



**TURUN  
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UNIVERSITY  
OF TURKU

# Investigating and Improving Cardiac Troponin Assays

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Selma Salonen





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# **INVESTIGATING AND IMPROVING CARDIAC TROPONIN ASSAYS**

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Selma Salonen

## University of Turku

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Faculty of Technology  
Department of Life Technologies  
Molecular Biotechnology and Diagnostics  
Doctoral Programme in Clinical Research (DPCR)

## Supervised by

---

Assistant Professor, Saara Wittfooth  
Biotechnology Unit  
Department of Life Technologies  
University of Turku  
Turku, Finland

PhD, Satu Lahtinen  
Biotechnology Unit  
Department of Life Technologies  
University of Turku  
Turku, Finland

Docent, Tapio Hellman  
Kidney Center  
Turku University Hospital and  
University of Turku  
Turku, Finland

## Reviewed by

---

Professor, Mikko Anttonen  
Department of Clinical Chemistry  
University of Helsinki  
Helsinki, Finland

Associate Professor, Kai Eggers  
Department of Medical Sciences  
Uppsala University  
Uppsala, Sweden

## Opponent

---

Professor, Paul Collinson  
City St George's University of London  
London, UK

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*To my family*

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## ABSTRACT

Cardiac troponins I (cTnI) and T (cTnT) are the preferred biomarkers of myocardial injury representing the diagnostic cornerstone of acute myocardial infarction (AMI). The implementation of high-sensitivity cardiac troponin (hs-cTn) assays has provided faster and more accurate detection of myocardial injury but also reduced clinical specificity for AMI. Current hs-cTnT assays targeting intact as well as degraded cTnT forms (i.e. total cTnT) can often detect hs-cTnT elevations in several other conditions, such as chronic kidney disease (CKD). Recently, intact and mildly degraded cTnT forms (i.e. long cTnT) have shown promise as a more specific biomarker for AMI. However, their detection requires particularly high analytical sensitivity. In addition to the specificity issues, hs-cTn assays are also prone to interferences caused by cardiac troponin-specific autoantibodies (cTnAAbs), which can lead to either false-positive or false-negative hs-cTn results.

The aim of this thesis was to develop a highly sensitive immunoassay for long forms of cTnT using upconversion luminescence (UCL). The analytical and diagnostic performance of the developed assay was evaluated, and long cTnT concentrations were measured in patients with advanced CKD not on dialysis to assess associations between long cTnT and adverse long-term outcomes. In addition, the effect of cTnAAbs on the elimination of cTn measured by five widely used commercial hs-cTn assays was investigated.

A highly sensitive UCL-based long cTnT assay with a limit of detection of 0.4 ng/L was successfully developed. The assay showed excellent discrimination between patients with non-ST elevation myocardial infarction and end-stage renal disease. Furthermore, long cTnT was independently associated with all-cause mortality in patients with CKD stage 4–5. The novel long cTnT assay can serve as a valuable tool for discovering the full potential of long cTnT as a biomarker for AMI. For the first time, cTnAAbs and the formation of large macrotroponin complexes were also shown to lead to reduced cTn clearance, which has been previously speculated to be the underlying mechanism of positive cTnAAb-mediated interference. Increased understanding of cTnAAb-related interferences is important both in clinical practice and in the development of new generations of hs-cTn assays.

**KEYWORDS:** acute myocardial infarction, cardiac troponin, biomarker, immunoassay, upconversion luminescence, autoantibody, assay interference

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## TIIVISTELMÄ

Sydänperäiset troponiinit I (engl. cardiac troponin I, cTnI) ja T (engl. cardiac troponin T, cTnT) ovat sydänlihaskvaurion biomerkkiaineita ja tärkeä osa sydäninfarktidiagnostiikkaa. Herkät troponiinitestit ovat mahdollistaneet sydänlihaskvaurion varhaisen ja tarkan tunnistamisen, mutta samalla spesifisyys sydäninfarktille on laskenut. Nykyiset cTnT-testit havaitsevat sekä kokonaisen että pilkkoutuneen cTnT:n (totaali cTnT) ja mittaavat kohonneita pitoisuuksia usein myös muiden sairauksien, kuten munuaisten vajaatoiminnan yhteydessä. Kokonaiset ja vähän pilkkoutuneet cTnT-muodot (pitkä cTnT) ovat osoittautuneet hiljattain lupaaviksi sydäninfarktin merkkiaineiksi, mutta niiden havaitseminen vaatii erittäin herkkää määritysmenetelmää. Spesifisyysongelmien lisäksi herkät troponiinitestit ovat alttiita troponiiniautovasta-aineiden aiheuttamille häiriöille, jotka voivat johtaa joko väriin positiivisiin tai väriin negatiivisiin testituloksiin.

Väitöstutkimuksen tavoitteena oli kehittää erittäin herkkä immunomääritys pitkille cTnT:n muodoille hyödyntäen käänteisviritteistä luminesenssia. Kehitetyn määrittelyn analyttistä ja diagnostista suorituskykyä arvioitiin, ja pitkän cTnT:n yhteyttä pitkän aikavälin haitallisiin lopputuloksiin tutkittiin vaiheen 4–5 munuaistautia sairastavilla potilailla ennen dialyysihoidon aloitusta. Lisäksi troponiiniautovasta-aineiden vaikutusta troponiiniin puhdistumiseen verenkierrosta tutkittiin viidellä eri kaupallisella testillä.

Kehitetty käänteisviritteiseen luminesenssiin perustuva pitkän cTnT:n määrittely saavutti 0,4 ng/L:n havaitsemisrajan ja kykeni erinomaisesti erottelemaan sydäninfarktipotilaat ilman ST-nousua loppuvaiheen munuaistautia sairastavista potilaista. Lisäksi pitkän cTnT:n havaittiin olevan itsenäisesti yhteydessä kuolleisuuteen predialyysipotilailla. Tulevaisuudessa määrittely on tärkeä työkalu tutkittaessa pitkän cTnT:n potentiaalia sydäninfarktin merkkiaineena. Tutkimuksessa todistettiin myös ensimmäistä kertaa troponiiniautovasta-aineiden muodostamien makrotroponiini-kompleksien johtavan troponiiniin pienentyneeseen puhdistumiseen verenkierrosta aiheuttaen siten kohonneita troponiini-pitoisuuksia. Autovasta-aineiden aiheuttamien häiriöiden parempi ymmärrys on olennaista sekä kliinisessä käytännössä että kehitettäessä uuden sukupolven troponiini-määrittelyksiä.

ASIASANAT: sydäninfarkti, sydänperäinen troponiini, biomerkkiaine, immunomääritys, käänteisviritteinen luminesenssi, autovasta-aine, määrittelyhäiriö

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# Abbreviations

|         |   |
|---------|---|
| aar     | amino acid residue  |
| ACS     | acute coronary syndrome   |
| AMI     | acute myocardial infarction   |
| AUC     | area under the curve  |
| BSA     | bovine serum albumin  |
| CABG    | coronary artery bypass grafting   |
| CAD     | coronary artery disease   |
| CI      | confidence interval   |
| CKD     | chronic kidney disease  |
| CKD-EPI | Chronic Kidney Disease Epidemiology Collaboration                         |
| CLSI    | Clinical and Laboratory Standards Institute                               |
| cTn     | cardiac troponin  |
| cTnAAb  | cardiac troponin-specific autoantibody                                    |
| cTnI    | cardiac troponin I  |
| cTnT    | cardiac troponin T  |
| CV      | coefficient of variation  |
| CVD     | cardiovascular disease  |
| DBU     | 1,8-diazabicyclo[5.4.0]-7-undecene  |
| ECG     | electrocardiogram   |
| EDTA    | ethylenediaminetetraacetic acid   |
| eGFR    | estimated glomerular filtration rate                                      |
| ESC     | European Society of Cardiology  |
| ESRD    | end-stage renal disease   |
| GFR     | glomerular filtration rate  |
| hs-cTn  | high-sensitivity cardiac troponin   |
| HR      | hazard ratio  |
| IC      | binary cTnI-TnC complex   |
| IFCC    | International Federation of Clinical Chemistry and Laboratory<br>Medicine |
| ITC     | ternary cTnI-cTnT-TnC complex   |
| KTx     | kidney transplantation  |

|        |   |
|--------|---|
| LiH    | lithium-heparin                                       |
| LoB    | limit of blank  |
| LoD    | limit of detection                                    |
| LoQ    | limit of quantitation                                 |
| mAb    | monoclonal antibody                                   |
| MACCE  | major adverse cardiovascular or cerebrovascular event |
| NOAF   | new-onset atrial fibrillation                         |
| NSTEMI | non-ST elevation myocardial infarction                |
| PAA    | poly(acrylic acid)                                    |
| PCI    | percutaneous coronary intervention                    |
| PEG    | polyethylene glycol                                   |
| ROC    | receiver operating characteristic                     |
| RT     | room temperature                                      |
| STEMI  | ST elevation myocardial infarction                    |
| TnC    | troponin C  |
| TRF    | time-resolved fluorescence                            |
| TSA    | tris-buffered saline with azide                       |
| UCL    | upconversion luminescence                             |
| UCNP   | upconverting nanoparticle                             |
| URL    | upper reference limit                                 |

# List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

- I Salonen SM, Tuominen TJK, Raiko KIS, Vasankari T, Aalto R, Hellman TA, Lahtinen SE, Soukka T, Airaksinen KEJ, Wittfooth ST. Highly sensitive immunoassay for long forms of cardiac troponin T using upconversion luminescence. *Clin Chem*, 2024; 8:1037–1045.
- II Salonen S, Kaipainen E, Hakamäki M, Liuhto N, Manni N, Toukola T, Virtanen J, Lankinen R, Metsärinne K, Vasankari T, Airaksinen KEJ, Järvisalo MJ, Wittfooth S, Hellman T. Association of long cardiac troponin T forms with adverse long-term outcomes in patients with advanced chronic kidney disease. (Manuscript)
- III Salonen SM, Kristensen JH, Simonen S, Hasselbalch RB, Strandkjær N, Østergaard M, Møller-Sørensen H, Dahl M, Bor MV, Frikke-Schmidt R, Jørgensen NR, Rode L, Holmvang L, Kjærgaard J, Bang LE, Forman J, Dalhoff KP, Bundgaard H, Iversen KK, Wittfooth S. Exploring the role of cardiac troponin-specific autoantibodies: prolonged cardiac troponin elimination, reduced clearance, and variable interference across 5 commercial assays. *Clin Chem*, 2025; 9:970–979.

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# 1 Introduction

Acute myocardial infarction (AMI) is a life-threatening condition and a major cause of death worldwide [1]. Fast diagnosis of AMI is crucial to allow timely initiation of the optimal treatment and to improve patient outcomes. Cardiac troponins (cTn) are the biomarkers of choice for the detection of myocardial injury, and they represent an important diagnostic cornerstone of AMI [2]. The implementation of high-sensitivity cardiac troponin (hs-cTn) assays has allowed more rapid and accurate detection of myocardial injury. However, hs-cTn assays can often detect cTn elevations in multiple cardiac and non-cardiac conditions, such as atrial fibrillation, heart failure, chronic kidney disease (CKD), stroke, sepsis, and after strenuous exercise, complicating the interpretation of hs-cTn results and reducing clinical specificity for AMI [3].

Recent strategies to improve specificity for AMI have focused on identifying fragmentation patterns of cTn in different conditions. Cardiac troponin T (cTnT) is highly susceptible to proteolytic degradation, but current hs-cTnT assays can detect intact cTnT as well as mildly and heavily degraded cTnT forms (i.e. total cTnT) [4]. Interestingly, intact and only mildly degraded forms (i.e. long cTnT) have been predominantly found in the blood of early presenting AMI patients, whereas smaller cTnT fragments seem to prevail later after AMI, in patients with end-stage renal disease (ESRD) and in marathon runners [4–10]. Thus, long cTnT forms have been suggested to be a promising and more specific diagnostic marker for AMI. To date, the methods used for the detection of long cTnT forms have mainly been laborious and/or lacked the sufficient analytical sensitivity [4–6,8]. Highly sensitive and simple tools are needed to reveal the full potential of long cTnT as a diagnostic biomarker for AMI.

In addition to the issues with clinical specificity, hs-cTn assays are also prone to analytical antibody-mediated interferences [11]. Cardiac troponin-specific autoantibodies (cTnAAb) are known to cause false-positive and false-negative hs-cTn results, which may even lead to erroneous clinical decisions. Endogenous cTnAAbs bind to circulating cTn and form large macrotroponin complexes, which can result in persistent hs-cTn elevations not related to an ongoing myocardial injury, potentially due to reduced clearance of macrotroponin [12,13]. However, direct

evidence of how cTnAAbs affect the elimination half-life and clearance of cTn has been lacking. On the other hand, cTnAAbs can also cause negative interference by masking critical epitopes and preventing assay antibodies from recognizing cTnT [14]. Increased understanding of these complex cTnAAb-mediated interferences in hs-cTn assays is needed both in clinical practice and in the development of new generations of hs-cTn assays with minimized susceptibility to interferences.

## 2 Review of the Literature

### 2.1 Acute myocardial infarction

#### 2.1.1 Definition and types

AMI is a life-threatening condition defined as an event of myocardial necrosis in the clinical setting of acute myocardial ischaemia (i.e. insufficient blood flow and oxygen supply to the heart muscle) [2]. AMI can be classified into ST-elevation myocardial infarction (STEMI) and non-ST elevation myocardial infarction (NSTEMI) based on the presence or absence of ST-segment elevation on the electrocardiogram (ECG). Further classification of AMI into types 1–5 is based on the cause of myocardial ischaemia and cardiomyocyte necrosis [2,15].

Type 1 AMI is caused by atherothrombotic coronary artery disease (CAD). Atherosclerotic plaque rupture or erosion promotes the formation of a thrombus that can block the coronary artery and consequently prevent the blood flow to the heart muscle resulting in myocardial ischaemia and cardiomyocyte necrosis [2]. A complete blockage in a coronary artery typically leads to STEMI, while a partially occluding thrombus may lead to NSTEMI or unstable angina defined as myocardial ischaemia at rest or on minimal exertion in the absence of myocardial necrosis [15,16].

Type 2 AMI is defined as an ischaemic myocardial injury that occurs due to imbalance in myocardial oxygen supply or an unmet need in myocardial oxygen demand without acute coronary atherothrombosis [2,17]. The myocardial oxygen supply-demand imbalance can be related to either reduced myocardial perfusion or increased myocardial oxygen demand. Reduced myocardial perfusion can be caused by various pathophysiological mechanisms, such as fixed coronary atherosclerosis without plaque rupture, coronary artery spasm, coronary embolism, coronary artery dissection, severe bradyarrhythmia, hypotension or shock, respiratory failure, and severe anaemia [2]. Furthermore, increased myocardial oxygen demand can be caused by sustained tachyarrhythmia or severe hypertension with or without left ventricular hypertrophy [2]. Due to multiple pathophysiological mechanisms contributing to myocardial ischaemia, the diagnosis and treatment of type 2 AMI can often be challenging [17].

Type 3 AMI is defined as a cardiac death that is likely caused by AMI but cTn measurements are not available or increases in cTn concentrations could not be detected before death [2]. Type 4 and 5 AMI are related coronary procedures: type 4 AMI is associated with percutaneous coronary intervention (PCI) and type 5 AMI with coronary artery bypass grafting (CABG) [2].

In the Fourth Universal Definition of Myocardial Infarction, AMI is defined as an acute myocardial injury with clinical evidence of acute myocardial ischaemia [2]. Myocardial injury is defined as an elevation of cTn values above the 99<sup>th</sup> percentile upper reference limit (URL), and it is considered acute if there is a rise and/or fall of cTn values in serial testing [2]. Thus, the criteria for types 1 and 2 AMI include the detection of a rise and/or fall of cTn values with at least one value above the 99<sup>th</sup> percentile URL [2]. In addition, at least one of the following criteria must be met: symptoms of acute myocardial ischaemia, new ischaemic ECG changes, development of pathological Q waves, imaging evidence of new loss of viable myocardium or new regional wall motion abnormality in a pattern consistent with an ischaemic aetiology, or identification of a coronary thrombus by angiography or autopsy (only for type 1) [2]. This thesis will mainly focus on type 1 AMI.

## 2.1.2 Diagnosis

Fast diagnosis of type 1 AMI is of utmost importance to enable timely initiation of the optimal treatment of the patient and improve patient outcomes. When a patient has evidence of acute myocardial ischaemia, the diagnostic and therapeutic pathway of acute coronary syndrome (ACS) is initiated [15]. ACS refers to a group of conditions caused by a sudden decrease in blood flow through the coronary arteries, including STEMI, NSTEMI, and unstable angina [15]. The diagnosis of type 1 AMI predominantly relies on the clinical presentation of the patient, ECG findings and cTn measurements. In addition, further investigations, including invasive and non-invasive imaging, can be performed before the final diagnosis is made [15].

The most common symptom of myocardial ischaemia is chest discomfort, which can be described as pain, tightness, pressure, heaviness, or burning. Other possible symptoms include dyspnoea, epigastric pain, fatigue, and pain in the left or right arm, neck or jaw [2,15]. These symptoms, however, are not specific for myocardial ischaemia. Furthermore, patients with myocardial ischaemia can also present with atypical symptoms or even without symptoms [2,15].

The 12-lead ECG has an integral role in the diagnostic work-up of patients with suspected ACS. If a patient presents with signs or symptoms suggestive of ACS, an ECG should be obtained and interpreted as soon as possible (preferably within 10 minutes) after first medical contact [2,15]. Clinical information and ECG findings allow initial evaluation and triage of patients with suspected ACS. Based on ECG,

patients with suspected ACS can be classified into two working diagnoses: STEMI and non-ST elevation ACS [15]. ECG monitoring is recommended in all patients with suspected ACS [15].

