing different commercial antibodies)	
6.	Mab-19C7 + Fab-9707 (50 + 33 ng/well)	Mab-8I7 ($3.75x10^8$ particles/well)
7.	Mab-19C7 + Fab-9707 (50 + 33 ng/well)	Mab-625 ($3.75x10^8$ particles/well)
8.	Mab-19C7 + Fab-MF4 (50 + 33 ng/well)	Mab-8I7 ($3.75x10^8$ particles/well)
9.	Mab-19C7 + Fab-MF4 (50 + 33 ng/well)	Mab-625 ($3.75x10^8$ particles/well)

*(Järvenpää *et al.*, 2012)

4.5 Assay evaluations

4.5.1 Methodological evaluation (I, III, IV)

Assay sensitivities were assessed from the dose-response curves and were calculated either as the concentration deviating three standard deviations from background signal (analytical detection limit; **II**, **IV**), or by calculating the assay limit of blank (LoB; **I**, **III**), limit of detection (LoD; **III**), and limit of quantitation (LoQ; **III**) according to the Clinical and Laboratory Standards Institute (CLSI) Guidelines EP17-A2 (Clinical and Laboratory Standards Institute, 2012). Within-run and within-laboratory imprecisions were assessed according to the CLSI Guideline EP5-A2 (**III**) (Clinical and Laboratory Standards Institute, 2004).

Assay recoveries were determined from heparin plasma samples fortified with a native troponin complex so that the added volume did not exceed 5% of the total sample volume (**III**). The determination of assay cross-reactivities was executed in a similar manner, but by using serum samples and skTnI (**IV**). The recovery percentages were calculated as the increase in cTnI concentration compared to the expected increase, and the percentual cross-reactivities were calculated as the observed skTnI-signal converted to cTnI concentration and compared to the added skTnI concentration. Assay linearity was assessed by diluting patient samples containing known amounts of cTnI with a plasma pool with a signal below the assay LoD (**III**).

4.5.2 Method comparisons (I, III)

Samples described in section 4.1 were used for methodological comparisons to commercial assays. For D-dimer the comparison assay was quantitative immunoturbidimetric latex agglutination STA Liatest D-dimer -assay (Diagnostica Stago, NJ, United States) (Waser *et al.*, 2005). For the method comparison, the developed D-dimer assay was performed with a 15-minute incubation (**I**). Assay version 5 for cTnI utilizing chimeric antibody fragments was compared to Siemens ADVIA Centaur® TnI-Ultra™ assay (Casals *et al.*, 2007) (**III**).

4.5.3 Assay interferences (II–IV)

Matrix-related interferences were evaluated by measuring the apparent cTnI concentrations of heparin plasma samples obtained from 32 apparently healthy volunteers with assay versions 1–4, where antibody 9707 was modified (**II**). The effect of using cFabs as both the solid-phase and detection antibodies was studied with the assay version 5 by measuring the cTnI values of 39 samples containing known amounts of triglycerides, bilirubin, RFs, or HAMA, and the results were then compared to those of the previously published version 2 of the same assay (Järvenpää *et al.*, 2012) (**III**). The effect of antibody selection on assay specificity was evaluated by comparing the calculated cross-reactivity values of four different assay versions (6–9) employing different assay antibodies, sold as cTnI-specific, without changing the assay epitope specificity determined by peptide mapping. Also, a clinical sample panel was measured with the four different assay versions. Clinical samples that were observed to have a deviating assay result with the assay version 6 were further measured with an unpublished research assay for skTnI (**IV**).

4.5.4 Statistical analyses (I–IV)

Statistical analyses were conducted using SAS Enterprise guide 3.0 and SAS software version 9.1 (SAS Institute; NC, USA) (**II**) and IBM SPSS statistics 21 (SPSS Inc., Chicago, IL) (**I**, **III**, **IV**). The two-tailed P-values <0.05 were considered to be statistically significant. Correlations between the different assays were studied with Spearman's rank correlation (**I–IV**). In the method comparison, Passing & Bablok or weighted Deming regression parameters were calculated with Analyze-it software (versions 2.30 (**I**,**III**) or 3.71.1 (**IV**), Analyze-it Software Ltd., Leeds, United Kingdom). Linear regression parameters were calculated with Origin Data Analysis and Graphing Software (version 8.0, OriginLab Corporation, Northampton, MA) (**II**). The agreement between different assays was assessed by Bland-Altman analyses (**I**,**III**) (Bland and Altman, 1986). Nonparametric Kruskal-Wallis and Mann-Whitney U-tests were used to compare the differences between the four different captures and between continuous variables of two different captures (nongaussian distribution) (**II**).

5 SUMMARY OF RESULTS

5.1 Nanoparticles as universal labels (I)

5.1.1 Adjustment of assay dynamic range

To study whether Eu nanoparticles can universally be applied in heterogeneous immunoassays requiring speed and wide measuring range, different approaches to extending immunoassay dynamic range were applied by using D-dimer and cTnI as model analytes. The ultimate goal was to develop assays that would be applicable to POCT. Therefore, in order to simulate simple microfluidic chip processing where the sample and detection antibodies are combined prior to their introduction to a solid-phase surface, a 30-second preincubation of the sample and particle solution was analyzed.

The desensitization process was analyzed so that the D-dimer and cTnI-specific antibodies (Mab-FDP14, F(ab')$_2$-8D3, Mab-9707, or Mab-8I7) were tested both as the solid-phase and detection antibodies. The effect of antibody configuration and the addition of free antibody on the upper limit of assay dynamic range, determined visually as the highest calibrator concentration in the linear part of the calibration curve, and LoB can be seen in **Table 6**.

Table 6. The calculated LoBs and dynamic ranges of the different assay versions with 5-minute assay incubation. Modified from (**I**). The universally used clinical cut-off level of D-dimer is 500 ng/mL (FEU) (Bounameaux et al., 2010).

	LoB (ng/mL)	Dynamic Range (ng/mL)
D-dimer		
F(ab')$_2$ -8D3 (solid-phase) / MAb-FDP14 (detection)		
0 µg/mL free Mab-FDP14/F(ab')$_2$-8D3	0.249	≤50
5 µg/mL free F(ab')$_2$-8D3	0.463	≤50
15 µg/mL free F(ab')$_2$-8D3	0.158	≤200
5 µg/mL free Mab-FDP14	2.73	≤1000
15 µg/mL free Mab-FDP14	13.6	≤10,000
Mab-FDP14 (solid-phase) / F(ab')$_2$ -8D3 (detection)		
0 µg/mL free Mab-FDP14/F(ab')$_2$-8D3$_2$	1.17	≤500
1 µg/mL free Mab-FDP14	2.75	≤10,000
5 µg/mL free Mab-FDP14	478	≤10,000
5 µg/mL free F(ab')$_2$-8D3	0.331	≤1000
15 µg/mL free F(ab')$_2$-8D3	0.603	≤3000
cTnI		
Mab-8I7 (solid-phase) / Mab-9707 (detection)		
0 µg/mL free Mab-8I7/ Mab-9707	0.0100	≤25
10 µg/mL free Mab-8I7	1.03	≤50
5 µg/mL free Mab-9707	0.0356	≤100
10 µg/mL free Mab-9707	0.0808	≤250
25 µg/mL free Mab-9707	0.0367	≤1000
Mab-9707(solid-phase) / Mab-8I7 (detection)		
0 µg/mL free Mab-8I7/ Mab-9707	0.00300	≤25
10 µg/mL free Mab-9707	1.06	≤50
5 µg/mL free Mab-8I7	0.00850	≤50
10 µg/mL free Mab-8I7	0.0154	≤100
25 µg/mL free Mab-8I7	0.290	≤1000

With D-dimer, the addition of free Mab-FDP14 moved the upper limit of dynamic range more than the addition of free F(ab')$_2$-8D3. At best, an addition of 15 µg/mL of free Mab-FDP14 caused a 200-fold increase in the upper limit of the dynamic range from 50 ng/mL to 10,000 ng/mL (F(ab')$_2$-8D3/Mab-FDP14-configuration), and a simultaneous, nearly 55-fold decrease in assay sensitivity (LoB 0.249 ng/mL vs. 13.6 ng/mL). With Mab-FDP14 as the solid-phase and F(ab')$_2$-8D3 as the detection antibody, as little as 1 µg/mL free Mab-FDP14 raised the upper limit of the dynamic range to 10,000 ng/mL. With cTnI, both antibody configurations reacted almost identically to the free antibody addition: the highest raise in the upper limit of dynamic range, from 25 ng/mL to 1000 ng/mL, was achieved when 25 µg/mL of free detection antibody was added.

