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CARDIAC TROPONIN T IN CLINICAL AND EXPERIMENTAL STUDIES

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ABSTRACT

The cardiac troponin T (cTnT) assay became commercially available in the 1990's, and together with cardiac troponin I (cTnI), cTnT is today the marker of choice for the detection of myocardial infarction. However, when the cTnT assay was introduced it was insufficiently evaluated in two important fields: in patients with renal disease and in experimental cardiovascular research. Therefore, the aims of this thesis were to evaluate cTnT in end-stage renal disease (ESRD) patients and in rat and mouse isolated heart preparations.

cTnT in clinical studies

Serum cTnT was elevated (≥0.10 μg/L) in a high proportion of both haemodialysis (HD) and peritoneal dialysis (PD) patients without symptoms of acute myocardial ischaemia. In HD + PD patients, chronic ischaemic heart disease was associated with higher levels of cTnT. In HD patients, concentrations of serum cTnT, creatine kinase MB (CKMB) and post-dialysis plasma big endothelin-1 were higher in patients with left ventricular hypertrophy (LVH). Furthermore there was a positive correlation between cTnT levels and left ventricular mass index. Also ESRD patients shortly before the onset of dialysis therapy had a high proportion (30%) of elevated serum cTnT, which correlated positively with CKMB, interleukin-6, C-reactive protein and was associated with the presence of cardiovascular disease. Elevated cTnT was a strong predictor of all-cause mortality in clinical outcomes studies.

In summary, these findings suggest that circulating cTnT may reflect LVH and/or subclinical myocardial cell damage in dialysis patients and suggest that cTnT in serum from dialysis patients originates from the heart. The present data support the hypothesis that cTnT is associated with inflammation and poor outcome in ESRD. Further, elevated cTnT levels seem to be associated to the uraemic syndrome rather than the dialysis treatment per se.

cTnT in experimental studies

The kinetics of cTnT release after 30 min of ischaemia, and importance of preanalytical sample handling in buffer perfused isolated rat hearts were studied. After start of reperfusion there was a short-lasting peak of cTnT release followed by a plateau phase. There was no consistent correlation between cTnT release and post-ischaemic dysfunction. cTnT concentrations in coronary effluent declined rapidly in room temperature. The cTnT loss was attenuated by adding bovine albumin to the samples. Studies on mice revealed no difference in cTnT content (μg cTnT/g heart) in hearts from wild type C57BL/6J and ApoE/LDLr knock out (KO) mice. Baseline serum cTnT was considerably higher in samples from the thoracic cavity than in those from the jugular vein. The cTnT content in skeletal muscle was <0.1% of the cTnT content in heart muscle for both C57BL/6J and ApoE/LDLr KO mice.

In summary, effluent cTnT after 20 min of reperfusion is complementary to haemodynamics in the evaluation of cardiac damage in the rat Langendorff preparation. Albumin should be added to coronary effluent to prevent cTnT loss and to increase precision of the assay. Hearts of C57BL/6J and ApoE/LDLr KO mice have similar cTnT content. Elevated baseline levels of serum cTnT in mice are not caused by troponin T from skeletal muscle.

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CONTENTS

LIST OF ABBREVIATIONS

ACS	Acute coronary syndrome
ApoE	Apolipoprotein E
CAC	Coronary artery calcification
CK	Creatine kinase
CKMB	Creatine kinase MB isoform
CRF	Chronic renal failure
CRP	C-reactive protein
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
DM	Diabetes mellitus
cTnI	Cardiac troponin I
cTnT	Cardiac troponin T
ECG	Electrocardiogram
ECLIA	Electrochemiluminescence immunoassay
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESRD	End-stage renal disease
ET	Endothelin
HD	Haemodialysis
IHD	Ischaemic heart disease
IL	Interleukin
IVSTd	Interventricular septal thickness at the end of diastole
HF	Heart failure
KO	Knock out
LD	Lactate dehydrogenase
LDLr	Low density lipoprotein receptor
LV	Left ventricular
LVH	Left ventricular hypertrophy
LVIDd	Left ventricular internal diameter at the end of diastole
LVMI	Left ventricular mass index
MEIA	Microparticle enzyme immunoassay
OR	Odds ratio
PD	Peritoneal dialysis
PWTd	Posterior wall thickness at the end of diastole
RIA	Radioimmunoassay
ROC	Receiver operator characteristic
SEM	Standard error of the mean
STAT	Short turn around time
sTnT	Skeletal muscle troponin T
TNF	Tumor necrosis factor
TNNT2	The human cardiac troponin T gene
TPA	Tripropylamine

1 INTRODUCTION

Cardiac troponin T (cTnT) and cardiac troponin I (cTnI) measured in serum or plasma are highly sensitive and specific markers of acute myocardial injury and are today recommended for the diagnosis of acute myocardial infarction *(1)*. By the year of 2000, cardiac troponin research had resulted in a consensus change in the definition of acute myocardial infarction with increased emphasis on cardiac marker testing with cardiac troponins as the new "gold standard". cTnT and cTnI are also of value for risk stratification in unstable coronary artery disease *(2)* and the role of cardiac troponins as markers of subsequent cardiac risk in patients with acute coronary syndrome (ACS) has become established.

However, although the immunoassays for cardiac troponins are highly specific for cardiac troponin isoforms and thereby myocardial injury, they lack the ability to discriminate between ischaemia caused by coronary atherotrombosis and other mechanisms of damage. Over the past 15 years cTnT and cTnI assays with increasing sensitivity has been developed making it possible to detect very small myocardial injuries. This has led to a growing list of disease states and clinical settings in which elevated concentrations of cardiac troponins have been measured without other evidence of overt ischaemic heart disease (IHD) *(3)*. These findings have generated new research fields including both preclinical and clinical studies, aiming to elucidate the causes of elevated cardiac troponin concentrations in various disease states other than acute myocardial infarction. All this new knowledge has brought us to the insight that we are only in the beginning of the cardiac troponin era, and much more is to be explored about cTnT and cTnI as protein markers of disease.

1.1 BIOCHEMISTRY AND MOLECULAR BIOLOGY OF TROPONIN T

The troponin complex consists of the three proteins TnT, TnI and troponin C (TnC) and is found on the myofibrillar thin filaments within the sarcomere of all types of striated muscle. TnT, with an unphosphorylated molecular weight of ~35 kDa, is the tropomyosin-binding subunit; TnI (~23 kDa) the inhibitory subunit and TnC (18 kDa) the Ca^{2+}-binding subunit *(4-6)*. The troponin complex appears to be divided into subdomains that are defined by boundaries that do not coincide at all with the boundaries of the three polypeptide chains *(7)*.

There are three major isoforms each of TnT and TnI. These isoforms are found in cardiac muscle, fast skeletal muscle, and in slow skeletal muscle, respectively, and are encoded by individual genes *(8)*. In adult healthy human the cardiac specific cTnT and cTnI genes are only expressed in cardiac muscle, whereas the cTnC gene is also expressed in slow skeletal muscle making cTnC less useful as a cardiac marker *(9)*.

The human cTnT gene (TNNT2) located on chromosome 1q32 is a relatively large gene that generates multiple alternatively spliced transcripts encoding distinct protein isoforms. The principal cTnT amino acid sequence encoded by the TNNT2 gene locus comprises 288 amino acids (Figure 1). In humans, four distinct cTnT isoforms (cTnT1-4) are generated via alternative splicing of exons 4 and 5 *(10-12)*, although at least 36

alternative splicing isoforms are theoretically possible. The various cTnT isoforms mainly differ in their NH_2-terminal structures *(13)*. In normal human ventricular myocardium the cTnT content is about 10.8 mg/g wet weight *(14)*. About 6-8% of cTnT is found as a free cytosolic component and the remaining cTnT is bound and compartmented in the myofibrils *(14-16)*.