Hs-cTn assays also play a key role in the diagnosis of AMI [2]. Particularly, the diagnosis of NSTEMI relies strongly on hs-cTn testing. If ACS is suspected, it is recommended to send a blood sample for cTn measurement immediately after admission [2,15]. However, elevated hs-cTn levels alone are not specific for AMI, as they can also occur in non-ischaemic myocardial injuries. Thus, myocardial injury is both a distinct entity defined by elevated cTn levels and a prerequisite for the diagnosis of AMI [2,15]. The European Society of Cardiology (ESC) recommends the use of rapid rule-in and rule-out algorithms (0 h/1 h or 0 h/2 h algorithms) involving serial measurement of hs-cTn levels within one or two hours. If a dynamic rising or falling trend of hs-cTn levels with at least one value above the 99<sup>th</sup> percentile URL is detected in serial testing, myocardial injury is considered acute. Thus, serial hs-cTn testing allows differentiation of NSTEMI from unstable angina and conditions associated with non-ischaemic myocardial injury [15]. However, timing of the sampling can substantially affect the ability to detect the changing pattern of hs-cTn values. It may be particularly difficult to detect changes in hs-cTn values, if serial samples are collected very early after symptom onset when hs-cTn levels are still low. Furthermore, it can be difficult to detect a rising or falling pattern when hs-cTn levels have reached a plateau phase [2].

Clinical presentation, ECG findings and hs-cTn measurements allow initial triaging and diagnosis of patients with suspected ACS [15]. To establish an accurate final diagnosis, patients triaged towards the rule-in pathway of AMI may require invasive coronary angiography or non-invasive imaging. Depending on the patient risk, immediate angiography often combined with PCI can be recommended. This concerns patients with suspected STEMI or non-ST elevation ACS with very high-risk features [15]. Non-invasive imaging methods including echocardiography, computed tomography, and cardiac magnetic resonance imaging, also play a central role in improving diagnostic accuracy and optimizing risk assessment [15].

### 2.1.3 Treatment

Treatment strategies for type 1 AMI aim at opening the coronary artery and restoring the blood flow and oxygen supply to the heart muscle. Reperfusion therapy options include fibrinolysis, PCI, and CABG [15]. Fibrinolytic therapy involves intravenous administration of a fibrinolytic agent that dissolves dangerous intravascular clots. Primary PCI (i.e. immediate angiography combined with PCI) is a minimally invasive catheter-based procedure used to open narrowed or blocked arteries. CABG is an open-heart surgery in which autologous arteries or veins are used as grafts to

bypass occluded coronary arteries. Currently, PCI is the preferred reperfusion strategy [15]. Patients with AMI should also receive antithrombotic therapy consisting of antiplatelet agents and anticoagulants [15].

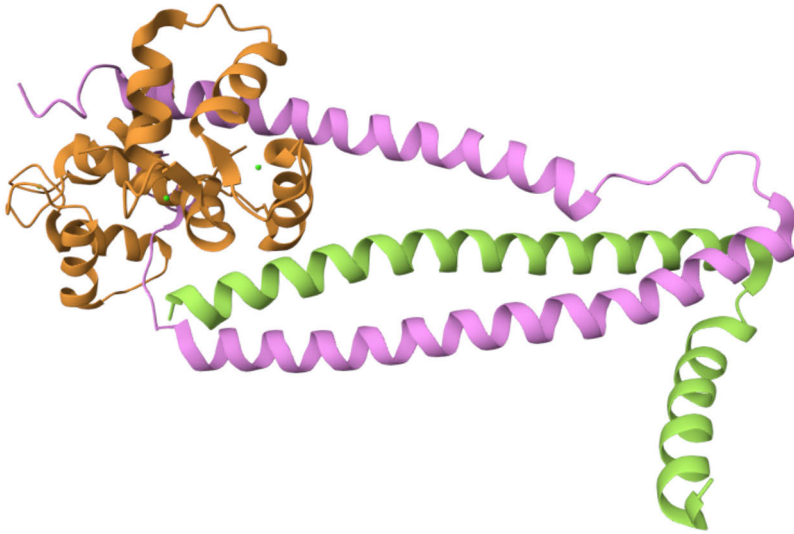
The selected treatment strategy and the urgency of treatment depend on whether the coronary artery is partially or completely occluded. For instance, patients with a working diagnosis of STEMI are recommended to be directed immediately to primary PCI or to fibrinolysis if primary PCI is not feasible [15]. Depending on the risk features of patients with suspected NSTEMI, there might be more time for further investigations to determine the optimal treatment for the patient [15].

## 2.2 Cardiac troponins

### 2.2.1 Structure and function

The cardiac troponin complex is a protein complex involved in the regulation of cardiac muscle contraction and composed of three subunits: troponin C (TnC, ~18 kDa), cardiac troponin I (cTnI, ~24 kDa) and cTnT (~36–37 kDa) [18]. Troponins are expressed in humans in cardiac muscle as well as in fast-twitch and slow-twitch skeletal muscles [18]. The cTnI and cTnT isoforms are almost exclusively expressed in the heart, although cTnT also seems to be occasionally expressed in diseased skeletal muscle [19–21]. In contrast, the same TnC isoform is present in both cardiac and skeletal muscle [18]. The mature cTnI molecule is 209 amino acids long, whereas the dominant cTnT isoform (TNT3) in the adult human heart consists of 288 amino acids [18,22,23]. Due to their superior sensitivity and cardiac tissue specificity, cTnI and cTnT are currently the preferred biomarkers of myocardial injury [2].

The ternary cTnI-cTnT-TnC (ITC) complex is a component of the thin filament within the cardiomyocyte sarcomere. The crystal structure of the core domain of the human cardiac troponin ITC complex in the  $\text{Ca}^{2+}$ -saturated form has been characterized by Takeda et al. [24]. The 46 kDa core consists of TnC (amino acid residues, aar 1–161), the central part of cTnI (aar 31–163) and the C-terminal part of cTnT (aar 183–288) (Figure 1). In the core domain, cTnI and cTnT form a rigid  $\alpha$ -helical coiled-coil domain, also known as IT-arm that is more resistant to proteolysis [24].



**Figure 1.** Three-dimensional crystal structure of the 46 kDa core domain of the human cardiac troponin ITC complex in the Ca<sup>2+</sup>-saturated form. TnC is depicted in orange, cTnI in pink, and cTnT in green. Small green spheres indicate Ca<sup>2+</sup> ions. The figure was obtained from the RCSB PDB (RCSB.org) of PDB ID 1J1D [24].

As a part of the contractile apparatus of cardiac muscle, each cTn subunit has a specific role in cardiac muscle contraction. TnC binds Ca<sup>2+</sup> ions, cTnI inhibits the actomyosin ATPase activity and consequently actomyosin complex formation at low intracellular Ca<sup>2+</sup> levels, and cTnT binds the cTn complex to tropomyosin. Following an action potential and increase in intracellular Ca<sup>2+</sup> concentration, Ca<sup>2+</sup> binds to TnC leading to a conformational change that enables the interaction of myosin with actin and initiation of muscle contraction [18,24]. The fine adjustment of cardiac muscle contraction is enabled by developmental isoform change, alternative splicing and posttranslational modifications, such as phosphorylation [18].

The majority of cTnT and cTnI is tightly bound to the sarcomere. A small fraction (6–8%) of unbound cTnI and cTnT has been proposed to be present in the cytoplasm of cardiomyocytes [25,26]. Recent evidence also suggests that there is a diffusible fraction of cTnT and cTnI within cardiomyocytes, but it is likely larger and not cytosolic. This easily releasable fraction consists of cTnT and cTnI that are reversibly bound to sarcomere [27].

## 2.2.2 Release into the bloodstream

Following myocardial injury, cTnI and cTnT are released into the blood from damaged cardiomyocytes [2]. The development of acute myocardial ischaemia in AMI results in irreversible cardiomyocyte necrosis which has been considered the

principal mechanism of cTn release. Myocardial necrosis leads to the destruction of cell membranes and organelles and the release of intracellular proteins, including cTn, into the blood [28,29].

Peak concentrations of cTnI and cTnT are typically observed in the early hours (approximately 8–12 hours) after admission in AMI patients with reperfusion [30]. Peak cTn values are also known to correlate with the infarct size [31]. Both cTnI and cTnT levels can remain elevated for days or even over a week after AMI, which could be explained by the fact that cTnI and cTnT are tightly bound to the sarcomere in cardiomyocytes resulting in a prolonged release from tissue [29,30,32]. In contrast, cytoplasmic cardiac damage biomarkers, such as myoglobin, seem to remain elevated for much shorter time periods (hours or a few days) following AMI [33].

Interestingly, the kinetics of hs-cTnI and hs-cTnT have been shown to differ from each other in AMI patients. First, cTnI levels can reach much higher peak concentrations than cTnT. Second, cTnI is eliminated from the circulation faster than cTnT. Third, cTnI exhibits monophasic kinetics, whereas cTnT exhibits prolonged biphasic kinetics after AMI [30,32]. The higher peak concentrations and faster clearance of cTnI might be explained by faster degradation and release of cTnI from damaged cardiomyocytes [34]. In contrast to cTnT, cTnI does not directly bind to insoluble thin filaments in the cardiomyocyte but to cTnT, which may allow the faster degradation and release of cTnI [35]. Furthermore, it has been speculated that some differences between the kinetics of cTnI and cTnT might be, in part, explained by different renal clearances and/or immunoreactivities [30,32]. However, renal clearances of cTnI and cTnT have been shown to be similar under physiological conditions [34].

The first peak of cTnT kinetics has been hypothesized to appear due to the fast release of cytosolic cTnT fraction, whereas the second peak appearing approximately 80 hours after symptom onset in AMI patients with reperfusion, might comprise cTnT that is tightly bound to the sarcomere [25]. However, it has also been suggested that the diffusible fraction of cTnT is larger than initially estimated and consists of cTnT that is reversibly bound to tropomyosin. Slow wash-out of this diffusible cTnT fraction might explain the sustained increase in cTnT after AMI [27]. A recent hypothesis also suggests that the first peak could be induced by acute myocardial ischaemia and subsequent necrosis, whereas the second peak could appear due to secondary events, such as inflammation of surrounding cardiac tissue, which may involve release mechanisms other than myocardial necrosis [7].

Myocardial injury is defined by an elevated cTn value, and it can be present also without myocardial ischaemia [2]. In addition to AMI, there are several other cardiac and non-cardiac conditions that can cause myocardial injury and are associated with hs-cTn elevations, such as heart failure, atrial fibrillation, pulmonary embolism,

CKD, sepsis, stroke, and strenuous exercise [36–42]. Furthermore, current hs-cTn assays can also measure detectable cTn levels in asymptomatic healthy individuals [43]. Traditionally, cTnI and cTnT have been considered as markers of myocardial necrosis. However, hs-cTn elevations have also been observed in conditions where myocardial necrosis is unlikely (e.g. healthy individuals after strenuous exercise) suggesting that there are alternative mechanisms that contribute to cTn release [28]. Cardiomyocytes have been shown to have a limited capacity to regenerate in the human heart. This cardiomyocyte renewal and normal turnover may explain detectable hs-cTn levels in healthy individuals [44–46].

Several alternative mechanisms, including programmed cardiomyocyte cell death (apoptosis and necroptosis) and reversible cardiomyocyte injury (cell wounds, bleb formation), have been proposed for cTn release [27]. Cardiomyocyte apoptosis and necroptosis have been mainly studied using animal models. The inhibition of apoptotic or necroptotic pathways has been demonstrated to reduce the size of myocardial injury in animal models of AMI [47–50]. Furthermore, apoptosis has been shown to be responsible for cTn release after brief ischaemia and preload-induced cardiomyocyte injury without ischaemia [51–53].

In addition to the cTn release from dead cells, cTn has also been proposed to leak into the bloodstream from living, reversibly injured cardiomyocytes through various mechanisms [54]. The cell membrane permeability of cardiomyocytes may transiently increase due to cell wounds formed in response to myocardial stress. Interestingly, cells seem to be able to repair holes larger than  $10\ \mu\text{m}^2$  within seconds in a cell wound repair process [55]. The formation and release of membranous blebs have also been suggested to contribute to the leakage of cTn into the blood [56]. Additionally, stimulation of stretch-responsive integrins has been demonstrated to induce cTnI release from viable cardiomyocytes [57]. Although there are various mechanisms that may potentially lead to cTn elevations, it remains incompletely understood which mechanisms contribute to cTn release in conditions other than AMI.

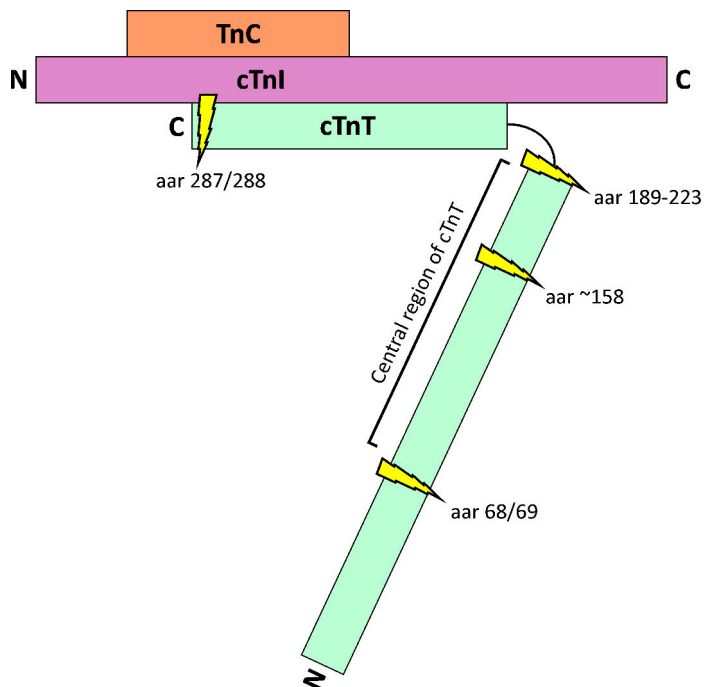
### 2.2.3 Fragmentation

The cTn complex is highly susceptible to proteolytic degradation that occurs in the setting of myocardial necrosis resulting in the presence of various cTnI and cTnT fragments in the blood of AMI patients [4–7,58–60]. The main circulating forms of cTn detected in AMI patients include full-size ITC complex consisting of intact or partially fragmented cTnT (37 kDa or 29 kDa) and cTnI (29 kDa or 27 kDa), low molecular weight ITC complex in which cTnT is present only as a C-terminal fragment (14 kDa), binary cTnI-TnC (IC) complex consisting of heavily fragmented cTnI (~14 kDa), and free cTnT fragments [6]. The composition of circulating cTn

has also been shown to change in a time-dependent manner following AMI, as full-size ITC complex seems to be most prominent in early presenting AMI patients. Over time, the proportion of binary IC complex and free small cTnT fragments increases [6].

The majority of cTnI has been found to be present as a part of the ITC or IC complexes and not as a free molecule in the circulation of AMI patients [6,61–66]. Proteolytic degradation of cTnI occurs at various sites both at the N-terminus and C-terminus. Early studies on cTnI fragmentation showed that the amount of cTnI fragments in the blood of AMI patients changes over time [58,59,67]. Recently, Katrukha et al. identified 11 major cTnI fragments in the blood of AMI patients and suggested that the most stable region of cTnI consists of aa 34–126 [60]. Interestingly, they also demonstrated that antibodies targeting the epitopes located within aa 23–196 were able to detect more than 80% of all cTnI in the samples of AMI patients within the first 36 hours after symptom onset. Additionally, the ratio of cTnI fragments was not observed to change in serial samples of AMI patients indicating that cTnI fragmentation may not follow a time-dependent pattern as clearly as initially thought [60]. In another recent study, proteolytic degradation of cTnI was also shown to correlate with the severity of ischaemic injury estimated by the total cTnI concentration, as AMI patients with the highest total cTnI concentrations were observed to have the lowest proportion of intact cTnI [68]. The enzymes that have been demonstrated to be capable of cleaving cTnI both at the N-terminus and C-terminus include calpains and matrix metalloproteinase-2 [69,70].

cTnT is also prone to N-terminal and C-terminal fragmentation [7]. Following AMI, cTnT is known to be released from damaged myocardium as a combination of intact cTnT (~37 kDa) and fragments of various sizes (~8–37 kDa) exhibiting a time-dependent degradation pattern. Intact and mildly degraded cTnT (29–37 kDa) seem to be the predominant form of cTnT release in early presenting AMI patients, whereas smaller heavily degraded cTnT fragments prevail at later time points [4–7,65,71]. Altogether, 23 different proteolytic cTnT fragments have been detected in blood of AMI patients, and the major N-terminal and C-terminal cleavage sites have been identified at aa 68–69 and 189–223, respectively [7]. The region between aa 189 and 223 contains multiple cleavage sites [7]. The schematic representation of the full-size ITC complex with the fragmentation sites of cTnT is shown in Figure 2.



**Figure 2.** Simplified schematic representation of full-size ITC complex. Lightnings indicate cTnT fragmentation sites. The site aar 189–223 consists of several cleavage sites. Figure not to scale.

The degradation of cTnT has been proposed to occur first at the N-terminus leading to the formation of the primary cTnT fragment (29 kDa). N-terminal cleavage is followed by cleavages at the C-terminus and the formation of secondary cTnT fragments (~15–20 kDa) [5–7]. Furthermore, all cTnT present in heparin plasma samples of AMI patients has been observed to have undergone C-terminal degradation at aar 287–288 in which the last C-terminal Lys amino acid is cleaved from the cTnT molecule [7]. Most intact cTnT and mildly degraded cTnT forms that still comprise the C-terminus seem to be part of the ITC complex [6,7].

Whether cTnT degradation occurs inside ischaemic cardiomyocytes, in the circulation, or both, remains a source of debate. Some recent evidence indicates that the degradation of cTnI and the ITC complex occurs at least to some extent in the circulation [72]. However, recent studies have suggested that most cTnT degradation may already occur in the damaged myocardium and not in the circulation of AMI patients [7,65]. Intracellular calpain-1 and caspase-3 enzymes are known to induce N-terminal cTnT cleavage at aar 68–69 [69,73–75]. Furthermore, thrombin, a coagulation enzyme, has also been shown to cleave cTnT at the same site [76,77]. Thrombin is generated during the preparation of serum samples explaining why intact cTnT can be found in lithium heparin plasma samples but not in serum samples

of AMI patients. Consequently, serum is not recommended to be used as a sample matrix in cTnT fragmentation studies [76–78]. Besides being a preanalytical factor, thrombin is also abundantly generated during intravascular blood clot formation in AMI patients and might thus also contribute to extracellular *in vivo* cTnT degradation [76,77,79]. However, multiple proteases are likely involved in cTnT degradation, and these proteases as well as whether cTnT degradation is intracellular or extracellular remain still largely unknown.