5.1.2 Performance of a 15-minute D-dimer assay

To test the performance of the D-dimer immunoassay with Mab-FDP14 as the solid-phase and F(ab')$_2$-8D3 as the detection antibody (with 1 µg/mL free Mab-FDP14, **Table 6**), the assay was run with 15-minute incubation and the results were compared to STA Liatest D-dimer assay. Deming regression analysis yielded a slope (95% confidence intervals, CI) of 0.0900 (0.0700–0.110) and a y-intercept of -7.79 (-17.9–2.29) ng/mL ($S_{y|x}$ 0.126 ng/mL) (**Figure 9A**). The mean relative difference (95% limits of agreement) with Bland-Altman agreement was 170% (131%–209%) (**Figure 9B**). The Spearman correlation coefficient was 0.906 (P<0.001). The measured median concentrations of the reference and the developed assays were (25th – 75th percentiles) 1700 ng/mL (575 ng/mL; 4485 ng/mL, FEU) and 111 ng/mL (36.5 ng/mL; 354 ng/mL), respectively.

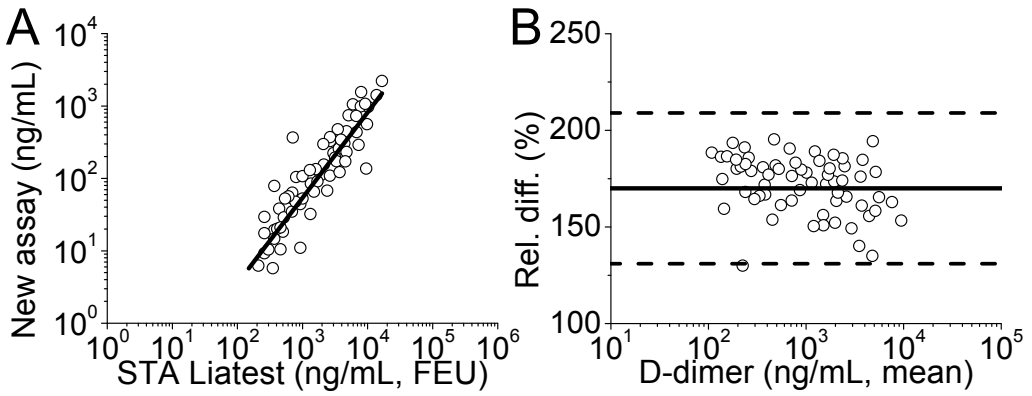

Figure 9. Method comparison of nanoparticle-based immunoassay for D-dimer (15-minute format) and STA Liatest D-dimer assay. (A) Correlation between the two assays (n=65, r=0.906). (B) Bland-Altman analysis of agreement. The mean difference of the two assays (170%) is presented with a solid horizontal line and the dotted lines represent the 95% limits of agreement (131%–209%). Modified from (**I**).

5.2 Analytical performance of immunoassays for cardiac troponin I

5.2.1 The effect of solid-phase antibody 9707 molecular form (II)

To test the effect of an antibody molecular form on the measured cTnI-levels, assay versions 1–4 were compared. The utilization of different antibody fragments did not significantly affect the assay signal level or its analytical sensitivity (**Figure 10**). The calculated analytical sensitivities (background + 3SD) were: assay version 1 (Mab) = 0.90 ng/L; assay version 2 (F(ab')$_2$) = 0.91 ng/L; assay version 3 (Fab) = 0.69 ng/L; and assay version 4 (cFAb) = 0.41 ng/L. All assays were linear up to 10,000 ng/L, and no high-dose hook was observed even when 1,000,000 ng/L cTnI was measured.

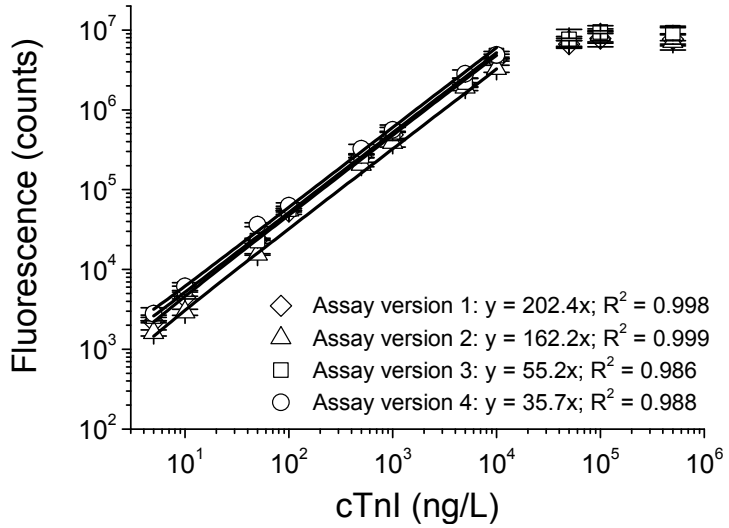

Figure 10. Dose-response curves of the assay versions 1–4 utilizing different forms of cTnI antibody 9707. The error bars represent the standard deviation from replicate wells. Modified from (**II**).

5.2.2 Chimeric recombinant antibody fragments (III)

To possibly further decrease different matrix-related interferences of the cTnI research assay, the detection antibody 8I7 was replaced with a cFab-11N11 in assay version 5. The assay version 5 was linear to 50,000 ng/L (R^2=0.993). The assay signal was increasing to 500,000 ng/L, and no high-dose hook was observed. Assay LoB and LoD were determined to be 1.35 ng/L and 3.30 ng/L, respectively. A typical dose-response curve of the assay 5 can be seen in **Figure 11**.

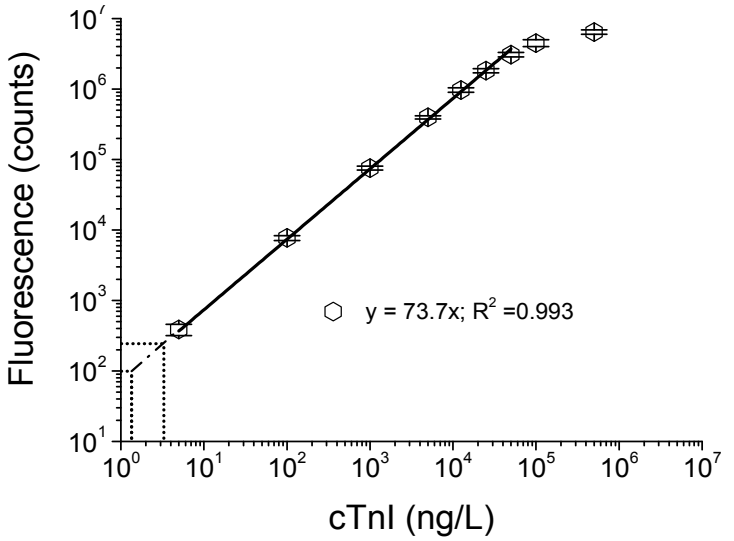

Figure 11. A typical dose-response curve of the assay version 5. The error bars represent the standard deviation from replicate wells. The dotted lines represent the calculated LoB (1.35 ng/L) and LoD (3.30 ng/L) of the assay. Modified from (**III**).