Nucleotide sequence (867 nucleotides):

ATGTCTGACATAGAAGAGGTGGTGGAAGAGTACGAGGAGGAGGAGCAGGAAGAAGCAGCTGTTGAAGAGC
AGGAGGAGGCAGCGGAAGAGGATGCTGAAGCAGAGGCTGAGACCGAGGAGACCAGGGCAGAAGAAGATGA
AGAAGAAGAGGAAGCAAAGGAGGCTGAAGATGGCCCAATGGAGGAGTCCAAACCAAAGCCCAGGTCGTTC
ATGCCCAACTTGGTGCCTCCCAAGATCCCCGATGGAGAGAGAGTGGACTTTGATGACATCCACCGGAAGC
GCATGGAGAAGGACCTGAATGAGTTGCAGGCGCTGATCGAGGCTCACTTTGAGAACAGGAAGAAAGAGGA
GGAGGAGCTCGTTTCTCTCAAAGACAGGATCGAGAGACGTCGGGCAGAGCGGGCCGAGCAGCAGCGCATC
CGGAATGAGCGGGAGAAGGAGCGGCAGAACCGCCTGGCTGAAGAGAGGGCTCGACGAGAGGAGGAGGAGA
ACAGGAGGAAGGCTGAGGATGAGGCCCGGAAGAAGAAGGCTTTGTCCAACATGATGCATTTTGGGGGTTA
CATCCAGAAGCAGGCCCAGACAGAGCGGAAAAGTGGGAAGAGGCAGACTGAGCGGGAAAAGAAGAAGAAG
ATTCTGGCTGAGAGGAGGAAGGTGCTGGCCATTGACCACCTGAATGAAGATCAGCTGAGGGAGAAGGCCA
AGGAGCTGTGGCAGAGCATCTATAACTTGGAGGCAGAGAAGTTCGACCTGCAGGAGAAGTTCAAGCAGCA
GAAATATGAGATCAATGTTCTCCGAAACAGGATCAACGATAACCAGAAAGTCTCCAAGACCCGCGGGAAG
GCTAAAGTCACCGGGCGCTGGAAATAG

Translation (288 amino acids):

MSDIEEVVEEYEEEEQEEAAVEEQEEAAEEDAEAEAETEETRAEEDEEEEEAKEAEDGPMEESKPKPRSF
MPNLVPPKIPDGERVDFDDIHRKRMEKDLNELQALIEAHFENRKKEEEELVSLKDRIERRRAERAEQQRI
RNEREKERQNRLAEERARREEEENRRKAEDEARKKKALSNMMHFGGYIQKQAQTERKSGKRQTEREKKKK
ILAERRKVLAIDHLNEDQLREKAKELWQSIYNLEAEKFDLQEKFKQQKYEINVLRNRINDNQKVSKTRGK
AKVTGRWK

Figure 1. Alignment of the principal adult human cTnT nucleotide and amino acid sequence. The National Center for Biotechnology Information (NCBI) reference sequence CCDS 30969.1, troponin T type 2, cardiac isoform 2. The standard one-letter codes denote the amino acid residues: A = Alanine, R = Arginine, N = Asparagine, D = Aspartic Acid, C = Cysteine, E = Glutamic Acid, Q = Glutamine, G = Glycine, H = Histidine, I = Isoleucine, L = Leucine, K = Lysine, M = Methionine, F = Phenylalanine, P = Proline, S = Serine, T = Threonine, W = Tryptophan, Y = Tyrosine, and V = Valine.

1.2 ONTOLOGY AND ISOFORM SWITCHING

In humans, three to five isoforms of cTnT are expressed in fetal cardiac muscle but neither fast nor slow skeletal muscle TnT (sTnT) is expressed *(10, 11, 17, 18)*. In human fetal skeletal muscle, fetal cTnT isoforms have been reported at both the transcriptional *(18, 19)* and translational levels *(20)*. A developmental down-regulation of cTnT and up-regulation of sTnT isoforms occurs in normal developing skeletal muscle, which leads to the absence of cTnT isoforms in nondiseased adult skeletal muscle *(10)*. For cTnI, human cardiac muscle appears to contain one single cTnI

isoform and cTnI does not seem to be expressed in healthy or diseased fetal and adult skeletal muscle at any time, including during development *(21, 22)*.

1.3 THE CARDIAC TROPONIN T IMMUNOASSAY

Since the different isoforms of the troponins make them antigenically different from each other, it was possible to develop antibody assays specific for cTnT and cTnI. The first immunoassay for cTnT was reported in 1989 *(23)*. This was an enzyme-linked immunosorbent assay (ELISA) using goat antihuman cTnT antiserum as capture antibody and mouse monoclonal antihuman cTnT antibody 1B10 labeled with horseradish peroxidase as detector antibody. There was a minor 1-2% cross-reactivity of the first manufactured assay with troponin T extracted from human or bovine mixed skeletal muscle and the assay had a detection limit of 0.5 µg/L *(24)*.

This assay was followed by the so called first generation cTnT immunoassay (ELISA troponin-T) using the mouse monoclonal capture antibody M7 and the mouse monoclonal labeled antibody 1B10. This improved assay had a detection limit of 0.1 µg/L and an overall cross-reactivity of 3.6% against sTnT *(25)*. In the second generation assay (Enzymun-Test Troponin-T) the cross-reactive MAb 1B10 was replaced by the cardiac specific monoclonal antibody M11.7 used as capture antibody and monoclonal antibody M7 was used as labeled antibody *(26)*. The epitopes of these antibodies are only six amino acid residues apart, a feature which makes the assay insensitive to proteolytic degradation of the antigen (Figure 2). Stability studies of cTnT in serum samples from patients with acute myocardial infarction showed no loss of immunological activity after 5 days' storage at room temperature *(27)*. The detection limit for cTnT was 0.012 µg/L and no cross-reactivity with purified sTnT up to 1000 µg/L was detected *(26)*.

The third generation cTnT test (Troponin T STAT) uses the same monoclonal antibodies (M11.7 and M7) as the second generation test, but is standardized with human recombinant cTnT instead of bovine cTnT *(28)*. The detection limit of the third generation cTnT assay is 0.01 µg/L. On the Elecsys and Modular instruments the ELISA was replaced by the electrochemiluminescence technology. Detection is accomplished by tris(2,2'-bipyridyl) ruthenium(II) ($Ru(bpu)_3^{2+}$) conjugated to the detector antibody. Initially $Ru(bpu)_3^{2+}$ and tripropylamine (TPA) are oxidized at the surface of an anode. TPA immediately loses a proton, becoming a powerful reducer. This causes $Ru(bpu)_3^{3+}$ to enter a high-energy state by a high-energy electron transfer from the electron carrier, TPA. The relaxation phase back to the ground state results in light emission detectable at 620 nm. $Ru(bpu)_3^{2+}$ may be oxidized and excited again due to the excess TPA used in the reagent *(29)*. In the third generation assay, heparin plasma was not recommended due to systematic lower cTnT results than in serum. Using the current fourth generation cTnT immunoassay, there is no systematic bias between cTnT measured in serum and heparin plasma *(30)*. Due to patent protection, only Roche Diagnostics (Basel Switzerland; formerly Boehringer Mannheim of Mannheim, Germany) has produced cTnT assays, while several manufacturers have produced assays for cTnI.