Recently, cTnT fragmentation has been of particular interest, as circulating molecular forms of cTnT have been observed to differ between AMI and other conditions associated with cTnT elevations. While intact and mildly degraded cTnT forms have mainly been detected in early presenting AMI patients, only heavily degraded, small cTnT fragments (<18 kDa) have been found in ESRD patients and in marathon runners, indicating different mechanisms behind cTnT release [9,10]. Thus, it is possible that the cTnT forms responsible for cTnT elevations in chronic myocardial injury or acute reversible damage of cardiomyocytes may be different from the cTnT forms released through myocardial necrosis in AMI. Targeting specifically intact and only mildly degraded cTnT forms might increase specificity for AMI and allow differentiation between cTnT elevations in AMI and other conditions [29]. Additionally, targeting different cTnT forms may also allow more reliable estimation of the ischaemic time window which could potentially improve the treatment and prognosis of AMI patients [66]. However, cTnT fragmentation studies have mainly been conducted with small numbers of patients and patient groups as well as with methods with limited sensitivity [4–6,8]. Thus, further research is required to better understand cTnT fragmentation in AMI and other conditions and the potential clinical implications.

## 2.2.4 Clearance

More than one mechanism is likely to contribute to the clearance of cTnI and cTnT. The current hypothesis is that cTnI and cTnT are cleared from the circulation via both renal and extrarenal mechanisms [80,81]. Extrarenal cTn clearance is assumed to occur via scavenger receptor-mediated endocytosis in the liver and reticuloendothelial system [80–82]. Scavenger receptors constitute a large, loosely defined group of cell surface receptors which can bind multiple unwanted proteins and promote their removal [83].

Mechanisms of cTn clearance have been predominantly studied in animal models. In the rat, fluorescently and radioactively labeled cTn preparations were observed to accumulate in the liver and kidneys [81]. As scavenger receptor-mediated endocytosis is known to become inefficient when only low levels of their targets are present in the circulation, it has been hypothesized that extrarenal

clearance dominates at high cTn concentrations, whereas renal clearance plays a significant role at low cTn concentrations [80,81]. Similar dual clearance system has also been proposed for myoglobin clearance [84–86]. The dual clearance system and the preferential cTn clearance via scavenger receptor-mediated endocytosis may explain why cTn clearance does not seem to significantly differ between patients with normal and reduced kidney function after a large AMI [81,87]. Reduced kidney function is likely to affect cTn clearance more in patients with small and stable cTn elevations [81,88]. Interestingly, reduced kidney function has also been speculated to affect cTnT levels to a greater extent than cTnI levels [89]. However, cTnT and cTnI seem to be cleared by the kidneys at similar rates [34].

In AMI patients with and without Q-waves, the elimination half-life of cTnI was reported to be 20.4 and 6.8 hours, respectively [90]. In another study, the estimated elimination half-lives of cTnI and cTnT in STEMI patients ranged from 12 to 17 hours when measured with five commercial hs-cTn assays [33]. However, it is impossible to determine the true elimination half-life of cTn in patients with an ongoing AMI due to simultaneous cTn release from ischaemic cardiomyocytes. Thus, cTn elimination has also been studied in animal models after an injection of exogenous cTn. The elimination half-life of injected cTnI has been reported to be 1.9 hours in dogs, 0.8 hours in rats and 0.5 hours in ponies [91,92].

Recently, a novel method based on autologous re-transfusion of plasma collected by plasmapheresis during AMI was used to determine the isolated cTn elimination kinetics in human [93]. After clinical recovery, patients returned to the hospital for autologous plasma re-transfusion and repeated blood sampling. As the method removed the effect of ongoing cTn release in AMI patients, it enabled more precise determination of the elimination half-lives of cTnI and cTnT. The estimated half-lives ranged from 134 min to 240 min and were clearly shorter than observed in patients with ongoing AMI [93].

## 2.3 Immunoassays for cardiac troponins

### 2.3.1 High-sensitivity assays

In clinical laboratories, cTnI and cTnT are measured using immunoassays which are based on specific antibody-antigen interactions. The first immunoassays for cTn were developed already in the late 1980s, whereas the first hs-cTn immunoassay was introduced in 2009 [94,95]. Currently, hs-cTn assays are widely used in clinical practice, and they also play an integral role in the fourth universal definition of myocardial infarction [2].

The definition of hs-cTn assay is based on two basic criteria. First, the assay should have a coefficient of variation (CV) of <10% at the 99<sup>th</sup> percentile URL of

healthy reference population which is the recommended diagnostic threshold for elevated hs-cTn levels [2,43]. Second, the assay should measure values above the limit of detection (LoD) in >50% of healthy individuals [43]. The implementation of hs-cTn assays has allowed faster and more accurate detection of myocardial injury. However, hs-cTn assays can often detect elevated cTn levels in multiple cardiac and non-cardiac conditions complicating the diagnosis of AMI [3,95].

The analytical characteristics of commercially available hs-cTn assays are summarized in the table provided by the Committee on Clinical Applications of Cardiac Biomarkers of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) [96]. The heterogeneity of molecular cTn forms in the circulation poses substantial challenges to the detection of cTn. Several manufacturers provide hs-cTnI assays, and these assays target different cTnI epitopes and consequently recognize different forms of cTnI [43,96,97]. The measurement of cTnI is known to be affected by various factors, including posttranslational modifications (proteolytic degradation and phosphorylation) and complex formation with TnC, heparin, heterophile or human antimouse antibodies, and cTnAAbs [43]. The lack of standardization remains a major challenge. Thus, hs-cTnI values obtained from different assays cannot be directly compared to each other, and assay-specific 99<sup>th</sup> percentile reference values should be used for each hs-cTnI assay [43,96].

In contrast to hs-cTnI assays, Roche Diagnostics GmbH was for a decade the only manufacturer providing commercial hs-cTnT assays due to international patents. These hs-cTnT assays target the stable central region of cTnT (aa 125–131 and 136–147) resulting in the detection of intact cTnT as well as mildly and heavily degraded cTnT forms (i.e. total cTnT) [4,96]. Following the expiration of the Roche hs-cTnT patents, other manufacturers have also begun to develop their own hs-cTnT assays [96].

### 2.3.2 Assays for specific forms of cardiac troponin T

Intact and mildly degraded cTnT forms have been predominantly found in early presenting AMI patients, whereas smaller heavily degraded cTnT forms seem to prevail in other conditions associated with cTnT elevations [4,6,7,9,10]. Thus, intact and mildly degraded cTnT forms have been proposed to be a promising and more specific biomarker for AMI. To date, cTnT fragmentation has mainly been studied using complicated, laborious and time-consuming methods with limited analytical sensitivity, such as gel filtration chromatography, western blot and mass spectrometry, which are not suitable for routine clinical practice. Due to these low-throughput methods, most cTnT fragmentation studies are based on very small sample sizes [4–7,9,10]. Importantly, as intact and mildly degraded cTnT forms

represent only a fraction of the total circulating cTnT, high analytical sensitivity is required for conducting reliable cTnT fragmentation studies in patients with different conditions.

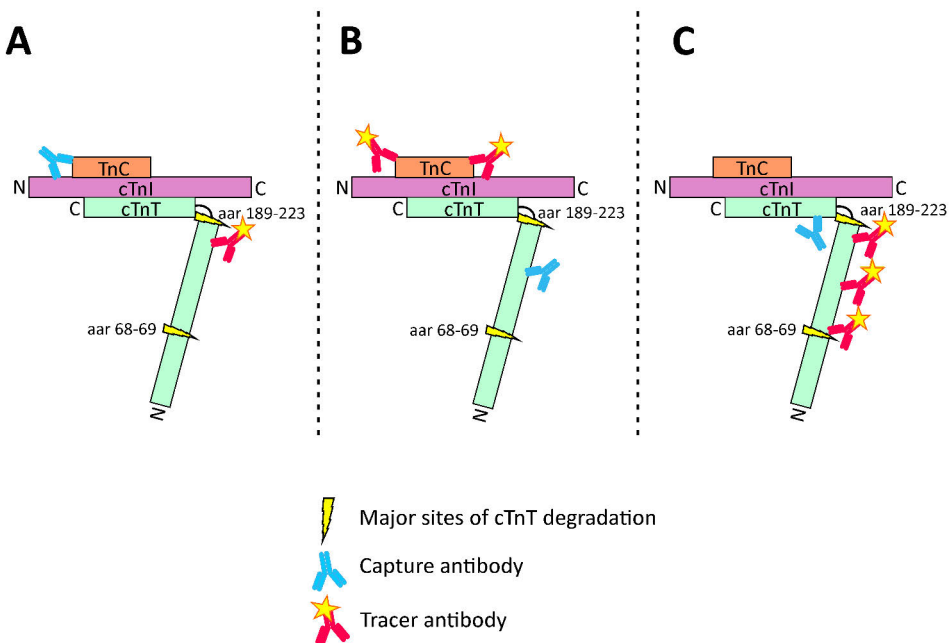
In one of the first studies to develop highly sensitive immunoassays for specific cTn forms, cTn composition was characterized in plasma samples collected from patients with type 1 AMI and patients with hs-cTnI elevations due to other etiologies (e.g. demand ischaemia and type 2 AMI) using three different immunoassays detecting either total cTnI, complexed cTnI (i.e. IC or ITC) or ITC complex with intact or mildly truncated cTnT [72]. These assays utilized a highly sensitive Pylon platform (ET Healthcare) [72,98]. Interestingly, no differences in cTnI composition were observed between the two patient groups. This study and a later study investigating coronary and peripheral cTn compositions in relation to culprit artery, severity of injury, and ischaemic time window in NSTEMI patients mainly focused on cTnI composition, although ITC containing intact or mildly truncated cTnT was also measured (assay principle shown in Figure 3A) [66,72]. Additionally, the number of patients was limited, and cTn compositions were characterized only in patients with type 1 AMI and in a heterogenous group of patients with hs-cTnI elevations due to other etiologies [66,72]. Timing of blood sampling after symptom onset affects cTn composition but in these studies, timing remained unknown or sampling occurred relatively late after symptom onset [66,72].

More recently, Li et al. used a similar approach to target different complexes and fragmentation forms of cTn [99]. They developed four highly sensitive immunoassays specific to the major cTn forms in the circulation using automated Mindray chemiluminescence immunoassay platform. The first assay detected 'long-cTnT ITC complex' in which cTnT is not degraded at aa 189–223 (Figure 3B), while the second assay also detected 'short-cTnT ITC complexes'. To measure the total cTnT concentration in the circulation, the third assay targeted the stable central part of cTnT similarly to the Roche hs-cTnT assay. The fourth assay measured the total cTnI concentration by targeting both binary IC and ternary ITC complexes [99]. These assays were used to characterize cTn composition and kinetics in patients with type 1 MI, patients undergoing cardiac surgeries and patients with chronic heart failure. Interestingly, the ratio of long-cTnT ITC complex to total cTnI, in particular, was observed to be significantly higher in patients with early-stage type 1 AMI and after acute cardiac surgery than in patients with chronic heart failure [100]. Thus, the results of the studies by Li et al. indicated that different cTn forms and their ratios may contribute to the differentiation between acute and chronic myocardial injuries [99,100].

A promising time-resolved fluorescence (TRF)-based immunoassay was also recently developed for the detection of intact and mildly degraded cTnT forms at the Department of Life Technologies of University of Turku using an alternative and

simpler approach by targeting only cTnT and not ITC complex [8]. The TRF long cTnT assay targeted the central and C-terminal parts of cTnT and detected cTnT forms that are not degraded at the major C-terminal cleavage site, aar 189–223 (Figure 3C). These forms were referred to as ‘long cTnT’. Three tracer antibodies labelled with europium chelates were used to maximize the analytical sensitivity [8].

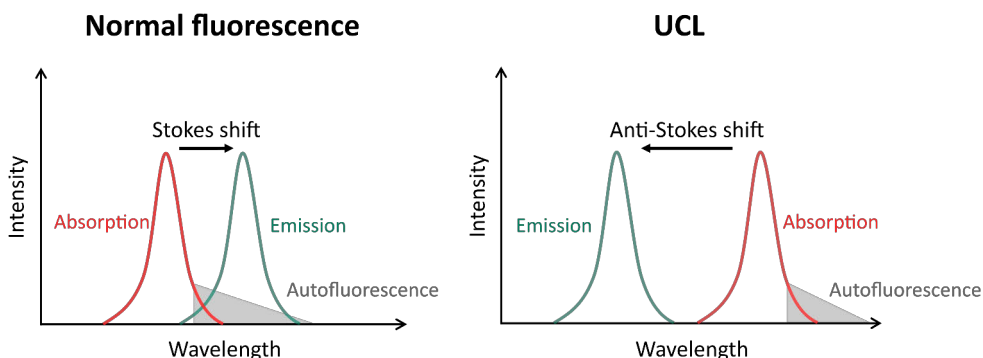
The TRF long cTnT assay showed improved specificity for AMI by discriminating between cTnT elevations in AMI and ESRD [8]. However, the assay had an LoD of 11 ng/L and limit of quantitation (LoQ) of 25 ng/L, which are not sufficiently low to allow reliable measurement of long cTnT concentrations in patients with small cTnT elevations and small fractions of long cTnT [8]. In comparison, the LoD and LoQ values for a widely used, commercial hs-cTnT assay by Roche Diagnostics are 3 ng/L and 5 ng/L, respectively [96]. To investigate the full potential of long cTnT as a promising and more specific biomarker for AMI, the analytical sensitivity of the TRF long cTnT should be improved.



**Figure 3.** Published investigational immunoassays targeting long cTnT forms not degraded at the major C-terminal cleavage site of cTnT, aar 189-223. (A) Large-size cTnTIC complexes assay published by van Wijk et al. [72]. The capture and tracer antibodies target complexed cTnI and the central part of cTnT, respectively. (B) Long-cTnT ITC complex assay published by Li et al. [99]. The capture antibody targets the central part of cTnT, while the two tracer antibodies target cTnI complex. (C) TRF long cTnT assay published by Airaksinen et al. [8]. The capture antibody targets the C-terminal part of cTnT, while the three tracer antibodies target the central part of cTnT.

### 2.3.3 Upconversion luminescence

Upconversion luminescence (UCL) is an attractive reporter technology for the development of highly sensitive immunoassays [101]. Upconverting nanoparticles (UCNPs) with a diameter of less than 100 nm are composed of inorganic crystalline host lattice doped with lanthanide ions (e.g.  $\text{Yb}^{3+}$  and  $\text{Er}^{3+}$ ) [102]. Unlike conventional fluorophores, which can absorb only one high-energy photon leading to the emission of a lower-energy photon according to the Stokes law, UCNPs have a unique ability to emit anti-Stokes shifted UCL by absorbing sequentially two or more low-energy photons for the emission of a single higher-energy photon. Thus, UCNPs can convert low-energy near-infrared excitation to high-energy emission at visible wavelengths, and they can be measured completely without autofluorescence background that originates from biological and other assay materials resulting in the excellent detectability of UCNPs (Figure 4) [101–103].



**Figure 4.** Diagrams of the Stokes shift in normal fluorescence and anti-Stokes shift in UCL. Autofluorescence usually interferes with the measurement of fluorescent signals. However, anti-Stokes shifted UCL can be measured completely without autofluorescence background.

Biological materials do not absorb infrared light used for the excitation of UCNPs, which allows the detection of UCNPs also in more challenging biological sample materials including whole blood [104]. UCNPs also have high photostability allowing repetitive measurements without photobleaching [101,105]. Furthermore, lanthanide dopants have many advantageous characteristics including narrow emission bands, large Stokes shift, and long emission lifetimes. These characteristics are also utilized in other lanthanide reporters, such as lanthanide chelates, that enable the measurement of TRF [101]. However, the instrumentation needed for the detection of UCNPs is simpler and more affordable, compared to the measurement of TRF [101,106]. The colour of the light emitted by UCNPs can be changed using

different lanthanide ion combinations, which enables the application of UCNPs also in spectral multiplexing [107].

Traditionally, the major challenges in applying UCNPs to bioaffinity assays have been related to the aggregation and non-specific binding of UCNP conjugates and the loss of structural integrity and decreased luminescence of UCNPs in water. However, these issues have been solved by the optimization of UCNP surface chemistry and assay conditions, which has allowed the development of highly sensitive immunoassays [108–110]. UCNPs have also been successfully utilized in highly sensitive investigational immunoassays for cTnI [106,109,110]. Raiko et al. developed an ultra-sensitive UCL-based immunoassay for cTnI that reached over 10-fold higher analytical sensitivity than reported for most of the current commercial hs-cTnI assays [110].

## 2.4 Cardiac troponins in chronic kidney disease

CKD is a condition with gradually and progressively declining kidney function. The most common causes of CKD include diabetes and hypertension, and the burden of CKD continues to grow globally [111–113]. According to current international guidelines, CKD is defined as abnormalities of kidney structure or function present for at least three months [111]. To meet the criteria for CKD, either of the following should be present for a minimum of three months: a glomerular filtration rate (GFR) of less than 60 mL/min/1.73 m<sup>2</sup> (GFR categories G3a–G5, see Table 1) or markers of kidney damage which include albuminuria, urine sediment abnormalities, persistent hematuria, electrolyte and other abnormalities due to tubular disorders, abnormalities detected by histology, structural abnormalities detected by imaging, and history of a kidney transplantation [111].