Assay recoveries were studied by spiking 64 plasma samples from young healthy individuals with 500 ng/L cTn complex. Assay recoveries, calculated as the measured cTnI from the 500 ng/L spiked cTnI, varied between 70% and 108% for samples found negative for troponin autoantibodies (average 83%, median 83%). For the 5 samples found as troponin autoantibody positive, the assay recoveries varied between 31% and 78% (average 53%, median 57%). The sample background was measured without added cTnI. Out of the 64 samples measured, 4.7% (n=3) gave cTnI values above the assay LoD of 3.3 ng/L (5.74 ng/L, 7.20 ng/L, and 11.8 ng/L).

Assay linearity was evaluated using six patient heparin plasma samples that were diluted 1–243-fold with a pool of normal plasma. The initial cTnI concentrations of the samples were: 22,262 ng/L; 14,540 ng/L; 10,145 ng/L; 2327 ng/L; 979 ng/L; and 718 ng/L. The assay showed good linearity (R^2=0.929–0.993) throughout the measured range (2.2 ng/L–22,261 ng/L) as seen in **Figure 12A**. The within-run and within-laboratory precisions determined by spiking a pool of cTnI negative plasma samples (UTU/BT) with endogenous cTnI obtained from patient samples can be seen in **Figure 12B**. The measured mean cTnI concentrations (twice a day for 20 days) were: 29 ng/L, 37 ng/L, and 2819 ng/L and the corresponding within-run imprecisions were 8.5%, 8.4%, and 7.5%. The within-laboratory precisions were 13.7%, 16.4%, and 15.9%, respectively. The assay LoQ calculated as functional sensitivity with within-laboratory precision of 20% was 17 ng/L.

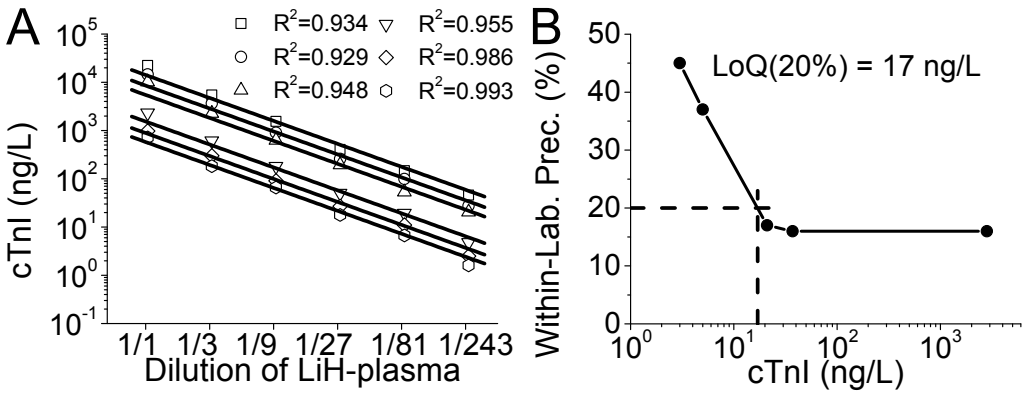

Figure 12. A) Linearity of the assay version 5 assessed with serial dilution (1–243-fold) of six clinical heparin plasma samples. B) Within-laboratory precision of the assay version 5, and the determination of assay LoQ (the lowest cTnI concentration measured with within-laboratory precision of 20%). Modified from (**III**).

To compare the cFab-based assay to a commercial cTnI assay, 265 leftover plasma samples from Oulu University Hospital were measured with the assay version 5 and the results were compared to those measured with Siemens ADVIA Centaur® TnI-Ultra™ assay. Out of the measured 265 samples, 17 were observed <LoD with

the assay version 5, and these were excluded from the method comparison analysis (6–300 ng/L; median, 20 ng/L). Passing & Pablok regression analysis yielded a slope of 0.180 (95% CI; 0.170–0.190) and a y-intercept of 1.94 (95% CI; -1.28–3.91) ng/L (**Figure 13A**). The mean relative difference (95% limits of agreement) with Bland-Altman agreement was 136% (62.3%–202%) (**Figure 13B**). The Spearman correlation coefficient was 0.965 (P<0.001). The measured median concentrations of the Siemens reference assay and the assay version 5 were (25th–75th percentiles) 1480 ng/L (202 ng/L; 6640 ng/L) and 241 ng/L (37.1 ng/L; 1290 ng/L), respectively.

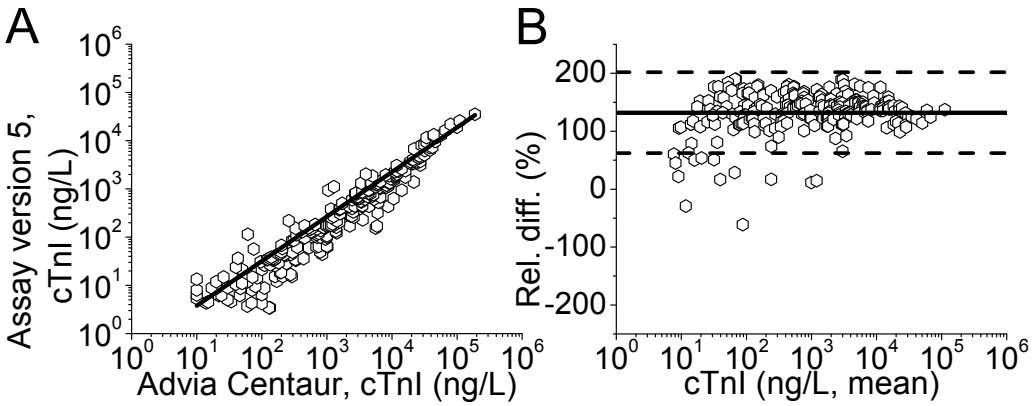

Figure 13. Method comparison. (A) Correlation between the cTnI assay version 5 and Siemens ADVIA Centaur® TnI-Ultra™ reference assay (n=248 r=0.965). (B) Bland-Altman analysis of agreement between the two assays. The mean difference (136%) is presented with a horizontal solid line. The dashed solid lines represent the 95% limits of agreement (62.3%–202%). Modified from (**III**).

5.2.3 Different commercial cTnI-specific antibodies (IV)

To test cTnI specificity, four different antibodies from three different manufacturers were tested in the research assay for cTnI, so that no changes in the antibody configuration and epitope specificity were made. The assay versions 6–9 all used Mab-19C7 as the first solid-phase antibody. With the combination of either Mab-8I7 or Mab-625 attached to intrinsically fluorescent nanoparticles, either Fab-9707 or Fab-MF4 was used as the second solid-phase antibody. The immunoassays with Mab-8I7 as the detection antibody (assay versions 6 and 8) were linear to ~15,000 ng/L, whereas the Mab-625-based assays (assay versions 7 and 9) were linear to ~50,000 ng/L. The signals were increasing, and no high-dose hook was observed with 500,000 ng/L cTnI (**Figure 14**). The calculated analytical sensitivities were: assay version 6, 1.25 ng/L; assay version 7, 2.34 ng/L; assay version 8, 1.82 ng/L; and assay version 9, 3.84 ng/L.