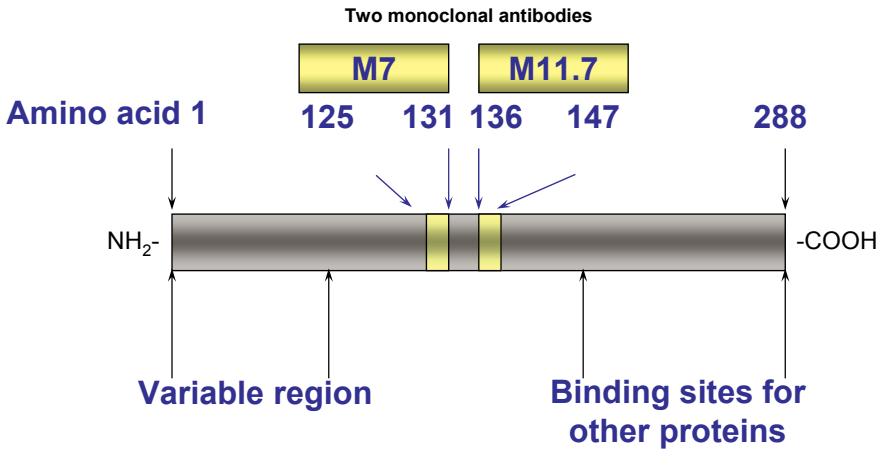

Figure 2. The two monoclonal antibodies in the cTnT immunoassay are directed against the central stable part of the human cTnT molecule. The amino-terminal (NH_2-terminal) part is variable and various cTnT isoforms mainly differ in their NH_2-terminal structure. Modified from Roche Diagnostics 2007.

1.4 CONDITIONS WITH ELEVATED CARDIAC TROPONINS

Cardiac troponins are today the biomarkers of choice for the detection of cardiac injury. However, an elevation of cTnT and cTnI indicates the presence of myocardial injury, but does not define the underlying reason and mechanism. Hence, acute myocardial infarction is only one of several potential diseases with elevated serum concentrations of cardiac troponins *(31)*.

1.4.1 Elevated troponin in acute myocardial ischaemia

Myocardial necrosis is accompanied by the release of proteins and other intracellular molecules into the cardiac interstitium. From the interstitial space, about 80% of the protein transport to the general circulation occurs directly via the microvascular endothelium *(32, 33)*. The remaining 20% of protein transport is effected by lymphatic flow.

Serum concentration of cTnT and cTnI begins to rise in average 3-4 hours after the onset of myocardial ischaemia. Typically in acute myocardial infarction cTnT has a biphasic release pattern with an initial peak concentration at 12 hours, followed by a plateau phase lasting about 48 hours, and a subsequent fall to undetectable levels after 10 days *(15, 34)*. The duration of the elevation however, is determined by the size of the myocardial infarction; after small infarctions cTnT may remain elevated for as little as a few days, and after large infarctions it may remain detectable for as long as 3 weeks. The release pattern of cTnI resembles that of cTnT but cTnI remains elevated for a somewhat shorter time than cTnT, and the biphasic pattern is less pronounced. The duration of cTnI elevation is typically 5-10 days and depends on infarct size *(35)*.

In patients with acute myocardial infarction, cTnT is released into the bloodstream mainly as free cTnT and as cTnT-I-C complex while cTnI is released in complexes of cTnT-I-C and cTnI-C *(36, 37)*.

Cardiac troponins are not only diagnostic markers of acute myocardial infarction, but are also important predictors of acute trombotic risk for patients with ACS (i.e. acute myocardial infarction and unstable angina). Randomised studies and meta-analyses have shown adverse prognostic risk associated with elevated cardiac troponins *(38, 39)*. Furthermore, patients with ACS and elevated cardiac troponins benefit from a more intense antitrombin or antiplatelet treatment. Troponin elevations identify a group of patients who will benefit from therapy with dalteparin *(40)* and enoxaparin *(41)*. Other studies have shown that elevated cardiac troponins identifies a group of patients in which the use of glycoprotein IIb/IIIa platelet inhibitors is useful *(42-44)*. Early coronary intervention also attenuates the adverse prognostic impact of cardiac troponin elevations *(45-49)*.

In addition to cardiac troponin elevation in spontaneous acute myocardial infarction, cTnT and cTnI are frequently slightly elevated after percutaneus coronary intervention *(50, 51)*, and always elevated after open heart surgery *(52)*. The mechanisms of these elevations are poorly defined.

1.4.2 Elevated troponin in other conditions

It is important to recognise that cardiac troponins may be elevated in a lot of different disease states without symptoms of IHD or abnormal ECG pattern *(31)*.

In the following conditions troponin levels may be elevated without overt IHD:

- Critical illness *(53)*
- Sepsis *(53, 54)*
- Acute pulmonary embolism *(55, 56)*
- Heart failure (HF), acute and chronic *(57-60)*
- Myocarditis *(61, 62)*
- Pericarditis *(63-65)*
- Renal failure *(66)*
- Heart transplant *(67)*
- Cardiac infiltrative disorders, amyloidosis *(68, 69)*
- Cardiotoxic chemotherapy *(70, 71)*
- Cardiac trauma, blunt cardiac trauma, cardiac contusion *(72-75)*
- Tachyarrhythmias
- Elective electrical cardioversion *(76, 77)*
- Direct-current defibrillator shocks *(78, 79)*
- High frequency ablation *(80-82)*
- Strenuous exercise *(83-85)*
- Cardiac rhabdomyolysis *(86, 87)*

Elevated cardiac troponins in these conditions most likely emanate from injured myocardial cells, and regardless of the release mechanism, elevated concentrations of cTnT and cTnI almost always imply a poor prognosis *(31, 88)*.

1.5 CARDIAC TROPONIN T IN END-STAGE RENAL DISEASE

Cardiac disease is the leading cause of morbidity and mortality in patients with end-stage renal disease (ESRD). The poor prognosis of patients with ESRD is to a large extent due to the high incidence of cardiovascular disease (CVD) accounting for more than half of all deaths. Twenty to thirty per cent of cardiac deaths in these patients are caused by myocardial infarction *(89)*. IHD appears to be only partially due to coronary atherosclerosis since in a group of dialysis patients with symptomatic IHD, more than 40% had patent coronary arteries *(90)*. In these patients, ventricular hypertrophy, interstitial myocardial fibrosis, and endothelial dysfunction, which all limit the coronary flow reserve during stress and which are frequent findings in ESRD, may play a pathophysiological role.

The diagnosis of ischaemic myocardial injury is difficult in the ESRD population. Several studies have demonstrated that the older cardiac marker creatine kinase (CK) and the more cardiac specific isoform creatine kinase MB (CKMB), are frequently elevated in ESRD patients without other signs of acute IHD *(91, 92)*. Since the cardiac troponins were shown to be much more cardiac specific than CK and CKMB in none-renal patients, they where believed to replace these old markers also in ESRD patients. However, increased serum concentrations of cTnT and, to a much lesser extent, of cTnI were reported in ESRD patients without any signs of acute myocardial ischaemia *(93)*. The mechanism of this troponin elevation is still unclear. It is possible that the cTnT assay is so sensitive that it detects subclinical myocardial cell injury or myocardial remodelling in left ventricular hypertrophy (LVH) whereas the cTnI assays are less sensitive. On the other hand, the elevated serum concentrations of cTnT could also originate from diseased skeletal muscle. One of the most important issues in the evaluation of cardiac troponins in ESRD has been to investigate whether or not elevated serum concentrations of cTnT are associated with cardiac disease.

Another important question to elucidate was the predictive power of cTnT in ESRD. Although cTnT measured in serum or plasma was known to be a highly sensitive and specific marker of acute myocardial damage and a predictor of adverse outcome in non-uraemic patients with unstable coronary artery disease *(94)*, the use of cTnT was questioned in ESRD patients since cTnT concentrations in these patients were shown to be elevated without other signs of acute myocardial damage *(95, 96)*. The mechanisms behind the increased cTnT concentrations were unclear, and they were by some regarded as spuriously elevated. Some studies showed that cTnT predicted cardiovascular mortality as well as all-cause mortality in renal patients treated with haemodialysis (HD) *(97, 98)*, whereas others reported no association with outcome *(99, 100)*.