GFR indicates the flow rate of the filtered fluid through all the functioning nephrons and is currently the recommended method for the assessment of overall kidney function [111,113]. GFR is either measured as the renal clearance of exogenous filtration markers (e.g. iothalamate, iohexol, diethylenetriamine pentaacetate, inulin) or estimated using endogenous filtration markers (creatinine or cystatin C) [111]. The preferred and most commonly used equation for calculating estimated glomerular filtration rate (eGFR) using serum creatinine was developed by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) [111]. The 2021 CKD-EPI creatinine equation incorporates creatinine, age, and sex, whereas the previous 2009 CKD-EPI creatinine equation also incorporated race [114,115]. CKD can be classified based on GFR (categories summarized in Table 1) [111]. When GFR drops below 15 mL/min/1.73 m<sup>2</sup> (stage 5), a patient has reached ESRD. At this point, kidneys can no longer function on their own, and the two main treatment options for ESRD patients include dialysis and kidney transplantation [113].

**Table 1.** Stages of CKD based on GFR.

| Stage | GFR (mL/min/1.73 m <sup>2</sup> ) | Description                      |
|-------|-----------------------------------|----------------------------------|
| G1    | ≥90                               | Normal or high                   |
| G2    | 60–89                             | Mildly decreased                 |
| G3a   | 45–59                             | Mildly to moderately decreased   |
| G3b   | 30–44                             | Moderately to severely decreased |
| G4    | 15–29                             | Severely decreased               |
| G5    | <15                               | Kidney failure                   |

Abbreviations: CKD, chronic kidney disease; GFR, glomerular filtration rate.

CKD is a substantial risk factor for morbidity and mortality [111,113]. Due to progressively reducing kidney function, a variety of substances begin to accumulate in the body. Some of these substances are called uremic toxins because of their adverse biological effects. Uremic toxins are thought to contribute to the progression and complications of CKD [113,116]. The risk of adverse outcomes, such as all-cause mortality and cardiovascular events, increases with lower eGFR [117]. Thus, early detection and management of CKD are important to delay disease progression and reduce associated complications [111]. However, CKD patients at earlier stages may often be asymptomatic or have non-specific symptoms delaying the diagnosis. Thus, CKD is often diagnosed either by chance after screening tests or when symptoms have already become severe [112,113].

Cardiovascular disease (CVD) is one of the major complications of CKD [111,113]. CVD and CKD share common risk factors, such as diabetes and hypertension, which may, in part, explain the increased cardiovascular risk in individuals with CKD. However, mechanisms specific to CKD have also been independently associated with cardiovascular risk [118–120]. The increased risk of death of CKD patients is also largely attributable to cardiovascular deaths. More CKD patients have been shown to die of cardiovascular disease attributed to reduced GFR than of ESRD [121].

Persistent hs-cTn elevations, particularly hs-cTnT elevations, are a common finding in CKD patients with eGFR below 60 mL/min/1.73 m<sup>2</sup> [39,122]. Consequently, the interpretation of hs-cTn results in CKD patients with suspected AMI might often be challenging [120]. As CKD is associated with an increased risk of CVD, elevated hs-cTn levels potentially reflect both increased cardiovascular burden and reduced renal elimination of cTn [39,80–82]. Although extracellular clearance of cTnT via scavenger receptor-mediated endocytosis has been proposed to dominate at high cTnT levels, kidney function seems to significantly contribute to cTnT clearance at low cTnT levels. Thus, reduced kidney function is also likely to cause persistently elevated cTnT levels often observed in CKD [80].

Patients with CKD even within the same GFR category may have very different individual risks of kidney failure, cardiovascular events and all-cause mortality. Thus, the development of risk prediction models might help in estimating individual risk and making personalized treatment decisions [111,123]. Interestingly, elevated hs-cTnT levels have been associated with adverse long-term outcomes in the general population as well as in CKD including ESRD patients receiving maintenance dialysis [39,124–130]. Recently, independent associations between hs-cTnT and all-cause mortality and new-onset atrial fibrillation (NOAF) have been described in patients with CKD stage 4–5 [131,132]. Commercial hs-cTnT assays measure intact cTnT as well as mildly and heavily degraded cTnT forms, but only small cTnT fragments seem to be present in patients with ESRD receiving maintenance dialysis [8,9]. Currently, there are no published studies assessing cTnT fragmentation in the setting of CKD stage 4–5 prior to dialysis initiation and thus, no data exist on the association between long cTnT forms and adverse long-term outcomes in patients with CKD stage 4–5.

## 2.5 Cardiac troponin-specific autoantibodies

### 2.5.1 Prevalence, formation and clinical significance

Autoantibodies are produced by the human immune system against the individual's own tissues, cells and molecules. Autoantibodies to cTn (i.e. cTnAAbs) have been detected in 2–20% of individuals with or without cardiac disease [133–142,12]. Approximately 10% of healthy individuals have been reported to develop cTnAAbs [138–140]. In comparison, the prevalence of cTnAAbs in patients with a non-ST elevation ACS at long-term follow-up has been shown to vary from 11% to 17% [137,141]. To date, the highest prevalences has been reported in patients with dilated or ischaemic cardiomyopathies [133–136].

Any release of cTn into the circulation following myocardial injury, such as AMI, may potentially trigger an autoimmune response and formation of cTnAAbs. Some AMI patients have been shown to become cTnAAb-positive only after AMI, which supports the hypothesis that large cTn release may lead to the formation of cTnAAbs [137,141]. However, the majority of AMI patients does not seem to develop cTnAAbs despite the release of large amounts of cTn into the circulation [137,141]. In animal models, immunization of mice with murine cTn has been demonstrated to lead to the production of cTnAAbs [143]. Furthermore, infection with cardiotropic viruses or bacteria is known to induce the formation of autoantibodies against cardiac antigens [144–146]. cTnAAbs are also relatively common in apparently healthy individuals, but the underlying mechanisms of the formation of cTnAAbs in healthy individuals remain unknown [147].

Clinical significance of cTnAAbs have been studied in animal models as well as in humans. However, the evaluation of the clinical significance is challenging, as cTnAAbs are present both in patients with cardiac disease and in healthy individuals. In animal models, the formation of cTnAAbs has been shown to result in adverse outcomes, such as myocardial inflammation and fibrosis, heart failure, and development of dilated cardiomyopathy [143,148–152]. In humans, the presence of cTnAAbs has been suggested to contribute to the pathological ventricular remodelling after AMI [135]. However, Lindahl et al. found no association between the presence of cTnAAbs and adverse long-term prognosis in a large cohort of non-ST elevation ACS patients [141].

In the bloodstream, cTnAAbs can bind to circulating cTn and form large macrotroponin complexes with a molecular weight of around 200 kDa [11]. The presence of macrotroponin complexes has been observed to cause persistent hs-cTn elevations that are not related to a recent or ongoing myocardial injury [12,13]. Interestingly, the presence of macrotroponin itself has been associated with improved overall survival in a cohort of community patients with elevated hs-cTnI values [153]. Furthermore, an earlier study revealed that the possible presence of macrotroponin was associated with favourable long-term all-cause and cardiovascular mortality in a cohort of patients with elevated cTnI values measured by a conventional cTnI assay [154]. This may be due to the fact that hs-cTn elevations detected in patients with macrotroponin may not reflect actual myocardial injury. Nevertheless, the clinical significance of cTnAAbs and macrotroponin remains unclear [147,155].

## 2.5.2 Interference with cardiac troponin assays

Hs-cTn assays, like all immunoassays, are susceptible to antibody-mediated interferences [11]. Endogenous cTnAAbs have been identified to be a common cause of discrepancy between different hs-cTn assays, and they are known to interfere with hs-cTn assays by causing either misleading hs-cTn elevations or false-negative hs-cTn results (Figure 5) [12–14,156]. These cTnAAb-related positive and negative interferences may potentially affect patient management and lead to erroneous clinical decisions [11].

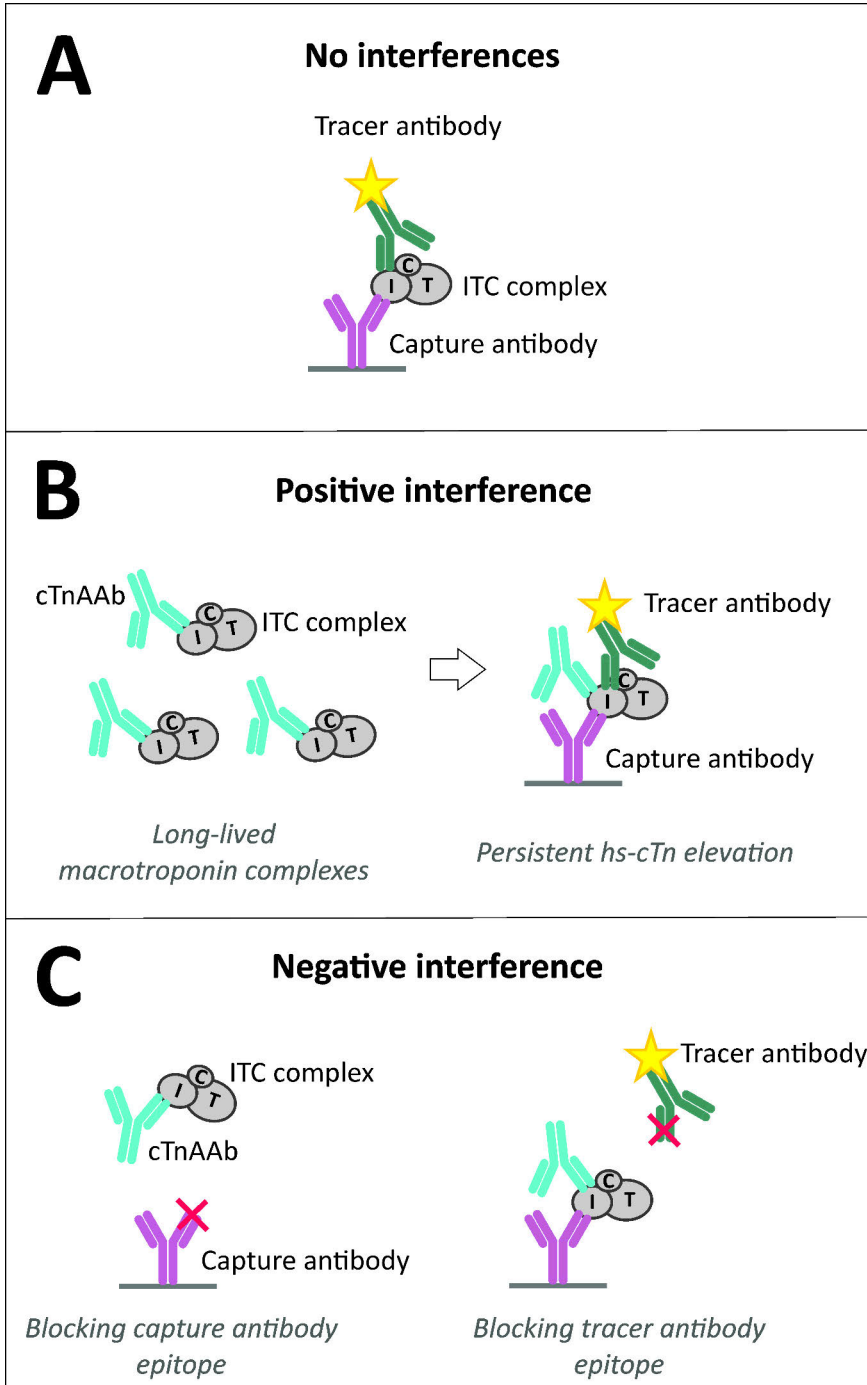
The presence of large macrotroponin complexes formed between cTnAAbs and cTn in the circulation may lead to persistent hs-cTn elevations that are inconsistent with the clinical presentation of the patient and can continue for weeks or even years [12,13,157–161]. These persistent hs-cTn elevations have been hypothesized to be attributable to reduced clearance of macrotroponin, as immunoglobulins are known to have a much longer elimination half-life than free cTn in the circulation (Figure 5B) [11]. Similar mechanism has also been reported for other macroanalytes, such

as macroenzymes and macroprolactin [162,163]. However, direct evidence of the reduced clearance of macrotroponin has been lacking.

Macro-troponin has been observed to be a common cause of positive interference in hs-cTn assays. For instance, Warner and Marshall identified macro-troponin in up to 5% of the patients with elevated hs-cTnI values measured with the Abbott Architect hs-cTnI assay [12]. A recent study by Strandkjær et al. investigated macro-troponin prevalence in healthy individuals with the highest hs-cTn levels (top 10%) measured using the Siemens Atellica hs-cTnI and Roche hs-cTnT assays [164]. They found macro-troponin in 76% of the plasma samples measured by the Siemens Atellica hs-cTnI assay but none in the plasma samples measured by the Roche hs-cTnT assay suggesting that positive macro-troponin interference may not affect the Roche hs-cTnT assay as prominently [164].

When cTnAAbs bind to circulating cTn, they can also cause negative interference by masking critical epitopes and preventing assay antibodies from recognizing cTn (Figure 5C) [14,156,165–168]. The target epitopes of cTnAAbs may vary considerably between cTnAAb-positive individuals [169]. However, a few studies with limited numbers of cTnAAb-positive patients have shown that cTnAAbs mainly seem to target epitopes located in the central region of the cTnI molecule (~aar 30–110) and C-terminal region of the cTnT molecule (aar 223–242) [156,169–171]. Furthermore, Vylegzhanina et al. demonstrated that cTnAAbs are specific to the structural epitopes formed by cTnI and cTnT polypeptide chains in the ITC complex, as cTnAAbs were not observed to interfere with the detection of the binary IC complex or free cTnI [171]. This may lead to the underestimation of cTnI levels particularly in the early samples of AMI patients, as ITC complexes have been predominantly detected in early presenting AMI patients [171].

Due to the susceptibility of cTnI to proteolytic degradation, conventional cTnI assays utilize antibodies targeting the stable central region of cTnI and are thus more susceptible to cTnAAb-mediated negative interference [58,43,172]. To avoid this interference, many of the current hs-cTnI assays utilize multiple capture or tracer antibodies targeting also epitopes outside the most stable central region of cTnI [96,156,169,173]. However, extensive evaluation of how cTnAAbs interfere with different commercial hs-cTn assays is needed.



**Figure 5.** cTnAAb-mediated interferences in hs-cTn immunoassays. (A) Sandwich-type hs-cTn immunoassay without any interference. (B) Persistent hs-cTn elevation due to macrotroponin. (C) False-negative hs-cTn result due to cTnAABs masking epitopes of the assay antibodies.

### 2.5.3 Detection

Positive or negative cTnAAb-mediated interference can be suspected when hs-cTn results are not consistent with the clinical presentation of the patient. However, false-negative hs-cTn results are rarely identified in clinical practice, whereas false-positive hs-cTn results can be suspected and identified more commonly [11]. To avoid unnecessary examinations and costs as well as erroneous clinical decisions, it is important for clinicians to understand the possibility that false hs-cTn results may occur due to cTnAAbs. When the presence of cTnAAbs is suspected, clinical laboratories are responsible for performing further investigations and identifying possible interferences [11].

Currently, there is no widely available assay that could be used for the direct measurement of cTnAAbs. If cTnAAb-related interference is suspected, the sample can be first analyzed with an alternative hs-cTn assay, as cTnAAb-related interferences are often assay-specific. However, cTnAAbs can potentially interfere also with the alternative assay [11]. Recently, Lam and Kyle introduced practical approaches to detect macrotroponin including support vector machine analysis that was shown to classify discrepancies between hs-cTn assays with high specificity [174]. However, this approach seemed to be suitable only for certain hs-cTn assay pairs [174]. Furthermore, comparison of hs-cTnI and hs-cTnT levels and calculating their ratio seem to be an effective method for detecting macrotroponin [164].

Other recommended methods for the investigation of cTnAAb-related interferences include polyethylene glycol (PEG) precipitation, immunoglobulin depletion, gel filtration chromatography, and sucrose gradient ultracentrifugation [11]. However, these methods are not specific for cTnAAbs or macrotroponin and cannot discriminate between cTnAAb-related interferences and interferences caused by heterophile antibodies. Furthermore, the latter two methods are also complex, labour-intensive and not available in routine clinical practice [11,147]. Over the years, various investigational immunoassays have also been developed for the measurement of cTnAAbs [14,135,142]. However, none of them have yet found their way in routine clinical use. Additionally, it is impossible to measure exact concentrations of cTnAAbs due to varying affinities of cTnAAbs and lack of defined standards [14,135,142].

### 3 Aims of the Study

The aim of this thesis was to develop a novel highly sensitive immunoassay for the detection of long molecular forms of cTnT and study cTnT fragmentation in different patient groups. In addition, the thesis aimed to improve understanding of cTnAAb-mediated interferences in hs-cTn assays.

The specific objectives of this thesis were:

- I. To develop a highly sensitive immunoassay for long cTnT forms using UCL and evaluate its analytical and diagnostic performance.
- II. To evaluate associations between long cTnT forms measured by the developed UCL long cTnT assay and adverse long-term outcomes in patients with CKD stage 4-5 not on dialysis.
- III. To study the effects of cTnAAbs on the elimination half-life and clearance of cTn measured by five commercial hs-cTn assays.

## 4 Summary of Materials and Methods

### 4.1 Patients

Clinical samples analyzed in the publications **I–III** are summarized in Table 2. All samples were stored at  $-70^{\circ}\text{C}$  until in-house cTnT and cTnAAb analyses.

#### 4.1.1 Patients with AMI or ESRD (I)

All clinical samples used in the original publication **I**, were collected for the troponin fragmentation in myocardial injury (Tropo-Fragm) study (ClinicalTrials.gov Identifier: NCT04465591). Lithium-heparin (LiH) plasma samples from NSTEMI patients ( $n=30$ ) were collected within 24 hours of symptom onset, whereas LiH plasma samples from ESRD patients ( $n=37$ ) were collected during a dialysis clinic visit before the hemodialysis. Serial LiH plasma samples were collected from STEMI patients ( $n=13$ ) at three varying time points after symptom onset. LiH plasma samples matched with either ethylenediaminetetraacetic acid (EDTA) plasma ( $n=9$ ) or serum samples ( $n=9$ ) were also collected from STEMI patients during the same draw. Furthermore, blood samples of 6 STEMI patients were divided into two aliquots before LiH plasma separation and either centrifuged immediately or after two hours at room temperature (RT).