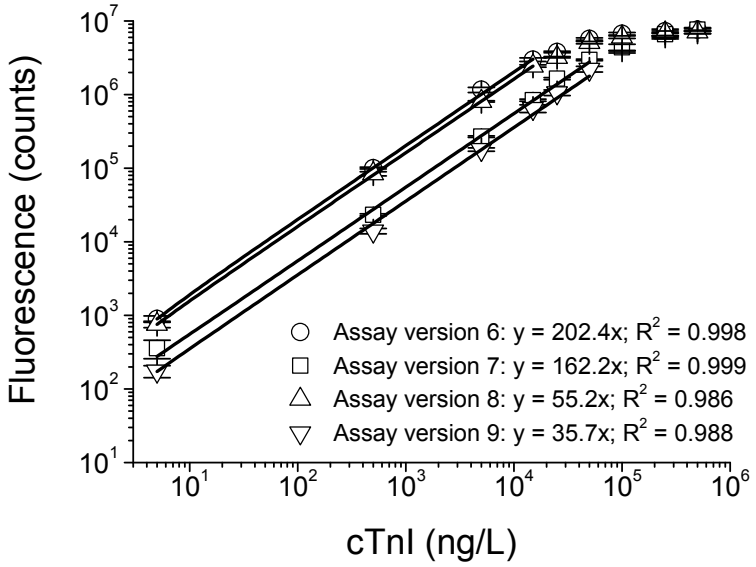

Figure 14. Dose-response curves of the four research assays versions utilizing different commercially available cTnI-specific antibodies. The error bars represent the standard deviation from replicate wells. Modified from (**IV**).

5.3 Cardiac troponin I immunoassay specificity

5.3.1 The effect of solid-phase antibody 9707 molecular form (II)

The effect of antibody 9707 molecular form (Mab, $F(ab')_2$, Fab and cFab) on the apparent cTnI levels were assessed by measuring 32 plasma samples from apparently healthy reference population with the assay versions 1–4. Minimum, maximum, median, and average values measured with cTnI assays exploiting the different molecular forms of antibody 9707 are presented in **Table 7.** and as a box-plot presentation in **Figure 15**. Values measured with assay version 1 correlated poorly with assay versions 2, 3, and 4: r=0.220, 0.0300, and 0.010 (P<0.001), respectively. The corresponding linear regression equations were: y=0.02166x+3.0937; y=0.002340x+2.7939; and y=0.0008870x+2.4106. The Spearman correlation coefficients for assay version 2 and versions 3 and 4 were significantly better r=0.770 (assay version 3) and r=0.780 (assay version 4). The corresponding linear regression equations were: y=0.6058x+0.7481 and y=0.6121x+0.3203, respectively. The correlation between the assay versions 3 and 4 was good: r=0.970 (y=0.9725x–0.3286).

Table 7. The measured cTnI concentrations of 32 plasma samples from apparently healthy individuals analyzed with the cTnI assay versions utilizing different molecular forms of antibody 9707. Modified from (**II**).

Assay version	Minimum (ng/L)	Maximum (ng/L)	Median (ng/L)	Average (ng/L)
1	2.64	116	7.28	15.9
2	1.45	11.1	2.60	3.44
3	1.10	11.2	2.51	2.83
4	0.746	10.6	1.80	2.42

Statistical analysis of the cTnI values measured with the four different assay versions showed significant differences between the four 9707 molecular forms ($P<0.001$, Kruskal-Wallis test). Mann-Whitney test also showed statistical differences between assay version 1 and the rest of the tested assay versions 2–4 ($P<0.001$). The difference between assay versions 2 and 3, as well as between versions 3 and 4 was insignificant: $P=0.38$ and $P=0.10$, respectively. However, the difference between assay versions 2 and 4 was observed to be statistically significant: $P=0.016$ (Mann-Whitney test).

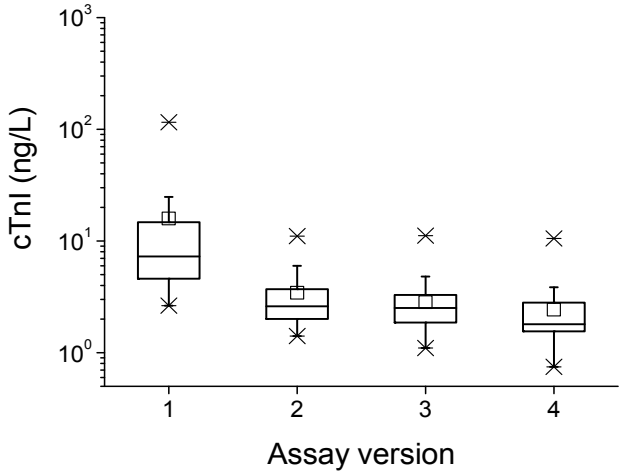

Figure 15. Box-plot presentation of the measured cTnI values (ng/L) from 32 apparently healthy volunteers measured with assay versions utilizing different molecular forms of 9707 antibody. The crosses indicate the minimum and maximum values; the boxes represent the 25–75th percentiles; the whiskers the 1–99th percentiles; the horizontal lines represent the median and the small boxes represent the mean. Modified from (**II**).

5.3.2 Chimeric recombinant antibody fragments (III)

The effect of utilizing chimeric antibody fragments (assay version 5) on matrix-related interferences was assessed by comparing measured cTnI levels from samples containing known amounts of triglycerides, bilirubin, RF, or HAMA to values measured with a previously published assay version 2 (Järvenpää *et al.*, 2012). The results from the two assays can be seen in **Table 8**.

Table 8. Comparison of plasma and serum samples containing known amounts of triglycerides, bilirubin, RFs, or HAMA measured with assay version 2 and assay version 5 employing cFab-fragments. Modified from (**III**).

		Assay version 2 (LoD = 2.0 ng/L)	Assay version 5 (LoD = 3.30 ng/L)
Concentration of possibly interfering substance	Interfering substance	measured cTnI concentration (ng/L)	measured cTnI concentration (ng/L)
5.73 g/L	Triglycerides	<LoD	<LoD
18.16 g/L	Triglycerides	<LoD	<LoD
10.55 g/L	Triglycerides	<LoD	<LoD
10.95 g/L	Triglycerides	<LoD	<LoD
48.19 g/L	Triglycerides	4.09	4.39
13.42 g/L	Triglycerides	<LoD	<LoD
11.02 g/L	Triglycerides	<LoD	<LoD
11.59 g/L	Triglycerides	6.30	3.27
10.57 g/L	Triglycerides	<LoD	<LoD
8.88 g/L	Triglycerides	<LoD	<LoD
7.53 g/L	Triglycerides	2.23	<LoD
10.41 g/L	Triglycerides	3.31	<LoD
0.002 g/L	Bilirubin	13.6	5.34
0.002 g/L	Bilirubin	<LoD	<LoD
0.05 g/L	Bilirubin	<LoD	<LoD
0.05 g/L	Bilirubin	<LoD	<LoD
0.11 g/L	Bilirubin	20.6	10.1
0.10 g/L	Bilirubin	10.4	<LoD
0.17 g/L	Bilirubin	4.75	<LoD
0.14 g/L	Bilirubin	7.39	<LoD
0.18 g/L	Bilirubin	7.64	3.92
0.32 g/L	Bilirubin	3.64	<LoD
0.32 g/L	Bilirubin	7.01	<LoD
0.14 g/L	Bilirubin	26.7	10.7
0.10 IU/L	RF	6.3	<LoD
0.10 IU/L	RF	<LoD	<LoD
0.03 IU/L	RF	<LoD	<LoD
0.34 IU/L	RF	<LoD	<LoD
0.34 IU/L	RF	3.81	<LoD
0.03 IU/L	RF	2.12	<LoD
1.06 IU/L	RF	7.43	<LoD
0.79 IU/L	RF	<LoD	<LoD
3.27 IU/L	RF	241	<LoD
1.00 IU/L	RF	3.04	<LoD
1.29 IU/L	RF	<LoD	<LoD
0.70 IU/L	RF	2.92	<LoD
0.30 g/L	HAMA	<LoD	<LoD
0.14 g/L	HAMA	<LoD	<LoD
0.18 g/L	HAMA	<LoD	<LoD

IU, international unit

In assay version 5, 15.4% (6/39) of the samples gave cTnI concentrations >LoD, when the corresponding value for assay version 2 was 51.3% (20/39). By removing the Fc-part from two out of three antibodies, interference from RF was totally eliminated in assay version 5: the highest value being 241 ng/L observed with assay version 2. Out of the 12 samples containing bilirubin, 33.3% (4) gave a measurable cTnI value >LoD with assay version 5, when the corresponding value was 75.0% (9) with assay version 2.