At the time of the recommendation of cTnT and cTnI as markers of myocardial infarction *(1)*, the role of cTnT as a prognostic tool in peritoneal dialysis (PD) patients had not yet been evaluated. Thus the value of cTnT determinations in patients with renal failure was not clearly established. Furthermore, the prevalence of elevated cTnT in predialysis chronic renal failure (CRF) patients had not been investigated and the predictive value of cTnT in these patients was not known. Accordingly one could not conclude whether the elevated cTnT in dialysis patients was caused by the renal

insufficiency with associated cardiac disease or induced by the dialysis treatment per se.

In patients with unstable angina pectoris, associations have been shown between cTnT and interleukin-6 (IL-6) and other inflammatory markers like fibrinogen and C-reactive protein (CRP) *(101)*. CRP may also be associated with elevated serum cTnT in HD patients *(102)*. Several studies have found a relationship between inflammation and mortality in ESRD patients *(103-105)*. Studies on correlations between cTnT and inflammatory markers, and the prognostic value of cTnT and IL-6 in predialysis patients were lacking in 2003 and therefore the association between cTnT and inflammatory markers in CRF was not clearly established at the time. Endothelin-1 (ET-1) is a potent vasoconstrictor with mitogenic, proinflammatory and antinatriuretic properties, and the endothelin system is involved in numerous cardiovascular disease states. Measurement of the precursor big ET-1, which has slower clearance, is an alternative approach for estimation of ET-1 release. Associations between endothelin and cTnT in ESRD had not been studied previously.

1.6 CARDIAC TROPONIN T IN RAT AND MICE

When cTnT emerged as possibly the best clinically available marker of myocyte injury such as in myocardial infarction and unstable coronary syndromes *(94, 106, 107)*, the analyte was considered of potential interest also for experimental studies. Of special interest was the finding that the accumulated cTnT release in patients was related to post-infarction cardiac dysfunction and infarct size *(108, 109)*. In animal studies cTnT was shown to be a sensitive and specifc marker of myocyte injury *(110, 111)*, and its release seemed to correlate with infarct size *(112)*.

One of the most frequently employed models in experimental heart research is isolated perfused hearts, in particular the rat heart. Isolated perfused heart preparations nowadays are largely based on adaptations and modifications of the method originally described by Langendorff over 100 years ago *(113)*. This model is often used for studies of global ischaemia with subtle, reversible injury and myocardial stunning. In studies of cardiac injury, for instance ischaemia-reperfusion, measurements of cardiac function and measurements of biochemical cardiac markers in the coronary effluent, are often used to evaluate the myocardial damage. The observation periods are usually short with reperfusion ranging from minutes to a few hours. The usefulness of cTnT as an early marker after shorter episodes of ischaemia was not clarified when the first commercially available cTnT assay was released. It was shown that cTnT is released from hearts within minutes after start of reperfusion both in studies on isolated perfused hearts *(111, 114)*, rat hearts in vivo *(112)* and clinically during coronary artery bypass surgery *(115)*. In the latter study, release of cTnT into the coronary sinus during the first hour of reperfusion correlated with the length of myocardial ischaemia. In another study, cTnT release correlated with the reduction of left ventricular developed pressure (LVDP) after four hours of reperfusion in isolated rat hearts *(116)*.

The immunoassays for cTnT and cTnI are designed for the detection of troponins in serum or plasma samples, and there may potentially be problems when analysing rat

troponins in coronary buffer effluents with low protein concentrations. For instance, the cTnT concentration was elevated in albumin-free modified Krebs-Henseleit buffer when using the first generation cTnT ELISA *(117)*, probably due to matrix effects. This has not been reported with the second and third generation cTnT assays. The magnitude of cTnT release after ischaemia-reperfusion injury in isolated, buffer-perfused hearts varies widely between research groups and discordant result have been reported *(116, 118)*. Others have shown inexplicable results for cTnT compared with other markers *(119)*. In most studies the procedures for preanalytical handling and the precision of the assays are not mentioned. Perfusate sampling and preanalytical handling may be crucial for accurate and reproducible measurements, and thus reliable experiments. Adsorption of the protein to the test tube walls is a potential error when measuring proteins at low concentrations in dilute solutions. This has been reported for both glass *(120)* and plastic surfaces *(121)*, and may give falsely low results and impair the precision of the assay.

With recombinant DNA technology, new animal models of atherosclerosis and IHD have been developed. One of these models is the apolipoprotein E/low density lipoprotein receptor knockout (apoE/LDLr KO) mouse, which develops atherosclerosis with a distribution pattern and composition of lesions similar to humans *(122)*. The apoE/LDLr KO mice fed an atherogenic diet are larger than wild types on standard chow, but the ratio between heart weight and body weight is similar *(123)*. In most unstimulated species (animals without induced myocardial damage) cTnT is undetectable (<0.01 μg/L) in serum. In mice, however, elevated baseline levels of cTnT have been reported both in serum with the first generation cTnT assay *(112)* and in plasma with the second generation cTnT assay *(124)*. This may hypothetically be due to some degree of cross-reactivity with skeletal muscle cTnT, or some as yet undetermined species-specific factors.

The sampling procedure may potentially influence cTnT levels in small animals as some degree of tissue injury may be unavoidable in the procedure. In mice, cTnT in samples from the abdominal aorta and the orbital plexus have been used for the detection of myocardial injury *(112, 124)*. Cardiac puncture on the other hand, which is practice for sampling a sufficient volume in mice, is not recommended since the procedure itself may cause myocardial damage. However, when we started to work with the apoE/LDLr KO mice in experimental models, there were no in vivo evaluations of blood sampling for murine cTnT measurement with the third generation cTnT assay available. In the isolated perfused mouse heart model with induced ischaemia, the cTnT release into the coronary effluent correlates well with infarct size in both C57BL/6J and apoE/LDLr KO mice *(123)*. Another approach to estimate the lesion size is to calculate the release of cTnT in coronary effluent per gram wet weight heart. However, the content of cTnT in hearts from apoE/LDLr KO mice had not been previously reported, and could differ from that of wild-type mice due to IHD.

2 AIMS

When the cTnT immunoassay became commercially available in the beginning of the 1990's it was insufficiently evaluated in two important fields: in patients with renal disease and in experimental cardiovascular research. Therefore, the general aims of this research were to evaluate cTnT in ESRD patients and in rat and mouse isolated heart preparations.

The specific aims of the *clinical studies* were to study:

- Associations between serum cTnT, other cardiac markers and cardiac disease in HD and PD patients.
- Correlations between cTnT, big ET-1 and ET-1 in HD patients.
- Associations between cTnT and the inflammatory marker CRP in PD patients.
- The prognostic value of serum cTnT and CRP in PD patients.
- The prevalence of elevated serum cTnT in predialysis patients.
- Associations between cTnT and inflammatory markers in predialysis patients ant the prognostic value of cTnT and IL-6 in these patients.

Hypothesis: Elevated cTnT is associated with morbidity and mortality in ESRD, and is not artifactual.

The specific aims of the *experimental studies* were to study and characterize:

- The kinetics of cTnT release after 30 minutes of global ischaemia in an isolated rat heart model.
- The relationship between cTnT release and heart dysfunction in isolated rat hearts.
- The importance of preanalytical handling: freezing and thawing; tube material; addition of albumin, to improve the stability of cTnT in the coronary effluent from isolated rat hearts.
- The cTnT content in heart and skeletal muscle of ApoE/LDL receptor double KO mice compared with C57BL/6J mice.
- The importance of the sampling procedure in mice for the measurement of serum cTnT.

Hypothesis: cTnT is a useful cardiac marker in rat and mouse isolated heart models. Preanalytical sample handling is important for the results.