#### 4.1.2 Patients with chronic kidney disease stage 4–5 (II)

The study cohort in the manuscript **II** comprised 137 patients with CKD stage 4–5 recruited to the Chronic Arterial Disease, quality of life and mortality in KIDney injury (CADKID) study (ClinicalTrials.gov Identifier: NCT04223726). LiH plasma samples were collected from the included patients within one year of recruitment to the CADKID study and prior to the initiation of dialysis treatment.

#### 4.1.3 Patients in elimination kinetics studies (III)

The study cohort in the original publication **III** comprised 20 STEMI patients who underwent plasmapheresis within 24 hours of revascularization (median 25 [25th–

75th percentile, 21–29] hours of symptom onset) to harvest plasma with a high cTn concentration. After at least three weeks of clinical recovery, patients returned to the hospital for a re-transfusion of autologous plasma followed by repeated blood sampling at fixed time points for 8 hours. Detailed information on the experimental design, patient eligibility criteria, and blood sampling are provided in the publication of Kristensen et al. [93].

#### 4.1.4 Ethics

The study protocols were approved by the local ethics committees. The studies were conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from all participants.

**Table 2.** Clinical samples used in the original publications I–III.

| Sample panel (ClinicalTrials.gov identifier) | Place and time of collection                                   | Study population (N)                                 | Sample matrix  | Publication  |
|--|--|--|--|--|
| Tropo-Fragm (NCT04465591)                    | Turku University Hospital, Turku, Finland 2020–2022            | Patients with NSTEMI (30), ESRD (37), and STEMI (37) | LiH plasma (+ matched EDTA plasma (n=9) and serum (n=9)) | <b>I</b><br>LoD, LoQ<br>Linearity<br>Sample matrix effect<br>Comparison of assays<br>Diagnostic performance<br>Time-dependent cTnT fragmentation |
|  | Biotechnology Unit, University of Turku, Turku, Finland, 2024  | Apparently healthy individuals (5)                   | LiH plasma   | <b>III</b><br>cTnAAb-negative control pool in epitope specificity studies  |
| CADKID (NCT04223726)                         | Turku University Hospital, Turku, Finland 2013–2017            | Patients with CKD stage 4–5 (137)                    | LiH plasma   | <b>II</b><br>Association of long cTnT with adverse long-term outcomes in CKD stage 4–5   |
| Troponin half-life (NA)                      | Copenhagen University Hospital, Copenhagen, Denmark, 2021–2022 | Patients with STEMI (20)                             | LiH plasma   | <b>III</b><br>Effect of cTnAAb on elimination half-life and clearance of hs-cTn  |

Abbreviations: NA, not available; STEMI, ST elevation myocardial infarction; NSTEMI, non-ST elevation myocardial infarction; ESRD, end-stage renal disease; CKD, chronic kidney disease; LiH, lithium-heparin; LoD, limit of detection; LoQ, limit of quantitation; cTnT, cardiac troponin T; hs-cTn, high-sensitivity cardiac troponin; cTnAAb, cardiac troponin-specific autoantibody.

## 4.2 Antibodies and human cardiac troponin ITC complex

Almost all monoclonal antibodies (mAb) as well as the native human cardiac troponin ITC complex used in the original publications **I–III** were obtained from HyTest (Finland). The cTnI-specific 8I7 mAb was purchased from International Point of Care Inc. (Canada). All mAbs, their antigens and epitopes are presented in Table 3 as reported by the manufacturer. The epitope of 8I7 mAb was corrected by Vylegzhanina et al. [175].

**Table 3.** Antibodies used in the original publications I–III.

| mAb  | Antigen   | Epitope (aar)        | Publication |
|------|-----------|----------------------|-------------|
| 7G7  | cTnT      | 67–86                | I           |
| 329  |           | 119–138              | I           |
| 300  |           | 119–138              | II          |
| 406  |           | 132–151              | II          |
| 1C11 |           | 171–190              | I, II       |
| 7E7  |           | 223–242              | I, II       |
| 916  | cTnI      | 13–22                | III         |
| 801  |           | 18–35                | III         |
| 4C2  |           | 23–29                | III         |
| M155 |           | 26–35                | III         |
| 19C7 |           | 41–49                | III         |
| 247  |           | 65–74                | III         |
| 560  |           | 83–93                | III         |
| 8E10 |           | 86–90                | III         |
| 84   |           | 117–126              | III         |
| M46  |           | 130–145              | III         |
| 441  |           | 148–158              | III         |
| 8I7  |           | 169–178 <sup>a</sup> | III         |
| 625  |           | 169–178              | III         |
| MF4  |           | 190–196              | III         |
| 7B9  |           | TnC                  |             |
| 3D3  | Human IgG |                      | III         |

mAb, monoclonal antibody; aar, amino acid residue; cTnT, cardiac troponin T; cTnI, cardiac troponin I; TnC, troponin C.

<sup>a</sup>Corrected epitope by Vylegzhanina et al. [175]. The epitope reported by the manufacturer is aar 137–148.

## 4.3 Preparation of antibody conjugates

Capture antibodies (**I–III**) were labelled with 30-fold molar excess of biotin isothiocyanate (Biotechnology unit, University of Turku, Finland) in 50 mM carbonate buffer (pH 9.8). The reaction mixture was incubated at RT in the dark for 4 h. Following the incubation, the biotinylated capture antibody was purified from free biotin, and the buffer was changed to tris-buffered saline with azide (TSA buffer, 50mM Tris-HCl, pH 7.75, 150 mM NaCl, 0.5 g/L NaN<sub>3</sub>) using NAP-10 and PD-10 columns (Cytiva, Massachusetts, USA). Bovine serum albumin (BSA, Probumin, Merck Millipore, Massachusetts, USA) was added to a concentration of 1 g/L and the biotinylated capture antibodies were stored at +4 °C.

Tracer antibodies used in the TRF immunoassays (**I** and **III**) were conjugated with 30- to 35-fold of intrinsically fluorescent europium chelate (Biotechnology Unit, University of Turku) as described previously [166]. Tracer antibodies used in the UCL immunoassays (**I** and **II**) were conjugated to oleic acid-capped NaYF<sub>4</sub>: 17% Yb<sup>3+</sup>, 3% Er<sup>3+</sup> UCNPs (Biotechnology Unit, University of Turku) [176]. Before conjugation, UCNPs were coated with poly(acrylic acid) (PAA) via two-step ligand exchange process using 1,8-diazabicyclo[5.4.0]-7-undecene (DBU, TCI, Japan) in the pH adjustment of PAA-coating reaction [110]. PAA-coated UCNPs were conjugated to tracer antibodies using EDC/sulfo-NHS chemistry following a previously published protocol [110].

## 4.4 UCL long cTnT assay (I, II)

### 4.4.1 Assay design and protocol

The selected capture antibody (7E7 mAb) and tracer antibody (1C11 mAb) of the UCL-based heterogenous sandwich-type immunoassay target aar 223–242 and 171–190, respectively (Table 4). Thus, the UCL long cTnT assay detects cTnT molecules that are not degraded at the major C-terminal cleavage site, aar 189–223. Native human cardiac troponin ITC complex is used as a calibrator. A schematic representation of the assay is shown in Figure 6C.

The UCL long cTnT assay was performed in C8 Lockwell LUMI White microtiter plates (Thermo Scientific, Massachusetts, USA), which were passively coated with 1 µg/well streptavidin (Biospa, Italy) as described previously [177]. Before initiating the assay, the tracer antibody (1C11 mAb-UCNP) was diluted to a concentration of 8 µg/mL in colorless assay buffer (Uniogen Oy, Finland) supplemented with 0.05 wt% PAA (MW 1200, Sigma-Aldrich, Missouri, USA), 1 mM KF, 0.2 wt% fat-free milk powder (Valio Oy, Finland), 0.8 g/L native mouse

IgG (Meridian Life Science, Tennessee, USA), and 0.05 g/L denatured mouse IgG (denaturation at 63°C for 30 min) and incubated for 30 min at RT.

After prewashing the microtiter plate with wash buffer (Uniogen), 200 ng of biotinylated capture antibody in 50 µL of colourless assay buffer was added to the wells and incubated at RT with slow shaking for 30 min. Following washing, 30 µL of sample or calibrator (ITC-complex in TSA buffer supplemented with 75 g/L bovine serum albumin [BSA, Probumin, Merck Millipore]) was mixed with 40 µL of sample buffer (13 mM Tris, pH 8, 175 mM NaCl, 0.13 g/L NaN<sub>3</sub>, 8.75 g/L BSA [Bioreba AG, Switzerland], 17.5 g/L D-trehalose [Sigma-Aldrich], 0.21 g/L bovine γ-globulin [Sigma-Aldrich], 0.28 g/L native mouse IgG, 0.0175 g/L denatured mouse IgG, 0.7 g/L casein [Calbiochem, Merck Millipore], and 13.13 U/mL heparin [Sigma-Aldrich]) and added to the wells in triplicates. Calibrators and samples were incubated at RT with slow shaking for 30 min followed by washing.

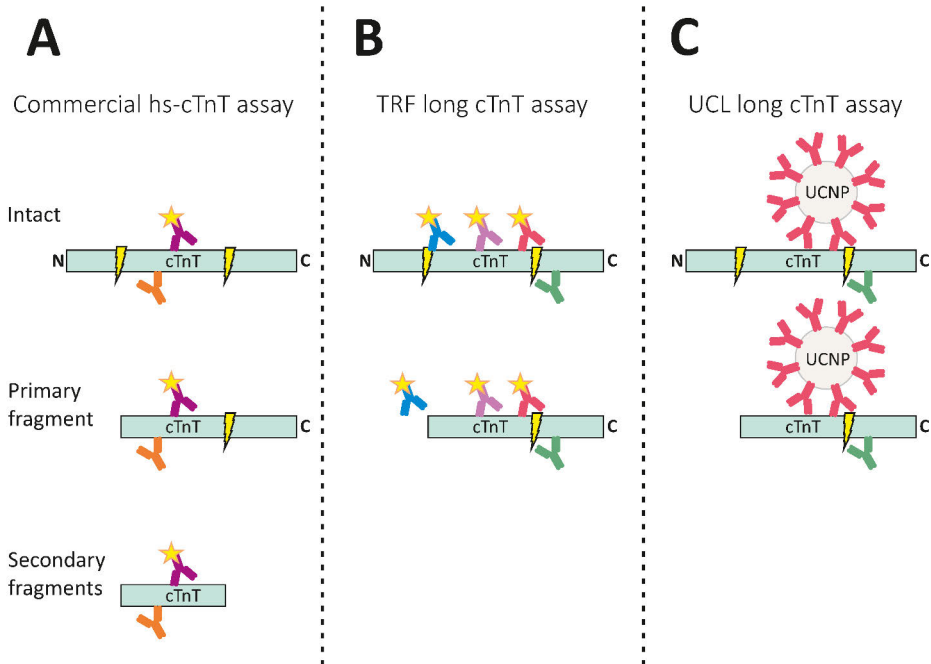
Before adding 50 µL of tracer antibody dilution to the wells, the dilution was sonicated using a vial tweeter sonicator (3 cycles, 0.5 s with 100% amplitude, Hielscher Ultrasonics GmbH, Germany). Following incubation at RT with slow shaking for 15 min, the plate was washed four times using pH-adjusted wash buffer (NaOH, pH 10.25) and left to dry at RT for 90 min. After drying, upconversion luminescence signal was measured from the bottoms of the wells at 540 nm using a modified Plate Chameleon microplate reader (Hidex Oy, Finland) equipped with a 980 nm laser [101].

#### 4.4.2 Evaluation of assay performance

The limit of blank (LoB, defined as the highest apparent analyte concentration likely to be observed for a blank sample) and LoD (defined as the lowest analyte concentration that can be reliably distinguished from a blank sample) of the UCL long cTnT assay were determined following the classical approach of the Clinical and Laboratory Standards Institute (CLSI) guideline EP17-A2 with minor modifications [178]. One batch of zero calibrator (7.5% BSA-TSA) was analyzed in 63 replicates over 18 days for nonparametric LoB. Clinical samples with low-level long cTnT concentrations ( $1-5 \times \text{LoB}$ ,  $n=20$ ) were analyzed in triplicates over 9 days for parametric LoD.

The LoQ (defined as the lowest analyte concentration that can be quantitatively determined with stated accuracy) was determined using the within-run precision profile of 113 clinical samples (measured in triplicates over 16 days) and an accuracy goal of 10% CV. To study possible sample matrix effects, LiH plasma samples collected from STEMI patients were compared to matched EDTA plasma ( $n=9$ ) and serum samples ( $n=9$ ). Dilution linearity was assessed by making 1/2 and 1/4 serial dilutions of LiH plasma samples collected from NSTEMI patients ( $n=6$ , initial long

cTnT concentrations 4–411 ng/L) in 7.5% BSA-TSA buffer. The diagnostic performance of the UCL long cTnT assay was preliminarily evaluated by measuring long cTnT concentrations in LiH plasma samples of NSTEMI (n=30) and ESRD (n=37) patients and comparing the results to a commercial hs-cTnT assay and a previously developed TRF long cTnT assay.



**Figure 6.** Molecular forms detected by the commercial hs-cTnT assay (i.e. total cTnT assay) (A), TRF long cTnT assay (B), and UCL long cTnT assay (C). Lightnings indicate the major N-terminal and C-terminal cleavage sites of cTnT, aa 68–69 and 189–223.

## 4.5 Other investigational in-house immunoassays

### 4.5.1 TRF long cTnT assay (I)

In the original publication I, a previously developed TRF long cTnT assay utilizing one capture antibody (7E7 mAb) and three tracer antibodies (7G7, 329 and 1C11 mAbs) labelled with europium(III) chelates was used as a reference method for the novel UCL long cTnT assay [8]. Similarly to the novel UCL long cTnT assay, the TRF long cTnT assay targets cTnT molecules that are not degraded at aa 189–223. The schematic representation of the TRF long cTnT assay is shown in Figure 6B. The LoD and target epitopes are presented in Table 4.

First, 200 ng of biotinylated capture antibody (7E7 mAb) diluted in 25  $\mu$ L of red assay buffer (Uniogen) was added to the wells of a yellow streptavidin plate (Uniogen) and incubated at RT for 1 hour. After washing the wells twice with wash buffer (Uniogen), 100 ng of each tracer antibody diluted in 40  $\mu$ L of tracer buffer (100 mM Tris, pH 7.75, 600 mM NaCl, 0.5 g/L NaN<sub>3</sub>, 25 g/L BSA [Probumin], 0.6 g/L bovine  $\gamma$ -globulin, 0.8 g/L native mouse IgG, 0.05 g/L denaturated mouse IgG, 4 g/L casein) were added to the wells. Sample or calibrator (native human cardiac troponin ITC complex) were added to the wells (volume 30  $\mu$ L) in triplicates. After 1 hour of incubation at +36°C with 900 rpm shaking (iEMS incubator/shaker, Thermo Fisher Scientific), the wells were washed six times and dried under a stream of hot air for 5 min. The TRF signal (excitation wavelength 340 nm, emission wavelength 615 nm, measurement delay 250  $\mu$ s, measurement window 750  $\mu$ s) was measured from the dried and cooled well bottoms using a Victor X4 Multilabel Counter (Revvity, Massachusetts, USA).

#### 4.5.2 UCL total cTnT assay (II)

In the manuscript **II**, total cTnT concentrations were measured with an UCL-based in-house immunoassay. The selected capture antibody (300 mAb) and tracer antibody (406 mAb) target the stable central region of cTnT, aa 119–138 and 132–151, respectively. The setup of the UCL total cTnT assay resembles the widely used Elecsys hs-cTnT assay manufactured by Roche Diagnostics (Figure 6A, Table 4), and thus the UCL total cTnT assay can also detect intact cTnT as well as mildly and heavily degraded cTnT fragments. Apart from the different antibody combination and the supplementation of the tracer antibody dilution buffer with 0.1 M NaCl, the UCL total cTnT assay was performed similarly to the UCL long cTnT assay protocol.

#### 4.5.3 cTnAAb assay (III)

In the original publication **III**, patient samples were analyzed for the presence of cTnAAbs using a previously published cTnAAb immunoassay [142]. Briefly, 150 ng of both biotinylated capture antibodies (M155 and 8I7 mAbs) were added to the wells of a yellow streptavidin plate (Uniogen) in 25  $\mu$ L of red assay buffer (Uniogen) and incubated at least for one hour at RT. During the capture antibody incubation, plasma samples were diluted 5-fold with insulating layer II buffer (Radiometer, Denmark) and divided into two aliquots, of which one was spiked with 30  $\mu$ g/L human ITC complex and the other remained unspiked. After incubating sample aliquots at +4°C for one hour, the wells were washed twice with wash buffer (Uniogen), and 30  $\mu$ L of sample aliquot and 200  $\mu$ L of red assay buffer supplemented

with 27 g/L NaCl were added to the wells in triplicates. Following one hour of incubation at +36°C with 900 rpm shaking (iEMS incubator/shaker), the wells were washed twice, and 40 ng of europium chelate labeled tracer antibody (3D3 mAb) was added to the wells in 200 µL of red assay buffer. After another one hour of incubation at +36°C with 900 rpm shaking, the wells were washed six times and dried under a stream of hot air for 5 min. The TRF signal was measured from the dried and cooled surface with a Victor X4 Multilabel Counter. Samples were determined to be cTnAAb-positive when the signal difference between ITC-spiked aliquot and unspiked aliquot was  $\geq 100$  counts, and the t-test gave a p-value  $< 0.05$ .