5.3.3 Different commercial cTnI-specific antibodies (IV)

Four different antibodies originally from three different manufacturers were tested as solid-phase – detector pairs in four different combinations (assay versions 6–9) so that the epitope specificity of the assay was retained. A total of 101 samples were analyzed with all of the four different assay versions. The Deming regression analyses for all six different comparisons with 95% CIs, and the Spearman correlation coefficients ($P<0.001$) can be seen in **Figure 16**. The measured median concentrations of the assays were (25th–75th percentiles) 583 ng/L (76.0 ng/L, 3290 ng/L, assay 6); 360 ng/L (55.0 ng/L, 2260 ng/L, assay 7); 390 ng/L (46.0 ng/L, 2570 ng/L, assay 8); and 268 ng/L (45.0 ng/L, 2240 ng/L, assay 9).

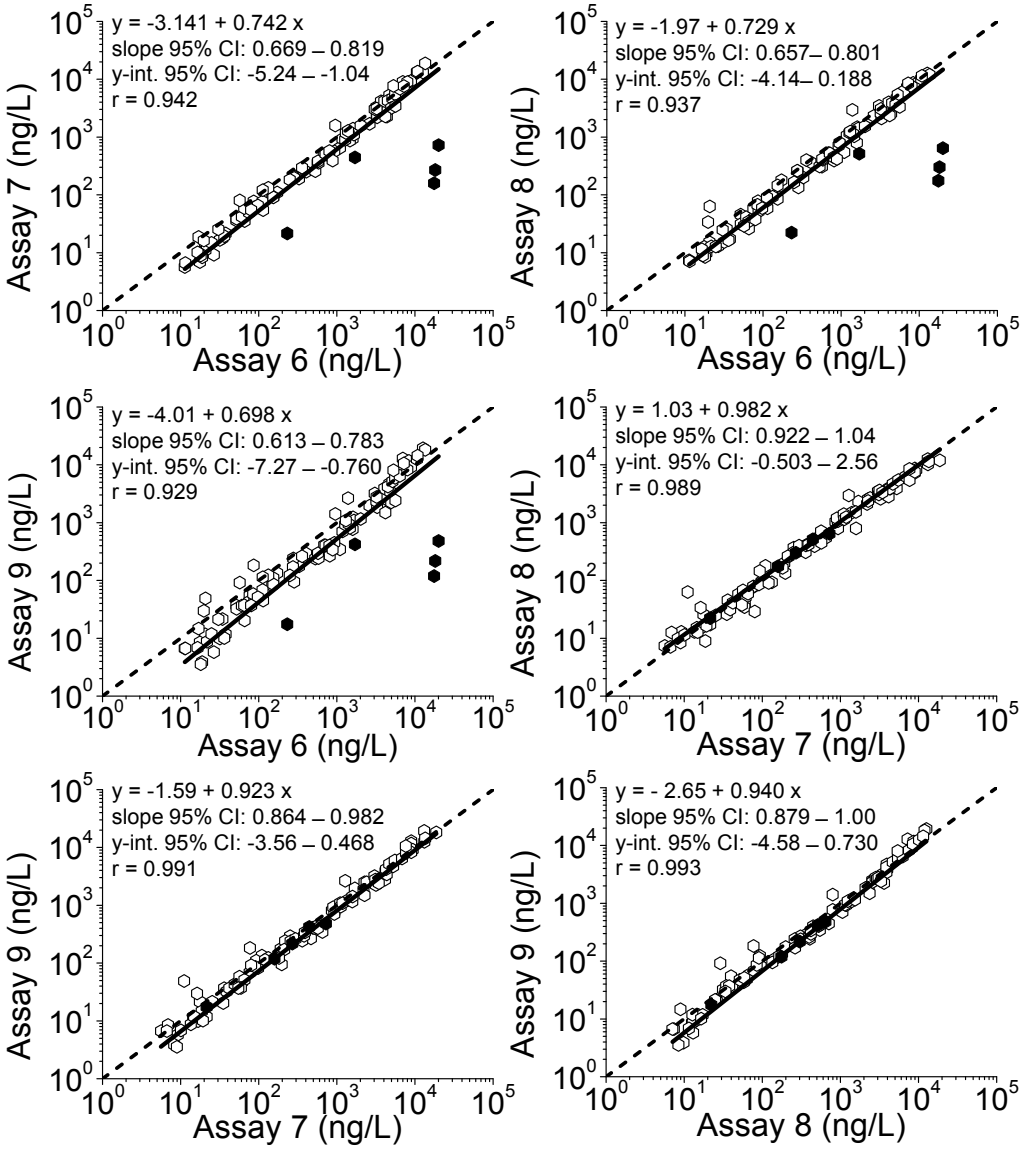

Figure 16. Clinical samples (n=101) tested with four different assay versions 6–9. Filled symbols represent samples that were observed to give unexpectedly high signals with assay version 6. Dashed lines represent the lines of identity. Modified from (**IV**).

Out of the 101 heparin plasma samples tested, five were observed to give unexpectedly high values with assay version 6, but were corrected with the other versions (filled symbols in **Figure 16**). When those samples were tested with an investigational research immunoassay for skTnI (unpublished), they were all observed to contain skTnI, the values ranging from 5500 ng/L to 702,000 ng/L.

When Fab-9707 and Mab-8I7 were used together as a solid-phase – detection pair in assay version 6, and 5000, 50,000, and 500,000 ng/L skTnI were added, a false cTnI signal up to 7867 ng/L was measured. Changing Mab-8I7 to Mab-625, or Fab-9707 to Fab-MF4 decreased the measured false cTnI signal to about one tenth (616–727 ng/L). The measured falsely elevated cTnI signals of each of the assay versions caused by skTnI additions can be seen in **Table 9**.

Table 9. Different spiked skTnI amounts measured as falsely elevated cTnI with different assay versions utilizing antibodies originally from different commercial manufacturers. Modified from (**IV**).

Assay version:	6	7	8	9
skTnI	Measured as ng/L cTnI			
5000 ng/L	17	10	7	10
50,000 ng/L	286	77	68	65
500,000 ng/L	7867	727	652	616

The calculated percentual cross-reactivities for different assay versions when 50 or 500 ng/L cTnI were combined with 50,000 or 500,000 ng/L skTnI can be seen in **Table 10**. Assay version 6 showed the highest cross-reactivity with up to 1.36%. All other versions had cross-reactivities ranging from 0.04% to 0.21%.

Table 10. The calculated cross-reactivities with different assay versions, when 50,000 or 500,000 ng/L skTnI were tested with 50 and 500 ng/L cTnI. Modified from (**IV**).

Assay version		6	7	8	9
cTnI	skTnI	Cross-reactivity			
50 ng/L	50,000 ng/L	0.42%	0.17%	0.12%	0.12%
	500,000 ng/L	1.36%	0.19%	0.12%	0.13%
500 ng/L	50,000 ng/L	0.44%	0.17%	0.04%	0.15%
	500,000 ng/L	1.27%	0.21%	0.13%	0.15%

6 DISCUSSION

As long as antibodies have been used in clinical assays, different interferences have been a matter of discussion. The estimation is that heterophilic interference is the reason for one false assay result for every 2000 assays performed (Levinson and Miller, 2002). Therefore, interferences are, from time to time, also observed in cTn diagnostics. Falsely elevated cTn levels emerging from heterophilic-type interferences have been reported during the past 10 years for both cTnT and cTnI in multiple assay systems (Lum *et al.*, 2006; Bionda *et al.*, 2007; Zhu *et al.*, 2008; Shayanfar *et al.*, 2008; Pernet *et al.*, 2008; Ghali *et al.*, 2012; Lippi *et al.*, 2013a). Negative interference from cTn-specific autoantibodies is also becoming increasingly recognized, although it was already discussed in the 1990s (Bohner *et al.*, 1996).