3 MATERIALS AND METHODS

3.1 SUBJECTS IN CLINICAL STUDIES

Paper I
A total of 62 patients: 36 HD patients and 26 PD patients without symptoms of acute myocardial ischaemia were investigated. In all patients, serum concentrations of cTnT, cTnI, CKMB and CK were determined, in HD patients before and after dialysis. Additionally, in HD patients, plasma ET-1 and big ET-1 were measured. In 27 out of 36 HD patients, left ventricular mass index (LVMI) was determined.

Paper II
For the 26 PD patients in paper I, clinical outcomes were evaluated by chart review after four years. cTnT and the inflammatory marker CRP were compared and the influence of the two analytes on survival was tested.

Paper III
ESRD patients (n=115), 29% of which had CVD defined by medical history, were studied shortly before the onset of dialysis therapy. After a mean follow-up period of 2.7 years, the prognostic value was calculated for cTnT, IL-6, age, CVD, malnutrition, diabetes mellitus (DM) and gender with all-cause mortality as the clinical end point. Sixty-four patients started PD as renal replacement therapy, while 49 started HD during the follow-up. One patient had transplantation shortly after the recruitment and for one patient dialysis was not started at the end of follow up.

3.2 ANIMAL MODELS

Paper IV and V: Rat heart perfusion
Male Sprague Dawley rats (200-300 g) were anaesthetised with diethyl ether, and 200 IU heparin was injected into the femoral vein. The hearts were then rapidly excised through a median sternotomy and placed in ice-cold buffer during preparation for aortic cannulation. The hearts were retrogradely perfused with gassed (5% CO_2, 95% O_2) Krebs-Henseleit buffer (NaCl 118.5 mmol/L, $NaHCO_3$ 25.0 mmol/L, KCl 4.7 mmol/L, KH_2PO_4 1.2 mmol/L, $MgSO_4 \cdot 7H_2O$ 1.2 mmol/L, Glucose·H_2O 11.1 mmol/L, $CaCl_2 \cdot 2H_2O$ 1.8 mmol/L) as a modified Langendorff preparation (Figure 3). The perfusion pressure (100 cm H_2O) was kept constant. Water jackets round the perfusate reservoirs and heart chamber kept the temperature at 37ºC throughout the experiments. A balloon was inserted into the left ventricle via the left atrium for isovolumetric recordings of left ventricular systolic (LVSP) and end-diastolic (LVEDP) pressures. LVDP was calculated as LVDP = LVSP - LVEDP. Coronary flow was measured by timed collections of the coronary effluent. After excision, the hearts were stabilized for 25 minutes before start of the experiments. Global ischaemia (30 minutes) was induced by clamping the inflow tubing. A total of 59 hearts were used. All hearts were treated equally and they were not exposed to pharmaceutical or any other additional manipulation.

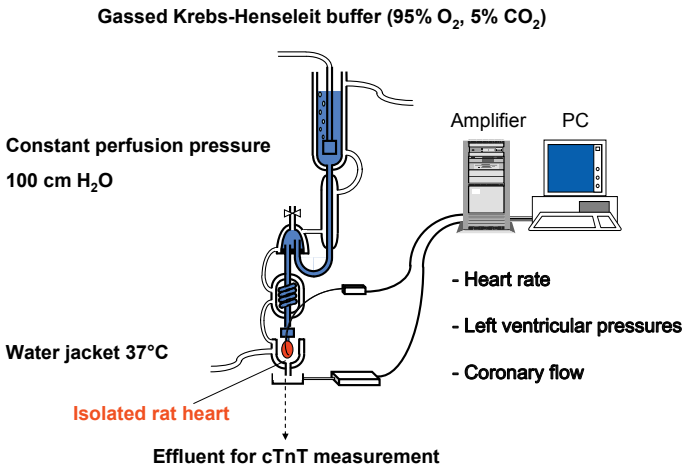

Figure 3. Computerized modified Langendorff model. The hearts were retrogradely perfused with gassed (5% CO_2, 95% O_2) Krebs-Henseleit buffer at a constant pressure and with a core temperature of the heart maintained at 37ºC. Left ventricular pressures and coronary flow was measured and heart rate was calculated. Coronary effluent was collected for measurement of cTnT.

Paper VI: Animal preparation

Male apoE/LDLr KO mice and wild type C57BL/6J mice were purchased from Taconic B&K, Ry, Denmark at an age of 4-6 weeks. For 6 months, apoE/LDLr KO mice (n=7) were fed an atherogenic diet containing corn starch, glucose, sucrose, cocoa butter, cellulose, minerals, vitamin mix, 0.15% cholesterol and 21% (wt/wt) total fat (Analyzen, Odal, Sweden). C57BL/6J mice were fed standard chow (Beekay Feeds, B&K Universal, Stockholm, Sweden) for 6 months (C57BL/6J 6 months) (n=4) or 10 weeks (C57BL/6J 10 weeks) (n=7). The mice were anesthetized with isofluorane, 1.2 ± 0.2% prior to sampling procedures.

3.3 SAMPLING PROCEDURES

Paper I

For analysis of serum from PD patients, peripheral venous blood was collected in tubes without additives. In HD patients, blood samples were taken from the venous side of the arteriovenous fistula. Tubes with ethylenediaminetetraacetic acid (EDTA) were used for the preparation of plasma and tubes without additives for the preparation of serum. Specimens were centrifuged and serum or plasma was aliquoted for subsequent analysis. Serum CKMB and CK were determined immediately. Serum for cTnT was stored at -20°C and analysed within 24 hours. Serum specimens for cTnI and creatinine were stored at -70°C and analysed in a batch manner. Samples for ET-1 and big ET-1 were immediately placed on ice until centrifuged at 4°C. Plasma was stored at -70°C until analysed.

Paper II

For analysis of serum cTnT, cTnI, CKMB, CK and CRP, peripheral venous blood was collected in tubes without additives. Serum CRP, CKMB and CK were determined immediately. Serum for cTnT assay was stored at -20°C and analysed within 24 hours. Serum specimens for cTnI and creatinine were stored at -70°C and analysed in a batch manner. Clinical characteristics at inclusion of the study, including serum concentrations of parathyroid hormone (Incstar N-tact PTH), albumin (Behring Nephelometer Analyzer) and blood haemoglobin (Technicon H2) were collected from patient files.

Paper III

After an overnight fast, venous blood samples were taken for analysis of serum albumin, CRP, plasma IL-6 (n=109), and fibrinogen (n=106). The serum levels of cTnT and CKMB were determined in the same samples.

Paper IV

In one series (n=7) coronary effluent (2 mL) was collected at 0.5, 1, 2.5, 10, 15, 20, 25 and 30 minutes of reperfusion. These samples were used to investigate the release kinetics of cTnT after global ischaemia. In another series (n=52) coronary effluent was collected at 20 minutes of reperfusion. Coronary effluent was collected in ice-cold plastic tubes and stored at -70°C until analysis. Samples were analysed 160-170 minutes after withdrawal from freezer. The samples were used to investigate the relationship between cTnT release and heart dysfunction after ischaemia.

Paper V

To investigate the effect of time, freezing and thawing, and if albumin could stabilise samples

After 20 minutes of reperfusion coronary effluent (1.0 mL) was collected in polystyrene tubes (n=10), or 0.2 mL of 200 mg/mL bovine albumin was added to polystyrene tubes before collecting 0.8 mL coronary effluent (final albumin concentration 40 g/L) (n=10). Samples were thawed in room temperature and analysed for cTnT; 60-70 minutes (T1), 150-160 minutes (T2) and 240-250 minutes (T3) after withdrawal from freezer. After 300 minutes at room temperature the samples were re-frozen and analysed again the next day, 150-160 minutes (T4) after withdrawal from freezer. Troponin T free Krebs-Henseleit buffer with bovine albumin (final albumin concentration 40 g/L) (n=14) was analysed for cTnT to evaluate any background disturbance.