#### 4.5.4 Assays for epitope specificity studies (III)

Epitope specificity studies were conducted on cTnAAb-positive patient samples in the original publication **III** to determine the binding sites of cTnAAb on the cTnI molecule. The analytical recoveries of ITC complex measured in cTnAAb-positive samples were compared to those measured in a cTnAAb-negative control pool (LiH plasma from five healthy individuals). Sandwich-type immunoassays using different cTnI-specific capture antibodies and the same troponin C-specific tracer antibody were performed to measure the fluorescence signal from both unspiked and ITC-spiked plasma samples as described previously [169]. In short, 300 ng of biotinylated capture antibody was added to the wells of a yellow streptavidin plate (Uniogen) in 25 µL of red assay buffer (Uniogen) and incubated at least for one hour at RT. Meanwhile, aliquots of plasma samples were spiked with 30 µg/L ITC complex and incubated at +4°C for one hour. After washing the wells twice with wash buffer (Uniogen), 20 µL of sample (unspiked and ITC-spiked aliquots) and 100 ng of europium chelate labeled 7B9 tracer antibody in 20 µL of insulation layer II buffer (Radiometer) were added to the wells in duplicates and incubated at +36°C with 900 rpm shaking (iEMS incubator/shaker) for one hour. After incubation, the wells were washed six times and dried under a stream of hot air for 5 min. The TRF signal was measured from the dried and cooled surface with a Victor X4 Multilabel Counter. Finally, the ITC-specific signals measured in cTnAAb-positive samples were compared to the ITC-specific signal measured in the cTnAAb-negative control pool to obtain the sample-specific recoveries for different capture antibodies.

## 4.6 Commercial cTn immunoassays (I, III)

In the original publication **I**, total cTnT concentrations were measured with the Elecsys hs-cTnT assay (Roche Diagnostics GmbH, Germany). The schematic representation of the Elecsys hs-cTnT assay (i.e. total cTnT assay) is shown in Figure 6A. In the original publication **III**, elimination kinetics of cTn in humans were

investigated using 5 commercial hs-cTn assays including the Elecsys hs-cTnT assay, the Atellica IM hs-cTnI assay (Siemens, Germany), the Dimension Vista hs-cTnI assay (Siemens), the Alinity i STAT hs-cTnI assay (Abbott Laboratories, Illinois, USA) and the Vitros hs-cTnI assay (Ortho Clinical Diagnostics, New Jersey, USA). The LoDs and target epitopes of the commercial hs-cTn assays are summarized in Table 4.

**Table 4.** Investigational in-house cTnT assays and commercial hs-cTn assays used in the original publications I–III.

| Assay   | LoD (ng/L)       | Epitopes                                       | Publication |
|---|------------------|--|-------------|
| <b>Investigational in-house immunoassays for cTnT</b> |                  |  |             |
| UCL long cTnT assay                                   | 0.4              | C: 223–242<br>T: 171–190                       | I, II       |
| TRF long cTnT assay                                   | 11               | C: 223–242<br>T: 67–86,<br>119–138,<br>171–190 | I           |
| UCL total cTnT assay                                  | 0.5 <sup>a</sup> | C: 119–138<br>T: 132–151                       | II          |
| <b>Commercial hs-cTn assays</b>                       |                  |  |             |
| Elecsys hs-cTnT (Roche Diagnostics)                   | 3                | C: 125–131<br>T: 136–147                       | I, III      |
| Atellica hs-cTnI (Siemens)                            | 1.6              | C: 41–50, 171–190<br>T: 29–34                  | III         |
| Alinity hs-cTnI (Abbott Laboratories)                 | 1.6              | ND   | III         |
| Vista hs-cTnI (Siemens)                               | 2.0              | C: 29–34<br>T: 41–50, 171–190                  | III         |
| Vitros hs-cTnI (Ortho Clinical Diagnostics)           | 0.43             | C: 87–91<br>T: 24–40,<br>41–49                 | III         |

LoD, limit of detection; cTnT, cardiac troponin T; UCL, upconversion luminescence; TRF, time-resolved fluorescence; hs-cTn, high-sensitivity cardiac troponin; ND, not disclosed.

<sup>a</sup> Analytical detection limit was defined as zero calibrator + 3 × standard deviation (n=40).

## 4.7 Statistical analyses

Most statistical analyses were performed with IBM SPSS Statistics software (versions 27 and 29, IBM Corp., New York, USA). All tests were two-tailed and p-values less than 0.05 were considered statistically significant. The normality of the

distribution of continuous variables was mainly assessed visually and by the Shapiro-Wilk test. Normally distributed continuous variables are reported as mean (SD) and skewed continuous variables as median [25th–75th percentiles].

In the original publication **I**, dilution linearity was assessed using linear regression analysis (Origin 8 Software, OriginLab, Massachusetts, USA). The TRF and UCL long cTnT assays were compared using Deming regression (Sigmaplot 15 Software, Inpixon, California, USA). The two different procedures for LiH plasma sample preparation were compared using the Wilcoxon signed ranked test. Measurements of cTnT (total cTnT, long cTnT, and the troponin ratio calculated by dividing the long cTnT result by the total cTnT result) in NSTEMI and ESRD patients were compared using the Mann-Whitney U-test. The diagnostic abilities of the investigated cTnT assays to discriminate between NSTEMI and ESRD patients were assessed with receiver operating characteristic (ROC) curve analyses. The optimal troponin ratio cutoff point was defined using the Youden index.

In the manuscript **II**, the associations between cTnT values (total cTnT, long cTnT, the troponin ratio) and adverse long-term outcomes (all-cause mortality, MACCE, NOAF, and the composite adverse outcome defined as all-cause mortality or MACCE) in patients with CKD stage 4–5 were examined using univariate and multivariable Cox proportional hazards models adjusted either for age, sex, and CAD or age, sex and kidney transplantation (KTx). Kaplan-Meier survival analysis and the log-rank test were also performed, and the optimal cutoff values of long cTnT and total cTnT for predicting all-cause mortality and the composite adverse outcome were defined using the Youden Index.

In the original publication **III**, elimination and distribution half-lives and clearance of cTnT were determined using an exponential 2-phase model (GraphPad Prism Software, version 9.4.1, Dotmatics, Massachusetts, USA). Detailed description of these analyses is provided in the publication by Kristensen et al. [93]. Hs-cTn concentrations and elimination kinetic parameters were compared between cTnAAb-positive and cTnAAb-negative patients with the exact Wilcoxon-Mann Whitney test. P-values were adjusted for multiple testing using the method of Benjamini and Hochberg, which controls the risk of false discoveries [179]. An adjusted p-value < 0.10 implies that less than 10% of the reported differences are expected to be false positives, while an adjusted p-value < 0.20 implies that less than 20% of the reported differences are expected to be false positives.

## 5 Results & Discussion

### 5.1 Development of the highly sensitive long cTnT assay (I)

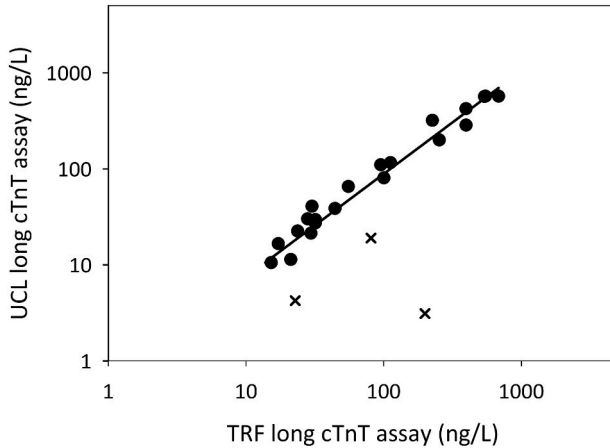
#### 5.1.1 Analytical performance of the UCL long cTnT assay

The developed UCL long cTnT assay reached an LoB of 0.15 ng/L, LoD of 0.40 ng/L and LoQ of 1.79 ng/L (10% CV). Compared to the previous TRF long cTnT assay, the LoD and LoQ values were approximately 28-fold and 14-fold lower, respectively [8]. Thus, considerably higher analytical sensitivity was achieved using UCNP reporters allowing more precise quantification of long cTnT forms in patients with small cTnT elevations and/or small fractions of long cTnT. The linearity range was sufficiently wide as calibration curve was found to be linear between 0.1 and 1000 ng/L ( $R^2=1.000$ ). However, the linearity range at high long cTnT concentrations was limited likely due to the large size of UCNP reporters. The assay also showed good linearity in LiH samples collected from NSTEMI patients ( $n=6$ ) and diluted in BSA-TSA buffer ( $R^2=0.993-1.000$ ). A 2-hour delay in the centrifugation of LiH plasma was not found to significantly influence the detection of long cTnT (mean change  $-3\%$  (SD  $7\%$ ),  $p=0.917$ ).

The UCL long cTnT assay was developed to detect long cTnT forms primarily in LiH plasma samples. The possible matrix effects of EDTA plasma and serum were studied with matched samples collected from STEMI patients. The results measured in EDTA plasma were median 82% [81%–91%] of the results measured in LiH plasma. Previously, EDTA has been observed to induce dissociation of the ITC complex into individual subunits [62]. Recent studies have supported this finding and also showed that gradual *in vitro* cTnT degradation appears to be more prominent in EDTA plasma than in LiH plasma [180,181]. In addition to the gradual cTnT degradation in EDTA plasma, the dissociation of the ITC complex may also affect the detection of long cTnT, as the C-terminal part of cTnT targeted by the capture antibody is involved in the formation of the ITC complex. The dissociation of the complex may change the conformation of the capture antibody epitope and partly explain the observed 18% decrease in long cTnT values in EDTA plasma.

The results measured in serum were 78% [69%–85%] of the LiH plasma results. Thus, the studied serum samples showed even more pronounced decrease in long cTnT concentration. Furthermore, the decrease was highly variable among the studied samples. The activation of thrombin during serum preparation is known to induce N-terminal degradation of cTnT at a well-characterized cleavage site, aa 68–69 [76,77]. However, this cleavage site is not located between the epitopes of the capture and tracer antibodies of the UCL long cTnT assay and should not affect the performance of the assay. Thus, it is possible that other cleavage sites related to thrombin or other yet unknown proteases present in serum and inactivated by heparin may explain the results [7,76]. The high variation in the results might be explained by differences in the sample processing times causing varying degrees of cTnT degradation in the serum samples. The matrix effects of EDTA plasma and serum were investigated in this study with a very small number of samples. Thus, these preliminary findings should be confirmed with larger sample panels in the future. Currently, LiH plasma remains the recommended sample matrix for long cTnT analyses.

The UCL and TRF long cTnT assays showed strong correlation with 21 patient samples with long cTnT concentrations above the LoDs of the assays ( $r=0.98$ ). Deming regression resulted in a slope of 1.03 (95% CI, 0.93–1.12) and y-intercept of  $-3.56$  (95% CI,  $-8.94$ – $1.83$ ) (Figure 7). The UCL long cTnT measured median 5% [–21%–7%] lower long cTnT concentrations than the TRF long cTnT assay. Thus, the two long cTnT assays produced very similar results within the dynamic range of the assays. However, three outliers with more than 70% decrease in long cTnT concentration measured by the UCL long cTnT assay were excluded prior to correlation analysis. The outlier results might be explained by interference caused by the use of multiple tracer antibodies in the TRF long cTnT assay. The higher number of assay antibodies may increase the likelihood of interference by cross-linking heterophilic antibodies. The troponin ratio calculated for one of these outlier samples was also extraordinarily high (200%) suggesting that positive interference might have affected the results obtained with the TRF long cTnT assay.



**Figure 7.** Comparison of the TRF and UCL long cTnT assays above the LoDs of the assays. Deming regression analysis of 21 samples (black circles) resulted in an equation of  $y=1.03x-3.56$  and a correlation coefficient of 0.98. Crosses indicate outlier samples ( $n=3$ ). Reproduced from original publication I.

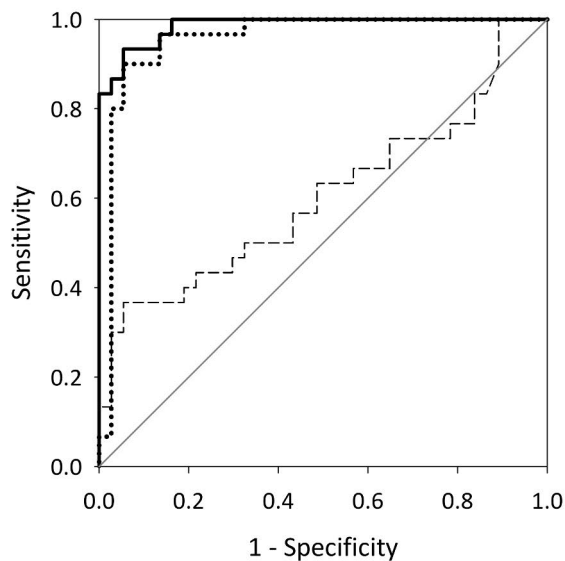
The recently published highly sensitive long-cTnT ITC complex assay developed by Li et al. utilizes capture and tracer antibodies targeting the stable central part of cTnT (aar 132–151) and the cTn complex, respectively [99]. While this long-cTnT ITC complex assay detects long cTnT (not degraded at aar 189–223) only in a complexed form, the UCL long cTnT assay can also detect free long cTnT. Thus, these two highly sensitive long cTnT assays are not quite equivalent. However, current evidence suggests that long cTnT is present in the circulation mainly in a complexed form indicating that these two highly sensitive long cTnT assays may, in principle, give rather similar results [6,7]. Compared to the long-cTnT ITC complex assay (LoD 0.17 ng/L and LoQ 0.37 ng/L), the UCL long cTnT assay reached relatively comparable analytical sensitivity [99]. However, while the long cTnT complex assay can be performed on an automated platform, our UCL long cTnT assay in its current form is not yet suitable for clinical practice at emergency clinics due to manual labor and 5 hours of total assay time.

### 5.1.2 Diagnostic performance of the UCL long cTnT assay with AMI and ESRD patient samples

The UCL long cTnT assay measured significantly lower long cTnT concentrations in ESRD patients (2.5 [1.9–3.9] ng/L) than in NSTEMI patients (28.4 [6.4–136.6] ng/L,  $p<0.001$ ), while total cTnT concentrations measured with the commercial hs-cTnT assay did not significantly differ between ESRD patients (76.0 [50.5–124.5] ng/L) and NSTEMI patients (103.0 [47.3–268.3] ng/L,  $p=0.144$ ).

Consequently, the troponin ratio calculated by dividing the long cTnT result by the total cTnT result was significantly lower in ESRD patients (3.3% [2.4%–4.5%]) than in NSTEMI patients (21.5% [12.5%–53.3%],  $p < 0.001$ ).

The diagnostic abilities of the assays to discriminate between ESRD and NSTEMI patients were evaluated using ROC analyses. The area under the curves (AUC) for long cTnT and the troponin ratio measured using the UCL long cTnT assay were 0.905 (95% CI, 0.824–0.985) and 0.986 (95% CI, 0.967–1.000), respectively (Figure 8). The AUC for total cTnT was significantly lower [0.605 (95% CI, 0.461–0.748),  $p < 0.001$  for both comparisons]. Furthermore, the AUC determined for the troponin ratio of the TRF long cTnT assay [0.955 (95% CI, 0.900–1.000)] was also numerically but not statistically lower than the respective value of the UCL long cTnT assay ( $p = 0.293$ ) (Figure 8). At the optimal troponin ratio cutoff point (7.5%) defined for the UCL long cTnT assay, the sensitivity and specificity values for separating ESRD and NSTEMI patients were 93% and 95%, respectively.



**Figure 8.** ROC curves illustrating diagnostic abilities of total cTnT (dashed line, AUC 0.605) and the troponin ratios determined using the TRF (dotted line, AUC 0.955) and UCL (solid line, AUC 0.986) long cTnT assays to discriminate between NSTEMI and ESRD patients. Reproduced from original publication I.

Similarly to the previous TRF long cTnT assay, the novel UCL long cTnT assay showed excellent performance in discriminating between patients with ESRD and NSTEMI. However, the UCL long cTnT assay was able to discriminate between these two groups with an even higher AUC due to the highly improved analytical sensitivity. Interestingly, the results are also in line with the results of the recent

study by Li et al. showing that the proportion of long-cTnT ITC complex is significantly higher in acute myocardial injury than in chronic disease [100]. However, these two long cTnT assays measure slightly different long cTnT forms. Additionally, the calculated ratios and the investigated patient groups were different from our study [100].

Recently, the novel UCL long cTnT assay has also shown promise in differentiating marathon runners and patients with Takotsubo syndrome from patients with AMI [182,183]. However, the clinical performance of the assay needs to be evaluated in much larger studies to reveal whether long cTnT has the potential to be a more specific biomarker for AMI. Currently, ongoing studies focus on the most relevant clinical setting: patients presenting to the emergency department with AMI-like symptoms.

### 5.1.3 Time-dependent cTnT degradation following AMI

Long cTnT and the troponin ratio followed an exponential decay function (mean  $R^2$  0.98 (SD 0.03) and 0.99 (SD 0.01), respectively) in serial samples of STEMI patients ( $n=13$ ). The mean half-lives estimated for long cTnT and the troponin ratio were 8.7 (SD 2.7) h and 10.5 (SD 2.9) h, respectively. The half-life of total cTnT could not be calculated, because the trends were varying and 4 out of 13 patients had increasing values in a later sample. Compared to total cTnT, the half-lives of long cTnT and the troponin ratio indicate a relatively rapid decrease of long cTnT forms in the circulation. These results are consistent with the existing knowledge, as total cTnT levels measured with the commercial hs-cTnT assay have been shown to remain elevated for days and exhibit biphasic kinetics after AMI [30,32]. The apparently short half-life of long cTnT suggests that long cTnT and the troponin ratio might also serve as potential biomarkers for reinfarction.

Several factors, including the progression of myocardial cell damage and blood flow perturbations in the infarcted tissue, may contribute to the release and decrease of long cTnT in the circulation. Moreover, the time from symptom onset to serial sampling varied considerably between the STEMI patients. Thus, the half-lives reported in this study should be considered as rough estimates of long cTnT kinetics rather than exact elimination constants. Further studies should follow to investigate long cTnT kinetics more thoroughly.