6.1 Immunoassays and their performance

6.1.1 Nanoparticles as labels (I)

Eu nanoparticles have proven their utility in immunoassay approaches, where assay sensitivity is highly valued (Järvenpää *et al.*, 2012; Valanne *et al.*, 2005; Soukka *et al.*, 2003). In original publication **I**, increasing the amount of free antibody resulted in less sensitive assay in a majority of the different assay versions. This can be explained by the assumption that increasingly few antigens were able to bind both solid-phase and detection antibodies, when the amount of free antibody was increased. The results also clearly emphasize the importance of carefully studying the configuration of antibodies in two-site immunoassays: with no addition of free D-dimer antibodies, almost a five-fold difference in assay LoB and a ten-fold difference in the upper limit of dynamic range were observed, with changing the antibody configuration. To further emphasize the importance of antibody orientation and the addition of free antibodies, a number of the tested assay versions would have been clinically useless with dynamic ranges well below the clinically interesting area of 500 ng/mL (FEU). For cTnI, the differences were clearly less significant. Specific reports focusing on the extension of assay dynamic ranges are very limited. These include the use of specific molecular spacers and plasmonic structures enabling an increase of up to 8 orders of magnitude in assay dynamic ranges, when compared to traditional assays (Zhou *et al.*, 2012). Also the application of multiple antibodies possessing a variety of affinities (Ohmura *et al.*, 2003) and the use of immunosensors with fluorescent labels (Renard and Bedouelle, 2004) have been introduced. Traditionally, the extension of immunoassay dynamic range is approached with the desire to achieve as sensitive an assay as possible, with the need to use only small amounts of the sample (Todd *et al.*, 2007). Extension of assay dynamic range by diluting the sample or decreasing the sample volume was not used here due to the assay being a model system for a commercial assay format with predetermined sample and buffer ratios. When time-resolved fluorescence is used, the application of excess

functional binders usually offers an opportunity for wide dynamic ranges (Lövgren *et al.*, 1984), as well as means to shorten the incubation time (Ekins, 1960).

In the critical care units and EDs, the average TAT expected by the physicians is 5–15 minutes (Harvey, 1999). Due to the high specific activity of the lanthanide-doped nanoparticles, it was possible to develop immunoassays with short 5- and 15-minute incubations. Therefore, it can be stated that nanoparticles can be used as universal labels, when developing novel immunoassays for POCT applications.

6.1.2 Immunoassay versions for cardiac troponin I (II–IV)

The current official recommendation of Clinical Chemistry and Laboratory Medicine for cTnI assays is that the utilized antibodies should target the stable midfragment of the analyte (Panteghini *et al.*, 2001). It has been reported, though, that approximately 10% of the patients with chest pain have cTnI autoantibodies in their circulation, thus possibly resulting in falsely decreased cTnI values (Savukoski *et al.*, 2014). This is caused by the fact that the circulating cTn autoantibodies are mainly bound to the stable midfragment of cTnI (Eriksson *et al.*, 2005b; Savukoski *et al.*, 2012; Savukoski *et al.*, 2013). Original publications **II–IV** were all based on an assay configuration, where all the three assay antibodies were selected on the basis of their ability to circumvent the inhibiting effects of the circulating cTn autoantibodies (Eriksson *et al.*, 2005b). However, when assay recoveries were studied in publication **III**, the recovery values were lower for cTn autoantibody-positive samples: the mean and median values were 53% and 57%, when the corresponding values for cTn autoantibody-negative samples were 83% and 83%, respectively. This difference between the recoveries from autoantibody-positive and negative samples can partly be explained with the avidity of the detection particles. By attaching multiple detection antibodies to the surface of the nanoparticles, we are increasing the binding site density through avidity, which has been shown to enhance the inherent affinity of the antibody (Soukka *et al.*, 2001a). Thus, the high density of the antibody renders the assay more vulnerable to all matrix-related analytical interferences, including those of circulating autoantibodies. It has also been shown that interference from cardiac autoantibodies may not be totally prevented even by using a cTnI assay designed to avoid the central parts of the cTnI molecule most affected by autoantibody interference (Eriksson *et al.*, 2005b).

The molecular form of the 9707 antibody did not affect the sensitivity of the cTnI assay. In original publication **II**, where Mab, $F(ab')_2$-, Fab-, and cFab-fragments of antibody 9707 were tested as a part of different versions (1–4) of a cTnI research assay, it was possible to reduce the antibody amounts of the 9707 antibody from 0.63 pmol (assay version 1) to 0.31 pmol (assay versions 3 and 4). Also, with the employment of recombinant 9707 fragments, the amount of the second solid-phase antibody Mab-19C7 was decreased to one fourth. These reductions in antibody amounts can most probably be explained by more oriented solid-phase

surfaces enabling a larger number of available antibody binding sites than can be achieved with traditionally biotinylated Mab and $F(ab')_2$-fragments (Peluso *et al.*, 2003). To highlight the nonexistent effect of antibody molecular form on assay sensitivity even more, the calculated LoD (3.30 ng/L) of assay version 5 (**III**) was in the same range as with the previously published assay 2 (2.0 ng/L) (Järvenpää *et al.*, 2012).

In original publication **III**, an assay version 5 utilizing cFabs was introduced. Probably due to the fact that the normal samples were obtained from young and apparently healthy individuals who are unlikely to present with increased cTn values commonly found in elderly people (Venge *et al.*, 2003; Eggers *et al.*, 2013), only 3 (4.7%) of the 64 measured samples gave signals >LoD. Altogether, the measured cTnI concentrations of assay version 5 were substantially lower than those measured with the commercial reference assay (Siemens ADVIA Centaur® TnI-Ultra™): the mean difference was 136%. Furthermore, out of the 265 patient samples measured with the assay version 5, 17 gave cTnI values <LoD. The reference assay utilizes remarkably different antibody epitopes than the cTnI research assay. The Siemens reference assay employs three Mabs with antibodies recognizing amino acid residues 27–40, 41–49, and 87–91 and an LoB of 6 ng/L (Casals *et al.*, 2007), while the cTnI research assay utilizes antibodies with epitope specificities to amino acid residues 41–49, 160–179, and 190–196. This difference in the utilized antibodies may explain the significantly different cTnI values measured with the two assays. It has been reported that both antibody affinities as well as the utilized epitopes can affect the measured signals even when the assays are tested with the same calibration material (Savukoski *et al.*, 2012). Therefore, the combination of used calibration material and different antibodies is the most probable reason for the assay bias and for the 17 samples with assay values <LoD. Another reason may be the fact that by replacing two of the three original antibodies with recombinant cFabs, the interferences caused by heterophilic-type interferences were decreased. This hypothesis is supported by the fact that the 17 samples for the most part represented low end values with the Siemens reference assay (6–300 ng/L, median 20 ng/L).