To investigate the role of the tube material

Coronary effluent was collected 20-27 minutes after start of reperfusion. The effluent was aliquoted into polystyrene tubes with and without albumin as described above, and into polypropylene tubes and siliconised plastic tubes (n=8 of each tube). Samples were analysed for cTnT; 60-70 minutes (T1) and 150-160 minutes (T2) after withdrawal from freezer. After 300 minutes at room temperature the samples were refrozen and analysed again the next day 160-170 minutes (T4) after withdrawal from freezer.

To determine the coefficient of variation (CV%) in effluent with and without albumin
CV% was calculated according to Aronson et al *(125)*. Within-series CV%, between-series CV% and total CV% were calculated for different tube materials and different concentrations of cTnT: in polystyrene tubes with cTnT 0.10 µg/L (mean) (n=42) and with cTnT 0.52 µg/L (n=34) to investigate the role of cTnT concentration in effluent without albumin; in polypropylene tubes with cTnT 0.39 µg/L (n=20) to compare polypropylene and polystyrene tubes; in polystyrene tubes with 40 g/L albumin with cTnT 3.25 µg/L (n=20). In this experimental setting it was not possible to get comparable cTnT concentrations in tubes with and without albumin. Samples were analysed 160-170 minutes after withdrawal from freezer.

To compare the stability of different markers of cardiac injury
Coronary effluent was collected in polystyrene tubes with and without albumin after 20 minutes of reperfusion as described above. The samples (n=7) were analysed for cTnT, lactate dehydrogenase (LD) and CK; 150-170 minutes after withdrawal from freezer.

To compare the second and third generation cTnT assays
The correlations between cTnT measured with ES 300 and Elecsys 2010 were tested, using coronary effluent with and without albumin (n=31). Samples were collected at different time points (i.e. at the end of stabilisation and after 20 minutes of reperfusion) to get a wide range of concentrations. Samples were analysed 160-170 minutes after withdrawal from freezer.

Paper VI
Serum from the jugular vein
Above musculus pectoralis descendence, the skin was removed to unveil the external jugular vein. The superficial pectoral muscle was penetrated with a sterican needle 0.40 x 20 mm 27 G Gr 20 and into the exposed vein. From each mouse approximately 500 µL of peripheral blood was withdrawn.

Serum from the thoracic cavity
A median sternotomy was performed, and the heart exposed. The right auricle was removed and blood collected from the thoracic cavity with a 1 mL syringe. After coagulation, serum was collected after centrifugation at 7500 rpm for 10 minutes. The hearts were collected and weighed. A skeletal muscle biopsy was harvested from the femoral muscle and weighed. All mice were sacrificed by an overdose of isofluorane. Serum samples and muscle tissue were kept at -70°C until analysis.

Homogenization and dilution
Frozen heart or frozen skeletal muscle biopsies (-70°C) were placed in liquid nitrogen, and when still frozen placed on a Chan Electro balance and weighed. The samples were then freeze-dried and re-weighed, the latter referred to as dry weight. The water content per gram muscle was calculated. The dried sample was powdered in an agate mortar and freed from flakes of blood and visible connective tissue. Approximately 10 mg of the muscle powder was weighed and placed in an Eppendorf tube. Wet muscle weight of the dry powder weight was recalculated from water content of the muscle.

Extraction procedure
Troponin T was extracted from the muscle powder (heart or skeletal) in 10 µL of high molar urea buffer/mg wet muscle weight (urea buffer; 5 mol/L urea, 1 mol/L KCl, 0.05 mol/L tris-HCl, 0.002 mol/L EDTA, pH 7.5) *(111)*. The tubes with the powdered sample were kept on ice and urea-buffer was added. The tubes were mixed on highest speed on a vortex mixer now and then during 30 min. After centrifugation (20 000 g, in cold) for 15 min the insoluble pellet was re-extracted in 5 µL urea-buffer/mg wet muscle-weight and again centrifuged. The two supernatants obtained after centrifugation were pooled and frozen (-70°C) until measuring cTnT.

Dilution for analysis of cTnT in homogenized heart and skeletal muscle
First dilution step (51 times); 0.010 mL of the homogenate was diluted with 0.500 mL of cTnT free serum. Second dilution step (101 times); 0.010 mL from the first dilution step was diluted with 1.000 mL cTnT free serum. Total dilution factor for the homogenate was 5151 times. Dilution for analysis of cTnT in homogenized skeletal muscle: 0.050 mL of the homogenate was diluted with 0.500 mL (11 times) cTnT free serum. By using these dilutions, cTnT concentrations in homogenates were within the measuring range (0.010-25.00 µg/L) of the assay.

3.4 LABORATORY METHODS

3.4.1 Biochemical assays

Cardiac troponin T
Serum cTnT was determined with the second generation troponin T ELISA (Enzymun-Test Troponin-T) on ES 300 system (Boehringer Mannheim GmbH, Mannheim, Germany) in paper I-II. This assay uses the two cardiac-specific monoclonal antibodies M11.7 and M7 *(26)*. The detection limit was 0.04 µg/L and the total CV of the assays was 8.0% at 0.16 µg/L. This assay was used to investigate the release kinetics of coronary effluent cTnT after global ischaemia, and the relationship between cTnT release and heart dysfunction after ischaemia in isolated rat hearts in paper IV. The second generation cTnT assay was also used to measure cTnT in coronary effluent with and without albumin in paper V.

In paper III, serum cTnT was analysed with the third generation troponin T test (Troponin T STAT). The third generation TnT test uses the same monoclonal antibodies (M11.7 and M7) as the second generation test but is standardised with human recombinant cTnT instead of bovine cTnT, and has a detection limit of 0.01 µg/L (Roche Diagnostics, Mannheim, Germany) *(28)*. This assay is an electrochemiluminescence immunoassay (ECLIA) and it was performed on the Elecsys 2010 immunoassay analyser (Roche Diagnostics). The principles of the assay are shown in Figure 4. The total CV of the assay was 4.8% at 0.05 µg/L. The third generation cTnT assay was also compared with the second generation cTnT assay for the measurement of cTnT in isolated rat heart coronary effluent with and without albumin in paper V.

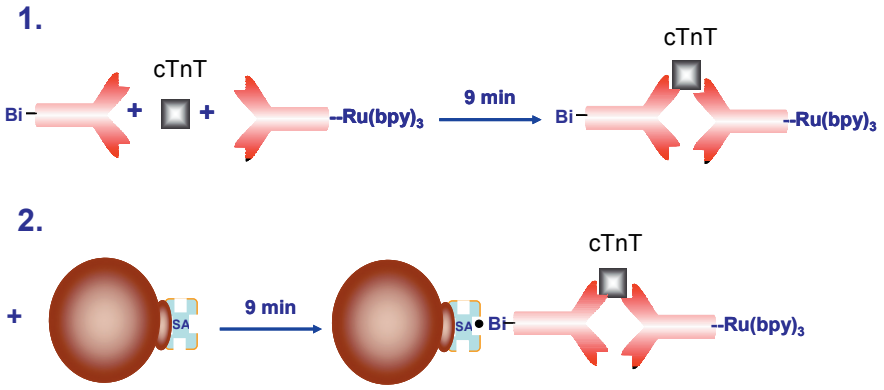

Figure 4. 1: During the first incubation, a biotinylated (Bi) monoclonal cTnT-specifik antibody and a monoclonal cTnT-specifik antibody labelled with a ruthenium complex ($Ru(bpy)_3$) reacts with cTnT to form a sandwich complex. 2: During the second incubation, the complex becomes bound to streptavidin (SA)-coated microparticles via interaction of biotin and streptavidin. In a measuring cell, application of a voltage induces chemiluminescent emission which is measured by a photomultiplier. Modified from Roche Diagnostics 2007.