## 5.2 Association of long cTnT with adverse long-term outcomes in patients with advanced CKD (II)

### 5.2.1 Patient characteristics and study outcomes

A total of 136 CKD stage 4–5 patients with a mean age of 61 (SD 13) years and a median eGFR of 12 [11–15] mL/min/1.73 m<sup>2</sup> were included in the final study cohort. Altogether, 47 (34.6%) of these patients were female. The median values for total cTnT, long cTnT and the troponin ratio were 37 [23–66] ng/L, 1.9 [1.3–3.0] ng/L and 5% [3%–7%], respectively. One patient of the 137 initially included patients was excluded as an outlier due to highly deviant total cTnT and long cTnT values inconsistent with the clinical presentation. Non-linearity was also observed in serial dilution of the outlier plasma sample indicating the presence of analytical interference.

After a median follow-up of 6.2 [4.6–7.7] years, 62 (45.6%) patients had died, 36 (26.5%) patients had experienced an incident MACCE, 28 (23.3%) patients had experienced an incident NOAF, and 76 (55.9%) patients either died or experienced a MACCE (i.e. experienced the composite adverse outcome). At the end of follow-up, 59 (43.4%) patients had started dialysis treatment, and 16 (11.8%) had not. Furthermore, 61 (44.9%) patients had received a KTx (after dialysis). The median time to KTx was 2.3 [1.5–3.8] years. Out of the 61 KTx recipients, 10 (16.4%) died during follow-up. Correspondingly, 52 (69.3%) out of the 75 patients who did not receive a KTx died during follow-up.

### 5.2.2 Associations between cTnT measurements and adverse long-term outcomes

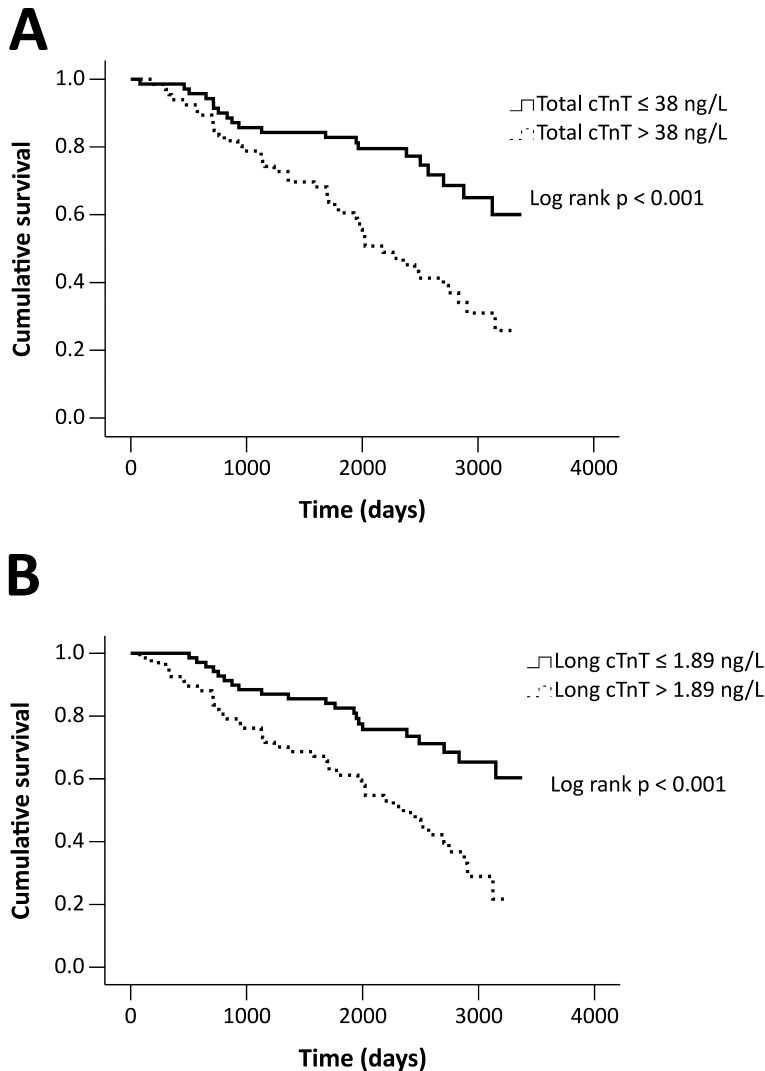
Hazard ratios (HR) and p-values of all univariate and multivariable Cox proportional hazard models exploring the associations between cTnT measurements (total cTnT, long cTnT, and the troponin ratio) and the study outcomes (all-cause mortality, MACCE, NOAF, and the composite adverse outcome defined as all-cause mortality or MACCE) are presented in Table 5. Briefly, total cTnT was associated with all-cause mortality, MACCE, NOAF and the composite adverse outcome in the univariate Cox models. These associations remained significant in the multivariable Cox models adjusted for age, sex and CAD. Interestingly, long cTnT was also associated with all-cause mortality, NOAF and the composite adverse outcome but not with MACCE in the respective univariate and multivariable Cox models. However, the troponin ratio was not associated with any of the study outcomes in the univariate Cox models. The binary cutoff values in predicting all-cause mortality

were determined for total cTnT and long cTnT at 38 ng/L and 1.89 ng/L, respectively. The Kaplan-Meier survival curves for all-cause mortality are presented in Figure 9.

**Table 5.** Univariate and multivariable Cox models exploring the associations between cTnT measurements and the study outcomes in advanced CKD. Adapted from manuscript II.

| Outcome   | Total cTnT             |        | Long cTnT              |       | Troponin ratio          |       |
|---|------------------------|--------|------------------------|-------|-------------------------|-------|
|   | HR (95% CI)            | p      | HR (95% CI)            | p     | HR (95% CI)             | p     |
| <b>Univariate Cox models</b>                                  |                        |        |                        |       |                         |       |
| All-cause mortality   | 1.008<br>(1.004–1.011) | <0.001 | 1.019<br>(1.005–1.033) | 0.007 | 1.598<br>(0.240–10.644) | 0.628 |
| MACCE   | 1.008<br>(1.004–1.012) | <0.001 | 1.015<br>(0.998–1.033) | 0.088 | 2.171<br>(0.258–18.281) | 0.476 |
| NOAF  | 1.007<br>(1.002–1.012) | 0.005  | 1.023<br>(1.008–1.039) | 0.003 | 2.605<br>(0.312–21.771) | 0.377 |
| Composite adverse outcome                                     | 1.008<br>(1.005–1.011) | <0.001 | 1.015<br>(1.002–1.028) | 0.021 | 1.491<br>(0.260–8.568)  | 0.654 |
| <b>Multivariable Cox models adjusted for age, sex and CAD</b> |                        |        |                        |       |                         |       |
| All-cause mortality   | 1.006<br>(1.003–1.010) | <0.001 | 1.019<br>(1.005–1.033) | 0.007 | -                       | -     |
| MACCE   | 1.008<br>(1.004–1.012) | <0.001 | 1.015<br>(0.997–1.033) | 0.099 | -                       | -     |
| NOAF  | 1.007<br>(1.001–1.012) | 0.014  | 1.024<br>(1.008–1.040) | 0.003 | -                       | -     |
| Composite adverse outcome                                     | 1.007<br>(1.004–1.010) | <0.001 | 1.015<br>(1.002–1.028) | 0.027 | -                       | -     |
| <b>Multivariable Cox models adjusted for age, sex and KTx</b> |                        |        |                        |       |                         |       |
| All-cause mortality   | 1.004<br>(1.000–1.008) | 0.056  | 1.013<br>(0.999–1.027) | 0.069 | -                       | -     |
| MACCE   | 1.005<br>(1.001–1.010) | 0.026  | 1.009<br>(0.991–1.027) | 0.310 | -                       | -     |
| NOAF  | 1.005<br>(0.999–1.011) | 0.109  | 1.020<br>(1.004–1.037) | 0.012 | -                       | -     |
| Composite adverse outcome                                     | 1.005<br>(1.001–1.008) | 0.007  | 1.009<br>(0.996–1.022) | 0.177 | -                       | -     |

cTnT, cardiac troponin T; HR, hazard ratio; CI, confidence interval; MACCE, major adverse cardiovascular or cerebrovascular event; NOAF, new-onset atrial fibrillation; CAD, coronary artery disease; KTx, kidney transplantation.



**Figure 9.** Kaplan-Meier survival curves for all-cause mortality according to total cTnT (A) and long cTnT (B). Reproduced from manuscript II.

The significance of the associations between cTnT measurements and the study outcomes weakened, when KTx was included as a covariate in the multivariable Cox models instead of CAD (Table 5). Significant associations were observed only between total cTnT and MACCE, total cTnT and the composite adverse outcome, and long cTnT and NOAF. Thus, risk prediction in KTx recipients seems to be more difficult, which is likely explained by the fact that KTx is the most important treatment modality for improving long-term survival in maintenance dialysis patients [184].

In multivariable Cox models adjusted for age, sex and CAD, the independent associations between long cTnT and the study outcomes apart from MACCE were highly significant and the HRs were comparable to those of total cTnT. Thus, it is possible that long cTnT representing only 5% of total cTnT in this study may carry a major part of the prognostic weight related to cTnT in the prediction of adverse long-term outcomes, in particular, in predialysis CKD stage 4–5 patients who are not eligible to receive a KTx. The proportion of these patients is high, as according to a recent national survey in the United States, only every fifth dialysis patient was wait-listed for KTx [185]. Therefore, long cTnT may have potential in the risk assessment of adverse long-term outcomes in the majority of ESRD patients. However, apart from NOAF, total cTnT had more significant associations with the study outcomes than long cTnT in this study, suggesting that total cTnT might currently be a more useful predictor of long-term survival and cardiovascular outcomes in CKD patients [124,126,186].

While long cTnT forms have been found in the circulation of early presenting AMI patients, small cTnT fragments seem to be responsible for cTnT elevations in reversible and/or more chronic myocardial injuries caused by, for example, strenuous exercise or CKD [8–10,182]. Long cTnT forms seem to be released mainly through acute myocardial necrosis, and unsurprisingly, long cTnT concentrations were observed to remain relatively low in CKD stage 4–5 patients in this study. Smaller cTnT fragments might potentially be released also through other mechanisms, which may, for example, increase cell membrane permeability of the cardiomyocytes [28]. Thus, it is possible that total cTnT comprising mainly small cTnT fragments in this study may better represent reversible myocardial injury, severity of cardiovascular burden, and uremic inflammation in CKD stage 4–5 patients.

The troponin ratio that represents the extent of cTnT fragmentation process at a given time point did not have any significant associations with the study outcomes. Therefore, the expected clinical implications of the troponin ratio may reside in the diagnostics of conditions with a sudden release of large amounts of cTnT rather than in the prediction of adverse long-term outcomes in chronic myocardial injuries [8].

The major limitations of the study include the post hoc nature of the study, small sample size and limited number of adverse events. The distribution of long cTnT values was also very narrow, which may affect the findings of the study. Furthermore, improved knowledge of the release and clearance mechanisms of cTnT is required to better understand the associations between different cTnT forms and adverse long-term outcomes in advanced CKD.

## 5.3 Effect of cTnAAbs on the elimination kinetics of cTn (III)

### 5.3.1 Presence cTnAAbs and elimination kinetics of cTn

Altogether, 2 out of 20 participants (10%) who had received an autologous plasma re-transfusion were found to be cTnAAb-positive (participants 8 and 15) when the samples collected before and after plasma re-transfusion were analyzed with a previously developed cTnAAb immunoassay. The hs-cTn concentrations of importance as well as the elimination kinetic parameters for cTnAAb-positive and cTnAAb-negative participants are shown in Table 6.

Prior to plasma re-transfusion, the Atellica, Alinity, Vista and Elecsys hs-cTn assays measured higher baseline concentrations in cTnAAb-positive (1.6–15.3 × cTnAAb-negative median) than in most cTnAAb-negative participants (Table 6). Following plasma re-transfusion, hs-cTn concentrations also appeared to decline more slowly in cTnAAb-positive than in cTnAAb-negative participants (Figure 10). At the last time points of the repeated sampling, hs-cTn concentrations were also considerably higher in cTnAAb-positive than in cTnAAb-negative participants (Table 6).

The cTnAAb-positive participant 15 had a particularly distinctive kinetic profile with the Atellica, Alinity, Vista and Elecsys hs-cTn assays exhibiting a clearly prolonged elimination half-life and slower clearance of cTn than most of the cTnAAb-negative participants (Figure 10B, Table 6). Similarly, the other cTnAAb-positive participant 8 also showed a prolonged elimination half-life and slower cTn clearance compared to most cTnAAb-negative participants when the Atellica and Vista hs-cTn assays were used (Table 6). However, the elimination kinetic parameters estimated using the Alinity and Elecsys hs-cTn assays did not noticeably differ between the cTnAAb-positive participant 8 and the cTnAAb-negative participants.

Interestingly, the results obtained with the Vitros hs-cTnI assay appeared to be notably different from the other 4 assays. The lowest Vitros hs-cTnI baseline concentrations were measured specifically in cTnAAb-positive participants (0.3 × cTnAAb-negative median) (Table 6). Compared to cTnAAb-negative participants, the Vitros hs-cTnI levels measured after plasma re-transfusion also remained rather low in cTnAAb-positive participants throughout the blood sampling (Figure 10, Table 6). Moreover, the Vitros hs-cTnI assay did not seem to be affected by the presence of cTnAAbs in terms of the estimated elimination half-life values (Table 6). The clearance values, however, were exceptionally high for both cTnAAb-positive participants. The estimated recovery of the injected cTnI dose in the circulation after plasma re-transfusion (using an approximation of 5 L for the human blood volume) was only 19% for the two cTnAAb-positive participants. The respective value for cTnAAb-negative participants was median 96% [91%–116%]

suggesting that the injected dose of endogenous cTnI was, to large extent, not detected by the Vitros hs-cTnI assay in cTnAAb-positive participants. As the other four hs-cTn assays did not show similar differences in the estimated recoveries between cTnAAb-positive (median 80% [66%–109%]) and cTnAAb-negative participants (median 97% [86%–110%]), the Vitros hs-cTnI assay was likely prone to negative interference caused by cTnAAbs.

**Table 6.** hs-cTn concentrations of importance and elimination kinetic parameters for cTnAAb-negative and cTnAAb-positive participants. Adapted from original publication III.

|   | cTnAAb-negative participants <sup>a</sup> | cTnAAb-positive participants |                     | p-value <sup>b</sup> | Adjusted p-value <sup>c</sup> |
|---|---|------------------------------|---------------------|----------------------|-------------------------------|
|   |   | Participant 8                | Participant 15      |                      |                               |
| <b>Atellica hs-cTnI</b>                 |   |                              |                     |                      |                               |
| Baseline (ng/L)                         | 16 (7–50)                                 | 34                           | 169                 | 0.018                | 0.063                         |
| 1st sample after re-transfusion (ng/L)  | 3075 (521–6830)                           | 4330                         | 7670                | 0.126                | 0.180                         |
| Last sample after re-transfusion (ng/L) | 149 (46–295)                              | 729                          | 4750                | 0.011                | 0.055                         |
| Distribution half-life (min)            | 23 (5–35)                                 | 18                           | 218                 | 0.758                | 0.842                         |
| Elimination half-life (min)             | 125 (62–221)                              | 248                          | $1 \times 10^{31}$  | 0.011                | 0.055                         |
| Clearance (mL/min)                      | 54 (32–93)                                | 32                           | $7 \times 10^{-28}$ | 0.029                | 0.067                         |
| <b>Alinity hs-cTnI</b>                  |   |                              |                     |                      |                               |
| Baseline (ng/L)                         | 12 (6–326)                                | 62                           | 60                  | 0.105                | 0.166                         |
| 1st sample after re-transfusion (ng/L)  | 2596 (412–6937)                           | 5090                         | 5206                | 0.059                | 0.118                         |
| Last sample after re-transfusion (ng/L) | 155 (43–492)                              | 972                          | 2285                | 0.011                | 0.055                         |
| Distribution half-life (min)            | 26 (3–52)                                 | 4                            | NA                  | 0.211                | 0.275                         |
| Elimination half-life (min)             | 213 (76–957)                              | 176                          | 434                 | 0.758                | 0.842                         |
| Clearance (mL/min)                      | 49 (35–94)                                | 30                           | NA                  | 0.143                | 0.195                         |
| <b>Vista hs-cTnI</b>                    |   |                              |                     |                      |                               |
| Baseline (ng/L)                         | 18 (10–67)                                | 66                           | 275                 | 0.026                | 0.065                         |
| 1st sample after re-transfusion (ng/L)  | 2746 (547–8018)                           | 4997                         | 10695               | 0.026                | 0.065                         |
| Last sample after re-transfusion (ng/L) | 191 (66–514)                              | 1219                         | 6832                | 0.011                | 0.055                         |
| Distribution half-life (min)            | 27 (20–45)                                | 12                           | 283                 | 1.000                | 1.000                         |
| Elimination half-life (min)             | 192 (94–259)                              | 309                          | $1 \times 10^{31}$  | 0.011                | 0.055                         |
| Clearance (mL/min)                      | 41 (25–80)                                | 15                           | $7 \times 10^{-28}$ | 0.019                | 0.063                         |