The basic quality requirements for the POCT of cTnI do not differ from other assays: ideally, POCT assays for cTnI should meet the current requirement of 10% within-laboratory precision recommended for cTnI-assays at MI decision limit (Wu *et al.*, 1999). Currently, there are nine POCT devices available for the detection of cTnI and cTnT that demonstrate considerable differences in their abilities to rule-in and rule-out MI (Palamalai *et al.*, 2013; Amundson and Apple, 2014). The cFab-based assay version 5 was conducted manually with a 15-minute assay incubation. Therefore, the assay signal was measured at a highly kinetic stage (13–41% of maximum signal), thus significantly hampering the evaluation of assay LoQ and precision. The assay was not able to meet the current precision requirements, but applying the assay as a part of an automated assay system would most probably facilitate better assay precision and enable hitting the 10% LoQ goal. To reduce the TAT, assays for POCT should also be able to use whole

blood. However, in this study, citrate and heparin plasma as well as serum samples were used, since all the assays were performed in a simple microtiter well format. Only by including the assays in a fully functional POCT-system, would it be possible to fully evaluate the POCT applicability of the developed nanoparticle-assisted immunoassays.

6.2 Matrix-related interferences

Matrix-related interferences were studied in original publications **II–IV**. Traditionally, interferences in cTnI and cTnT-testing have not been considered to be a significant problem, but as the assay LoDs are decreasing with the new generation of high sensitivity assays, the need to re-evaluate the issue has raised its head (Morrow and Antman, 2009). The exact incidence of interferences occurring in cTn assays is unknown, but it is expected to be higher than previously thought (Zaidi and Cowell, 2010). In a recent study, it was observed that 1.01% of all measured cTnI values were falsely elevated (Li *et al.*, 2014). In studies **II** and **III,** mouse Mabs, mouse Fabs, and cFabs were utilized. As the assay interferences decrease with the Fc-region removal and antibody chimerization, the next step in reducing the matrix-related interferences would be the humanization of the used cFabs by replacing the remaining mouse framework parts in the V_H and V_L sequences with the corresponding parts of human origin (Gonzales, 2003).

As stated previously, the cTnI research assay configuration (**II–IV**) is based on three antibodies: two solid-phase antibodies and one detection antibody. This type of three-site immunoassay approach have been shown to be over twice as prone to heterophilic antibody interference as traditional two-site assay approaches (Zhu *et al.*, 2008). One of the cTnI assay solid-phase Mabs represents subclass IgG2 (19C7), and the remaining (9707, 8I7, and 625) belong to subclass IgG1, which have been found to be highly susceptible to heterophilic-type interferences (Bjerner *et al.*, 2005). Therefore, the interferences observed in original publication **II**, especially with assay version 1, are probably stemming from heterophilic interferences. As with other analytes, heterophilic-type interferences are extremely difficult to predict with cTnI. Interferences between 0.1% and 3.1% have been reported in a normal population, and an interference rate as high as 50% was observed in a population with certain type of pneumonia (Yeo *et al.*, 2000; Uettwiller-Geiger *et al.*, 2002; Kim *et al.*, 2002; Fleming *et al.*, 2002; García-Mancebo *et al.*, 2005). Other causes than heterophilic and similar antibodies for possible spuriously increased cTnI values cannot be ignored, as falsely elevated cTn values have also been connected to fibrin and to the presence of microparticles (Li *et al.*, 2014).

The normal range values measured with the different assay versions (**II**, **III**) utilizing antibody fragments were in the same range as reported previously with different research versions of high sensitivity assays. Venge *et al.* (2009) reported that a prototype of Beckman Coulter Access high sensitivity cTnI assay was able to measure normal range cTnI values between 1.1–7.9 ng/L (median 3.2 ng/L).

Instead, Wu *et al.* (2009) have reported a median value of 1.72 ng/L for Singulex high sensitivity assay. In publication **II**, assay versions 2, 3, and 4 had median normal range values of 2.60 ng/L, 2.51 ng/L, and 1.80 ng/L, respectively. The corresponding minimum/maximum values were: assay version 2, 1.45/11.1; assay version 3, 1.10/11.2 ng/L; and assay version 4, 0.746/10.6 ng/L. Even the normal values above the assay LoD measured with the version 5 of the research assay (5.74 ng/L, 7.20 ng/L, and 11.8 ng/L) fall within the same range as published for other research assays (**III**). Nevertheless, it must be noted that due to differences in the used calibrators and antibodies, the measured values between different assays are not directly comparable from assay to assay (Christenson *et al.*, 2006).

When assay versions 2 and 5 were compared with samples containing known amounts of triglycerides, bilirubin, RF, or HAMA, clear reductions in the cTnI levels of measured samples were observed (**III**). However, no comparison cTnI-value was available for these samples, so it cannot be concluded that the samples were from apparently healthy persons with a low expectancy of measurable amounts of cTnI. The only difference between assay versions 2 and 5 was the removal of the Fc-portion in two out of three antibodies used, and antibody chimerization. Thus, it can be concluded that the majority of differences in the observed cTnI values were stemming from the utilization of recombinant antibody fragments. Values very little >LoD with the assay version 2 and <LoD of the assay version 5 may be due to the small differences in assay LoDs rather than the reduction of assay interference. The greatest observed difference was as high as 241 ng/L vs. <LoD. The differences should not be of this magnitude when using two different versions or the same assay, unless the other is more susceptible to matrix-related interferences. When possible interferences are found, the recommendation is that recovery and linearity studies be conducted to confirm that true interference is being detected (Sturgeon and Viljoen, 2011; Ismail, 2007). However, the volume and number of the samples obtained from ProMedDx was limited, so that only cTnI values with the two versions of the research assay were measured. The low number (n=3) of HAMA containing samples resulted in none of the HAMA containing samples having a cTnI concentration >LoD with assay version 2. This means that the HAMAs present in the three samples was most probably a subtype that does not affect the antibodies in assay versions 2 or 5. As expected, the RF interference was totally eliminated in assay version 5. Also the samples containing bilirubin showed decreases in the measured cTnI. This was unexpected, since false negative cTnI values have usually been associated with samples containing bilirubin (ver Elst *et al.*, 1999; Dasgupta *et al.*, 2001). However, spuriously increased cTnI values have been reported in samples containing free hemoglobin the generation of which is often accompanied by increased bilirubin values (Hawkins, 2003; Masimasi and Means, 2005). On the other hand, since no reference cTnI value was obtained for the interference samples, it cannot be concluded for certainty whether the measured cTnI value with assay version 2 in triglyceride and bilirubin containing samples represents false elevations.

Although high sensitivity cTn assays offer superior sensitivity and precision when compared to contemporary assays (Christ *et al.*, 2011; Wu and Christenson, 2013; Korley and Jaffe, 2013), a problem with lowered assay specificity has emerged. Actually, high sensitivity cTn assays are able to detect positive troponin values (>99th percentile) in a variety of different non-ACS-related conditions including a wide range of non-ischemic clinical conditions, as well as in sepsis and even in extreme exertion (Hamm *et al.*, 2002; Agewall *et al.*, 2011). Melanson *et al.* (2008) reported that when an old cTn assay from Siemens Healthcare Diagnostics was replaced with a more sensitive TnI-Ultra in 2007, a doubling of the amount of positive cTn results in the collected samples was observed, although no change in the actual number of final diagnoses were found.

Assay cross-reactivity issues with cTns have not widely been addressed since the cross-reactivity problems were observed with the first-generation cTnT assay. The affected assay employed a detection antibody that had 12% cross-reactivity with skeletal troponin T (skTnT) (Katus *et al.*, 1992; Gaze and Collinson, 2008). The cross-reactivity was eliminated in the second-generation assay (Ricchiuti *et al.*, 1998), but it has re-emerged as a concern during the past few years (Jaffe *et al.*, 2011). Cross-reactivity studies are mandatory for commercial assays, but not all cross-reactivity values are openly reported. According to instructions for use -leaflets, high sensitivity cTnI assays have varying cross-reactivities determined with 1,000,000 ng/L skTnI: Abbott Architect 0.07% and Beckman Access 2 0.034%. This means that, theoretically, 50,000 ng/L circulating skTnI would give rise to a 35 ng/L apparent cTnI concentration with the Abbott Architect assay, which would exceed the determined assay 99th percentiles (women 11.4 ng/L, men 27 ng/L, and both 19.3 ng/L)(Krintus *et al.*, 2014). Wu *et al.* (2009) have reported that no cross-reactivity was observed for Singulex Erenna high sensitivity cTnI assay. It must be noted, however, that skTnI was assayed over a range of 0.1–100 ng/L. Normal population are reported to have circulating skTnI concentrations of approximately 5500 (±5200) ng/L measured before exercise and 89,500 (±71,400) ng/L after anaerobic exercise (Chapman *et al.*, 2013). Ultimately as high as 990,000 ng/L skTnI has been measured in a patient with an inflammatory muscle disease polymyositis (Kiely, 2000). Therefore, with the increasingly sensitive troponin assays, even small increases in skTnI may potentially be a source of falsely increased troponin values.