In paper VI, the third generation cTnT assay was used to measure cTnT content in heart muscle, in skeletal muscle and in serum from mice. The content of cTnT was calculated as µg cTnT/g wet heart muscle and µg cTnT/g wet skeletal muscle. The within-series CV for analysing cTnT in homogenized heart diluted in serum as described above was 2.6% at 0.75 µg/L (n=10) and 2.4% at 4.6 µg/L (n=10). The error of the entire procedure was calculated from duplicate (n=18) analyses of cTnT extracted from muscle powder using the following formula:

$$CV = 100 \cdot \sqrt{\sum [2 \cdot (a-b)/(a+b)]^2 / 2n}$$

where a and b are the cTnT concentrations in the two samples from the same powder and n is the number of duplicates. At a mean cTnT concentration of 4.47 µg/L this CV was 12.5%.

Cardiac troponin I

Serum cTnI was measured with the first generation Opus Plus Troponin I assay (Behring Diagnostics, Westwood, MA, USA) performed on the Opus analyser in paper I-II. This assay used two monoclonal goat antibodies against cTnI. The detection limit was 0.5 µg/L and the total CV of the assay was 8.4% at 7.1 µg/L.

Creatine kinase MB

Serum CKMB mass concentration was analysed by microparticle enzyme immunoassay (MEIA) technology with AxSYM system (Abbott Diagnostics, Abbott Park, IL, USA) in paper I-II. The total CV was 8.3% at 3.8 µg/L. A relative index (%CKMB) for CKMB defined as [CKMB (µg/L)/CK (U/L)] × 100 was calculated in paper I.

In paper III, CKMB was analysed with an ECLIA using two monoclonal antibodies (CKMB STAT, Roche Diagnostics) on the Elecsys 2010 immunoassay analyser (Roche Diagnostics).

Endothelin-1 and big endothelin-1
Plasma ET-1 and big ET-1 were measured by radioimmunoassay (RIA) technique: Plasma aliquots (1 mL) were extracted with acid ethanol and dried under a nitrogen stream. For determination of big ET-1, big ET-1 antiserum (B6) and ^{125}I-labelled big ET-1 (Amersham, England) were used. The detection limit was 0.78 fmol per tube. Expressing the big ET-1 value as 100%, the cross reactivity of the used antiserum (B6) was <0.007% for ET-1 (1-21), <0.03% for ET-2, <0.03% for ET-3 and 35% for big ET-1 fragment (22-38). The intra-assay variation was 5.6% at a plasma concentration of 50 pmol/L *(126)*. For determination of ET-1, rabbit ET-1 antiserum (E1), which was diluted to give a specific binding of 35-40%, and ^{125}I-labelled ET-1 (Amersham, England) were used. The detection limit for the assay was 0.39 fmol per tube. The cross-reactivity for the E1 antiserum when the ET-1 (1-21) value is expressed as 100% was: ET-1 (16-21), 16%; ET-2, 27%; ET-3, 8%; and big ET-1, 0,45%. No cross-reactivity with big ET-1 (22-38) was observed at concentrations up to 1 µmol/L. The intra- and inter-assay variations were 6 and 14%, respectively *(126)*.

Creatine kinase
Serum CK was measured with dry chemistry using Ektachem 950ICR System (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY, USA) in paper I-II. This assay had a total CV of 8.6% at 192 U/L.

C-reactive protein
Serum CRP was measured with dry chemistry using Ektachem 950ICR System (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY, USA) in paper II. The detection limit for CRP was 7 mg/L and the total CV of the assay was 14% at 21 mg/L. In paper III, CRP concentrations were measured at the Department of Clinical Chemistry, Huddinge University Hospital with a nephelometric method used in clinical routine.

Interleukin-6
Plasma IL-6 was measured by a commercially available photometric ELISA (Boehringer Mannheim, Mannheim, Germany).

Creatinine
Creatinine was measured with dry chemistry using Ektachem 950ICR System (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY, USA) in paper I-II.

Albumin
Serum albumin concentrations were measured at the Department of Clinical Chemistry, Huddinge University Hospital with a bromcresol purple method used in clinical routine.

Fibrinogen
Serum fibrinogen concentrations were measured at the Department of Clinical

Chemistry, Huddinge University Hospital with a turbidimetry method used in clinical routine.

3.4.2 Decision limits for cardiac markers

Paper I
Values exceeding the upper reference limits were considered elevated: cTnT ≥0.10 µg/L, cTnI ≥0.5 µg/L, CKMB ≥5 µg/L, CK (males) ≥174 U/L , and CK (females) ≥144 U/L. The discrimination limits for acute myocardial infarction were cTnT ≥0.20 µg/L, cTnI ≥0.5 µg/L, CKMB >15 µg/L and %CKMB >5%.

Paper II
The reference limits used in clinical routine were: cTnT <0.10 µg/L, cTnI <2.0 µg/L, CKMB <5 µg/L, CK (males) <174 U/L, and CK (females) <144 U/L. The influence of cTnT on cumulative survival was calculated by using the decision limits ≥0.04 µg/L and ≥0.10 µg/L.

Paper III
For survival analysis, patients were divided according to the cTnT serum levels < or ≥0.10 µg/L.

In the experimental studies (paper IV-VI) cTnT concentrations were compared between groups and no discrimination limits were used.

3.4.3 Electrocardiogram

In all 36 HD patients in paper I, 12-led electrocardiogram (ECG) was performed within 2 hours after the end of a HD session. The ECG was used to exclude patients with signs of acute myocardial ischaemia. Evidence of myocardial ischaemia was: New or presumed new ST segment elevation at the J point in two or more contiguous leads with the cut-off points ≥0.2 mV in leads V1, V2, or V3 and ≥0.1 mV in other leads (contiguity in the frontal plane is defined by the lead sequence aVL, I, inverted aVR, II, aVF, III); new ST segment depression; new T wave abnormalities only. Evidence of loss of electrically functioning cardiac tissue was: Any QS complex in leads V1 through V3; abnormal Q wave in lead I, II, aVL, aVF or V4 through V6 in any two contiguous leads and at least 1 mm in depth.

3.4.4 Echocardiographic measurements

In 27 HD patients in paper I, echocardiography was performed within 2 hours after the end of a HD session. Echocardiographic measurements at rest were obtained from standard projections (left parasternal and apical positions) with the subjects in the left lateral decubitus position using an Acuson Sequoia 512 with a 3.5 MHz transducer (Siemens, Erlangen, Germany). Measurements were taken from two-dimensional recordings, M-mode measurements and with pulsed and colour flow Doppler.

The following left ventricular (LV) dimensions were measured at the end of diastole: interventricular septal thickness (IVSTd), posterior wall thickness (PWTd) and internal diameter (LVIDd). LV mass in grams was determined using the Penn-cube formula:

LV mass = 1.04 x [(IVSTd + LVIDd + PWTd)^3 – LVIDd^3] - 13.6 g *(127)*. When corrected for body surface area, LVMI was determined. LVH was defined as LVMI >125 g/m^2 *(128)*.

3.5 STATISTICAL ANALYSIS

Most quantitative variables in paper I-III were not normally distributed and therefore values are given as median and interquartile range, or median and range where appropriate. For some variables in paper III mean ± standard error of the mean (SEM) is given. In paper IV most data were normally distributed and data are presented as mean ± SEM. In paper V-VI data are given as median and interquartile range due to the relatively low number of observations.

3.5.1 Parametric methods

Paired *t*-test was employed for comparing changes in haemodynamic variables at 20 minutes of reperfusion to baseline values in paper IV.

A simple regression analysis was used to calculate regression coefficients between cTnT release and haemodynamics or cTnT concentrations and coronary flow in paper IV.

3.5.2 Non-parametric methods

Correlations between variables were tested by Spearman rank correlation test where Rho (ρ) is the correlation coefficient in paper I, III, IV and VI.

The Mann-Whitney U-test was employed to compare unpaired interval (quantitative) data between groups in paper I, II, III, V and VI, and the Wilcoxon rank sum test was used to compare paired data in paper I and V.