**Table 6.** (continued)

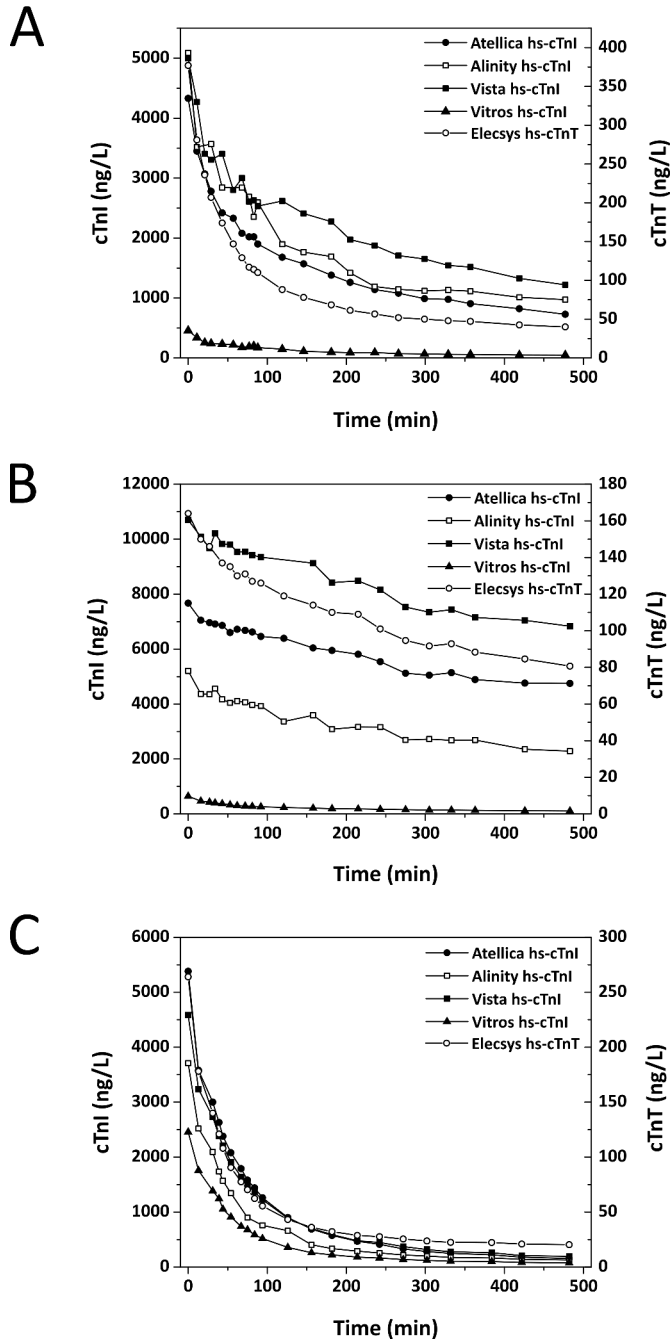
|   | cTnAAb-negative participants <sup>a</sup> | cTnAAb-positive participants |                | p-value <sup>b</sup> | Adjusted p-value <sup>c</sup> |
|---|---|------------------------------|----------------|----------------------|-------------------------------|
|   |   | Participant 8                | Participant 15 |                      |                               |
| <b>Vitros hs-cTnI</b>                   |   |                              |                |                      |                               |
| Baseline (ng/L)                         | 7 (2–24)                                  | 2                            | 2              | 0.011                | 0.055                         |
| 1st sample after re-transfusion (ng/L)  | 1759 (320–3903)                           | 457                          | 646            | 0.095                | 0.166                         |
| Last sample after re-transfusion (ng/L) | 82 (25–206)                               | 48                           | 109            | 0.958                | 1.000                         |
| Distribution half-life (min)            | 28 (17–51)                                | 8                            | 23             | 0.063                | 0.118                         |
| Elimination half-life (min)             | 240 (94–649)                              | 146                          | 306            | 1.000                | 1.000                         |
| Clearance (mL/min)                      | 47 (31–106)                               | 204                          | 118            | 0.015                | 0.063                         |
| <b>Elecsys hs-cTnT</b>                  |   |                              |                |                      |                               |
| Baseline (ng/L)                         | 12 (8–19)                                 | 19                           | 19             | 0.033                | 0.071                         |
| 1st sample after re-transfusion (ng/L)  | 237 (80–379)                              | 377                          | 164            | 0.643                | 0.772                         |
| Last sample after re-transfusion (ng/L) | 19 (13–73)                                | 40                           | 81             | 0.021                | 0.063                         |
| Distribution half-life (min)            | 22 (17–39)                                | 21                           | 26             | 0.589                | 0.736                         |
| Elimination half-life (min)             | 130 (88–622)                              | 168                          | 486            | 0.126                | 0.180                         |
| Clearance (mL/min)                      | 78 (7–127)                                | 45                           | 16             | 0.100                | 0.166                         |

cTnAAb, cardiac troponin-specific autoantibody; hs-cTnI, high-sensitivity cardiac troponin I; hs-cTnT, high-sensitivity cardiac troponin T; NA, not available.

<sup>a</sup>Data presented as median (minimum–maximum).

<sup>b</sup>Comparisons between cTnAAb-negative and cTnAAb-positive participants were performed using the exact Wilcoxon-Mann-Whitney test.

<sup>c</sup>p-values were adjusted for multiple testing using the method of Benjamini and Hochberg, which controls the risk of false discoveries. An adjusted p-value < 0.10 implies that less than 10% of the reported differences are expected to be false positives, while an adjusted p-value < 0.20 implies that less than 20% of the reported differences are expected to be false positives.



**Figure 10.** Elimination of cTnI and cTnT in cTnAAb-positive participant 8 (A), cTnAAb-positive participant 15 (B), and representative cTnAAb-negative participant 16 (C). Reproduced from original publication III.

Endogenous cTnAAbs are known to be a relatively common interfering factor and cause of discrepancy between hs-cTn assays [12,13,142]. Due to different assay characteristics, the degree of analytical cTnAAb interference and the reactivity to macrotroponin have been observed to vary greatly between hs-cTn assays [13,187]. In this study, 4 out of 5 widely used commercial hs-cTn assays (Atellica hs-cTnI, Alinity hs-cTnI, Vista hs-cTnI, Elecsys hs-cTnT assay) measured clearly elevated baseline concentrations still several weeks after AMI and showed prolonged cTn elimination after autologous plasma re-transfusion at least for one cTnAAb-positive participant, while one of the assays (Vitros hs-cTnI assay) did not. Additionally, the Atellica and Vista hs-cTnI assays seemed to be affected most prominently by the presence of macrotroponin in the cTnAAb-positive participant 15. The fact that Atellica and Vista hs-cTnI assays target the same epitopes and are both manufactured by Siemens likely explains the similarity of these results.

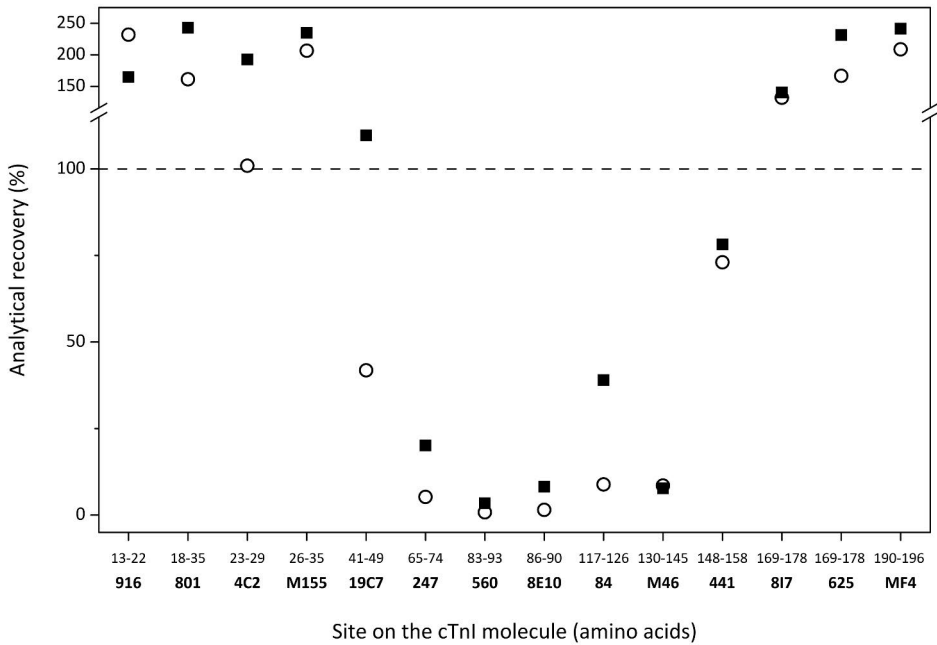
Concentrations as well as affinities and specificities of cTnAAbs are known to vary extensively between cTnAAb-positive individuals [12,169,187]. Although similar elimination kinetic trends could be seen for the two cTnAAb-positive participants in this study, the presence of cTnAAbs was observed to affect the elimination of cTn to varying degrees. The cTnAAb-positive participant 15 exhibited highly distinctive elimination kinetic profile when measured by the Atellica hs-cTnI, Alinity hs-cTnI, Vista hs-cTnI, and Elecsys hs-cTnT assay, whereas the cTnAAb-positive participant 8 could not be distinguished from cTnAAb-negative participants as clearly. Thus, these findings further highlight the interindividual variability in the concentrations and/or characteristics of cTnAAbs.

The major limitation of this study is the small cohort consisting of only two cTnAAb-positive and 18 cTnAAb-negative patients. However, this unique study provided first-time evidence of slower clearance of macrotroponin. Moreover, the effect of cTnAAbs and macrotroponin formation on cTn clearance was assessed across five different commercial hs-cTn assays. The findings of this study also build on and provide valuable insights into the previous study by Kristensen et al. [93].

### 5.3.2 Epitope specificity of cTnAAbs

Epitope specificity studies were conducted to determine the target epitopes of cTnAAbs on the cTnI molecule. Binding of the sample cTnAAbs to the same epitope as the capture antibody used in the assay leads to a lower analytical recovery of the spiked human cardiac troponin ITC complex. The cTnAAbs present in the samples of participants 8 and 15 were mainly found to bind to the central region of the cTnI molecule, as the lowest recoveries of the ITC complex (less than 40%, median 8%, [3%–12%]) were obtained for the epitopes of mAbs 247 (aar 65–74), 560 (aar 83–93), 8E10 (aar 86–90), 84 (aar 117–126) and M46 (aar 130–145) (Figure 11).

Moreover, the ITC recovery for the epitope of mAb 19C7 (aar 41–49) was relatively low for participant 8 (42%) but normal for participant 15 (110%). The recoveries obtained for other N-terminal and C-terminal cTnI epitopes ranged from 73% to 243% (Figure 11). Although cTnAAbs predominantly seemed to target the central part of cTnI, some differences were also observed in the epitope specificities of cTnAAbs between the two cTnAAb-positive participants, highlighting the interindividual variation in the characteristics of cTnAAbs.



**Figure 11.** Analytical recoveries of ITC complex for different cTnI epitopes in cTnAAb-positive samples. Open circle and square indicate participants 8 and 15, respectively. Reproduced from original publication III.

The results of the epitope specificity studies are consistent with the previous knowledge and recommendations not to target the central part of cTnI due to the possibility of cTnAAb-related negative interference [156,169]. Interestingly, the only capture antibody used in the Vitros hs-cTnI assay targets aar 87–91. This region corresponds to the epitope of mAb 8E10 that appeared to be almost completely blocked by the cTnAAbs in the recovery studies indicating that cTnAAbs present in the studied samples interfered with the Vitros hs-cTnI assay by preventing the capture antibody from binding to its epitope. Thus, this negative cTnAAb

interference likely explains the discordantly low cTnI concentrations measured with the Vitros hs-cTnI assay in the two cTnAAb-positive participants.

Previously, cTnAAbs have been reported to be present in approximately 10% of healthy individuals [138,188]. Thus, the negative cTnAAb interference raises concerns, as it may delay the increase of cTnI concentration to detectable levels and reduce the applicability of early rule-out algorithms in cTnAAb-positive AMI patients. However, the negative interference present in the samples of the two cTnAAb-positive participants in this study may not apply to other cTnAAb-positive individuals, as the characteristics of cTnAAbs are known to vary between individuals.

According to the previous findings of this study, the other three hs-cTnI assays did not seem to be susceptible to negative cTnAAb interference, as they detected macrotroponin and showed reduced cTn clearance in the two cTnAAb-positive participants. In line with these elimination kinetics results, the target epitopes of the Atellica and Vista hs-cTnI assays located in the N-terminal and C-terminal regions of cTnI (aar 29–34, 41–50, 171–190) were not found to be major targets of cTnAAbs in the recovery studies, although the epitope of the mAb 19C7 (aar 41–49) also seemed to be slightly blocked by cTnAAbs in the cTnAAb-positive participant 8. These two Siemens assays utilize either two capture or two tracer antibodies targeting aar 41–50 and 171–190, so that one of the capture/tracer antibodies can likely bind to cTnI if the other cannot. The target epitopes of the Alinity hs-cTnI assay are not provided by the manufacturer. However, the Alinity hs-cTnI assay was evidently also able to detect macrotroponin indicating that the critical epitopes were not masked by cTnAAbs. Thus, these three hs-cTnI assays are prone to clinically false-positive cTnAAb-related interference, which may lead to falsely elevated baseline hs-cTnI results in cTnAAb-positive individuals. However, positive interference can be more easily detected in clinical practice and is less dangerous for the patient than negative interference that, in the worst case, can result in a false-negative AMI diagnosis.

## 5.4 Clinical implications

It has been hypothesized that the measurement of specific forms of circulating cTn may allow differential diagnosis of diseases causing hs-cTn elevations and increase the clinical specificity of cTn for type 1 AMI. In this thesis, a highly sensitive UCL long cTnT assay was developed to allow accurate and precise measurement of long cTnT forms which have been previously proposed to be a promising biomarker for AMI. The novel UCL long cTnT assay will be a valuable tool in the future studies of cTnT fragmentation. If the future studies demonstrate that long cTnT is indeed a more specific biomarker for AMI and has clinical value, this highly sensitive assay

could be commercialized after assay automation and adopted for routine clinical use. Then the measurement of long cTnT by a highly sensitive long cTnT assay might allow faster diagnosis of AMI and timely initiation of optimal treatment, improve patient outcomes and reduce unnecessary use of medical resources. Moreover, the observed independent associations of long cTnT with all-cause mortality and NOAF in patients with CKD stage 4–5 indicate that long cTnT might potentially also have some prognostic value for adverse long-term outcomes in CKD.

In this thesis, the presence of cTnAAbs was shown for the first time to prolong cTn elimination and reduce cTn clearance explaining why persistent cTn elevations are often observed in cTnAAb-positive individuals. Moreover, these effects were studied across five widely used commercial hs-cTn assays revealing both positive and negative cTnAAb-mediated interferences. The increased knowledge of how cTnAAbs and macrotroponin may interfere with different hs-cTn assays is important in clinical practice and may help clinicians and laboratory professionals in interpreting hs-cTn results inconsistent with the clinical presentation. These results may also aid in developing next-generation hs-cTn assays with minimized susceptibility to cTnAAb-mediated interferences.

## 5.5 Future directions of research

To date, cTn fragmentation has been studied in few patient groups with limited number of patients. Although long cTnT measured by the novel UCL long cTnT assay has shown promise in discriminating AMI patients from ESRD patients, marathon runners, and Takotsubo patients, its true potential as a more specific biomarker for AMI remains to be investigated [8,182,183]. cTnT fragmentation needs to be studied in various patient populations, and the clinical performance of the UCL long cTnT assay needs to be evaluated in larger study cohorts consisting of patients who present to the emergency department with AMI-like symptoms. Moreover, before the UCL long cTnT assay could be commercialized and used in clinical practice, it should be applied on automated platforms to be more rapid and robust. The potential prognostic value of long cTnT in patients with CKD or other conditions also requires extensive research. Additionally, larger studies are needed to further increase understanding of cTnAAb-mediated interferences in different hs-cTn assays as well as the clinical significance of cTnAAbs. Solutions should be developed to prevent cTnAAb-related interferences in future hs-cTn assays.

## 6 Conclusions

The measurement of cTn represents a cornerstone in the diagnostics of AMI. However, hs-cTn elevations can be also detected in several other conditions associated with acute or chronic myocardial injuries, which may often complicate the interpretation of the hs-cTn results and the diagnosis of AMI. Recently, long cTnT forms have been proposed to provide improved specificity for AMI. As long cTnT forms represent only a fraction of the circulating total cTnT measured by the current commercial hs-cTnT assays, high analytical sensitivity is a prerequisite for reliable investigation of cTnT fragmentation.

In addition to issues with clinical specificity, hs-cTn assays are also prone to cTnAAb-mediated interferences. Large macrotroponin complexes comprising cTn and cTnAAb may cause persistently elevated hs-cTn results not related to myocardial injury, potentially due to reduced clearance of macrotroponin. However, direct evidence of how cTnAAbs affect the elimination kinetics of cTn has been lacking. On the other hand, cTnAAbs can also cause falsely decreased results by masking the target epitopes of the hs-cTn assay.

In this thesis, the aims included the development of a more sensitive immunoassay for long cTnT forms and the investigation of associations between long cTnT forms and adverse long-term outcomes in patients with CKD stage 4–5 not on dialysis. Furthermore, the effect of cTnAAbs on elimination kinetics of cTn was evaluated with five commercial hs-cTn assays.

The main conclusions of this thesis based on the original publications are the following:

- I. A highly sensitive immunoassay was successfully developed for the detection of long molecular forms of cTnT using UCL technology. The developed highly sensitive UCL long cTnT assay allows accurate and precise measurement of long cTnT forms in patients with small total cTnT elevations and exhibits excellent performance in discriminating between NSTEMI and ESRD patients. In the future, this novel highly sensitive UCL long cTnT assay can serve as a valuable research tool for investigating cTnT

fragmentation in different patient groups and discovering the full potential of long cTnT as a specific biomarker for AMI.

- II.** The associations between long cTnT measured with the highly sensitive UCL long cTnT assay and adverse long-term outcomes were described for the first time in a prospective cohort of CKD stage 4–5 patients. Long cTnT was found to be independently associated with all-cause mortality and NOAF indicating that long cTnT might be useful in the prediction of adverse long-term outcomes in CKD stage 4–5. To support these findings, further research using larger study cohorts is needed.
- III.** For the first time, evidence was provided that endogenous cTnAAbs and the formation of macrotroponin result in prolonged elimination and reduced clearance of cTn. While the Atellica hs-cTnI, Alinity hs-cTnI, Vista-hs-cTnI, and Elecsys hs-cTnT assays detected macrotroponin and showed reduced cTn clearance, the Vitros hs-cTnI assay was prone to negative cTnAAb interference. These complex effects of cTnAAbs on hs-cTn assays should be taken into account in clinical practice and in the development of new generations of hs-cTn assays with minimized susceptibility to cTnAAb-mediated interferences.

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Turku, October 2025

A handwritten signature in black ink that reads "Selma Salonen". The script is cursive and elegant, with the first letters of "Selma" and "Salonen" being significantly larger and more decorative than the rest of the letters.

*Selma Salonen*

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