In study **IV**, the combination of antibodies 9707 and 8I7 as solid-phase – detection pair (assay version 6) caused clearly the strongest cross-reactivity to skTnI (up to 1.36%). By replacing either 9707 or 8I7 with MF4 or 625 antibody, markedly lowered the cross-reactivity values. As it has become obvious that increased falsely elevated cTn values can be measured without MI (Lippi *et al.*, 2013a), more attention should be directed to assay cross-reactivities. Many manufactures sell Mabs as cardiac-specific without any documentation from possible specificity studies. Therefore, the selection of assay antibodies becomes crucially important in attempts to develop the next generation of high sensitivity assays. Antibodies

sold as cardiac-specific can detect trace amounts of skTnI, as was observed in publication **IV**. Vylegzhanina *et al.* (2013) recently reported that Mab-8I7 has 70% cross-reactivity with skTnI. Results from study **IV** support the observation, but also highlight the importance of the other antibodies used in the assay setup. Mab-8I7 showed extensive cross-reactivity with skTnI only when used together with Fab-9707 (0.42%–1.36%, assay version 6). Assays employing only 8I7 or 9707 had cross-reactivities between 0.04%–0.21% (assay versions 7 and 8). Also, the assay version 9 that employed antibodies with no reported cross-reactivity with skTnI (from HyTest Ltd.), had cross-reactivities in the same range (0.12%–0.15%). In theory, even a 0.04% cross-reactivity, could cause falsely elevated cTnI values. In practice though, only assay version 6 with both 9707 and 8I7 antibodies was clearly affected by skTnI present in the patient samples. Nevertheless, since assay versions 7–9 showed identical cross-reactivities, the specificity of assay antibodies should not be ignored, when developing new generations of high sensitivity assays.

The specificity issues around high sensitivity cTn assays should not be ignored, since they are routinely being implemented in clinical use. The lowered assay specificity has led to discussions about the validity of the 99th percentile values in clinical decision making (Morrow and Antman, 2009; Khalili and de Lemos, 2014; Aakre *et al.*, 2014; Simpson *et al.*, 2014). Apple *et al.* (2012b) have reported that up to 32-fold differences can be found in the assay 99th percentiles, when determined for 19 cTnI and cTnT assays with the same reference population used. Thus, the question about the way normality should be determined has become highly topical. In the future, the determination of assay 99th percentiles will, therefore, probably be based on strict recommendations and on the extensive medical examinations of the study participants (Koerbin *et al.*, 2013). Although the current recommendation of MI diagnosis emphasizes the importance of a rise and/or fall in the measured cTn value (Thygesen *et al.*, 2012), an analyte value above the determined diagnostic threshold may, in some cases, be the only basis for diagnosing an ischemic cardiac disease (Eggers *et al.*, 2012; Lippi *et al.*, 2013b). In the worst case scenario, a false positive, or false negative, cTn-result may therefore result in a wrong clinical decision.

As there is no single blocking agent that could remove all heterophilic-type interferences, and most commercial immunoassays for cTnI are currently using intact monoclonal or polyclonal antibodies (Tate, 2008), employing recombinant antibody fragments – chimeric or humanized in particular – should be highly encouraged in the future generations of high sensitivity assays for cTnI and TnT. Additionally, since the trend has been toward increasing sensitivity, and a cTn assay is to be regarded as a high sensitivity assay only when >50% (ideally 95%) of people in a normal population have detectable cTnI values (Apple *et al.*, 2012a), it should be carefully studied, whether these high sensitivity assays are actually measuring cTn, and not something matrix-related.

7 CONCLUSIONS

Increasingly sensitive assays are being developed for cTnI, which has resulted in significantly lowered assay specificities. Therefore, the selection of assay antibodies plays an important role in the development of assays with high specificity to cTnI. In addition, the employment of recombinant antibody fragments, chimeric fragments or, ultimately, humanized antibodies possess high potential for developing immunoassays minimally prone to matrix-related interferences possibly present in all blood samples.

The main conclusions based on the original publications are:

I The selection of correct antibody orientation as well as the exploitation of free solid-phase or detection antibodies in the capture solution enabled the extension of the upper limit of assay dynamic range up to 200-fold in nanoparticle-assisted immunoassays. The extension of dynamic range was carried out with two different analytes requiring very different sensitivities and assay ranges. Therefore, it can be concluded that Eu-doped intrinsically fluorescent nanoparticles are universally applicable to immunoassays requiring either high sensitivities or wide dynamic ranges around a specific predetermined analyte value.

II A possibly interference-prone subclass IgG1 antibody was tested in a research assay for cTnI in four different molecular forms: Mab, $F(ab')_2$, Fab, and cFab. Utilizing the Mab form caused significantly higher observed cTnI values in apparently healthy study subjects. Ultimately, the recombinant fragments showed the lowest measured cTnI values, calling for a broader evaluation of the advantages of recombinant antibody fragments in assays requiring high sensitivities.

III Highly sensitive assays for cTnI have emerged in the critical care testing scene during the past few years. Increasing the sensitivity has ultimately meant decreases in assay specificities, thus causing concerns in establishing and utilizing correct assay 99^{th} percentiles. A novel immunoassay for cTnI based on chimeric Fab-fragments enabled the rapid measurement of cTnI as well as significantly lowered the prevalence of spuriously elevated cTnI values when compared to a previous version of the assay. This highlights the results of study **II**, and calls for thorough and active measures in applying chimeric antibody fragments when the next generations of high sensitivity cTnI assays are being developed.

IV Antibodies claimed as cTnI-specific are widely sold by different antibody manufacturers. However, these antibodies may cause falsely elevated signals through cross-reactivity with skeletal TnI, especially when they are employed in nanoparticle-assisted immunoassays, where an avidity-related enhancement of the assay signal may induce even low-affinity matrix-

related interferences to cause complications. Different antibodies were tested without changing the assay epitope specificity as evidenced by peptide mapping. Clear cross-reactivity issues were observed with an assay configuration utilizing Mab-8I7 and Mab-9707 together, but not when only one or the other was used. Since current high sensitivity assays often rely on different signal amplification methods, which potentially expose the assays to low affinity-related interferences, the issue of antibody specificity should not be ignored even when antibodies with no apparent cross-reactivities are being used.

In conclusion, Eu nanoparticles can be employed as the detection system in immunoassays requiring specific properties: sensitivity or a wide dynamic range around a pre-determined cut-off value. All the developed assays were implemented as 5- or 15-minute assays, enabling their possible incorporation into a POCT assay system. Using recombinant antibody fragments clearly showed superiority over intact antibodies by being significantly less prone to low-affinity matrix-related interferences. Additionally, the selection of assay antibodies is highlighted in the hope of developing highly specific assays for cTnI. This thesis, therefore, emphasizes the need to thoroughly study the effects that the utilization of recombinant antibody fragments and the selection of assay antibodies have on the next generation of assays for cTnI. This is supported by the fact that high sensitivity assays have strongly been advocated to be used in the acute cardiac triage and the evaluation of long term cardiac risk.

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