The Kruskal-Wallis test was used to compare multiple independent groups in paper VI.

The relationships and differences between groups for nominal variables were analysed with Fisher exact two tailed test in paper II and III.

The 97.5th percentiles for cTnI, cTnI, CKMB and %CKMB were determined in paper I.

3.5.3 Survival analysis

The influence of different parameters on survival was analysed with Cox's proportional hazard regression. cTnT and other covariates were entered simultaneously into the regression to test if cTnT was an independent risk marker, and exponent beta (hazard ratio) and standard error were calculated in paper II.

The effects of age, gender, CVD, diabetes, serum IL-6, and serum cTnT on survival were determined with the Cox proportional hazard model and the hazard ratio of death was calculated in paper III.

Kaplan-Meier curves were used to describe the cumulative survival based on elevated cTnT in paper II and III. Survival was measured beginning with the day of sample collection until death or censoring, which was done at the end of the follow-up. For the survival analysis, patients were divided according to the cTnT serum levels: < or ≥0.04 μg/L and < or ≥0.10 μg/L in paper II and < or ≥0.10 μg/L in paper III.

Statistics were performed using the following data analysis softwares:

- Statistica 5.5 (Stat soft, Inc., USA) in paper I-II and V.
- Statistica 6.0 (Stat soft, Inc., USA) in paper VI.
- StatView 5.0 (SAS Institute Inc., Cary, NC, USA) in paper III.
- StatView J-4.5 Power PC Version (Abacus Concepts, Cary, NC, USA) in paper IV.

Statistical significance was defined as $P<0.05$.

3.6 ETHICAL CONSIDERATIONS

Study protocols for the studies included in this thesis were approved by the local ethics committee at Huddinge University Hospital (paper I-III) and the regional ethics committee for animal research of northern Stockholm (paper IV-VI). Informed consent was obtained from all patients and control subjects.

4 RESULTS AND DISCUSSION

4.1 CLINICAL STUDIES

The prevalence of elevated serum cTnT in dialysis patients without symptoms of acute myocardial ischaemia (paper I)

The numbers of patients with elevated cardiac markers are shown in Table 1. In HD + PD patients taken as one group (n=62), cTnT concentration was ≥0.10 in 28 of 62 (45%), ≥0.20 in 15 of 62 (24%) and ≥0.50 µg/L in 4 of 62 (6%) patients. None of the dialysis patients had CKMB >8 µg/L. The 97.5th percentiles (range) were: cTnT 0.54 µg/L (<0.02-1.6 µg/L), cTnI 1.3 µg/L (<0.5-5.7 µg/L), CKMB 8 µg/L (<1-8 µg/L) and %CKMB 28% (<1-44%).

Table 1. Number of haemodialysis (HD) patients and peritonealdialysis (PD) patients with elevated serum concentrations of markers of myocardial damage.

Parameter	Value[a]	HD Patients		PD patients	
cTnT	≥0.10 µg/L	20/36	(56%)	8/26	(31%)
cTnI	≥0.5 µg/L	3/36	(8%)	1/26	(4%)
CKMB	≥5 µg/L	3/36	(8%)	2/26	(8%)
CK	m≥174 U/L f≥144 U/L	0/36	(0%)	2/26	(8%)
%CKMB	>5%	25/36	(70%)	11/26	(42%)

[a]Elevated values were defined as values exceeding the upper reference limits.

The rate of 56% HD patients with elevated serum cTnT above the decision limit for acute myocardial infarction is consistent with other studies using the second generation cTnT assay, where up to 53% of asymptomatic patients with ESRD had an elevated serum concentration *(97, 102, 129-136)*. In studies using the first generation assay up to 75% had elevated cTnT *(93, 98, 129, 137-139)*. This assay gave higher concentrations of serum cTnT than the second generation assay in ESRD patients *(129, 137)*.

It is obvious that the prevalence of elevated cTnT will depend on the characteristics of the study population and the cut-off values used for the definition of an elevated concentration. In a large study on 773 ESRD using the third generation cTnT assay, three different cut-off criteria were used to define elevated cTnT: the 99th percentile of a reference population (0.01 µg/L), the lowest concentration to give a 10% CV (0.03 µg/L), and the receiver operator characteristic (ROC) curve determined value optimized for the detection of myocardial infarction (0.10 µg/L) *(66)*. The proportion of patients

with elevated cTnT in that study was 82% using 0.01 µg/L, 53% using 0.03 µg/L, and 20% using 0.10 µg/L as cut-off limit. In another study on 90 chronic HD patients, 27% had cTnT values >0.10 µg/L and 43% had values >0.05 µg/L *(140)*. Concentrations of circulating cTnT in ESRD patients may change over time. Over a one year period cTnT was increased in 36% of HD patients with no adverse outcome and in 63% of patients with other than cardiac deaths *(132)*.

Since cTnT is frequently elevated in ESRD, conventionally used decision limits for the diagnosis of acute myocardial infarction cannot be used. However in most ESRD patients without acute myocardial ischaemia, cTnT is only slightly elevated with a majority of the results below 1.0 µg/L, and concentrations above 3.0 µg/L are extremely rare. Furthermore, many dialysis patients have no changes or fluctuating concentrations of cTnT after at least 1 year *(132)*. A critical question is how to distinguish these cTnT elevations from those caused by ACS. On way is to obtain a baseline value for comparison. Thus for example in an ESRD patient with a baseline level of cTnT 0.80 µg/L, an increase of 100% to 1.6 µg/L within 24 hours strongly suggests acute myocardial infarction.

As we expected, cTnI with the first generation Opus Plus assay was elevated less frequently than cTnT in dialysis patients. Other studies using this assay showed elevated cTnI ≥0.5 µg/L in 6% *(134)*, and elevated cTnI >1.6 in 15% *(100)* of HD patients. In a study on 80 patients with renal dysfunction, Opus plus cTnI was elevated in 21% of the patients with increasing frequency in CRF, HD and acute renal failure patients groups compared with the chronic renal impairment group *(137)*. In a study on 101 HD patients where cTnI was measured with the more sensitive second generation Opus Plus immunoassay, cTnI was positive (>0.15 µg/L) in each of five measurements over a year in 14%, and sometimes positive and sometimes negative during the year in another 15% of the patients *(141)*.

Using the older cTnI assays it is well documented in published data that cTnI is positive in a considerable smaller proportion of dialysis patients than is cTnT. Up to 82% of ESRD patients without signs of ACS have elevated cTnT *(31, 66)*, while between 4 and 21% have elevated TnI *(138, 139, 142-145)*. This is not the case in patients without renal failure where cTnT and cTnI have similar diagnostic capacity, and the same ability to predict adverse advents *(146, 147)*. In contrary, some of the new cTnI assays seem to have a higher ability than cTnT to predict mortality in non-renal patients with suspected ACS *(148-151)*.

Since there are several important differences in cellular biology, protein chemistry and assay technologies between cTnT and cTnI, there may be many possible explanations for the different prevalence of the two cardiac troponins in ESRD:

- The cytosolic unbound fraction of cTnI is approximately half (3.5% vs 7%) of that of cTnT *(152)*. The free cytosolic proteins are released earlier than proteins bound within the sarcomeres after myocardial damage *(15)*.
- The cTnT content per gram of myocardium *(14)* is roughly twice of cTnI *(153, 154)*.
- The dialysis procedure may differently affect serum concentrations of cTnT and

cTnI. The cTnI molecule is more positively charged than cTnT and may have a higher affinity for the negatively charged dialysis membrane. cTnI may be decreased by up to 86% from pre to post dialysis *(96, 135, 155)*.

- In acute myocardial infarction cTnT and cTnI are released in different forms *(37)*. cTnT is released as intact cTnT:I:C complex, free cTnT and smaller fragments. cTnI is identified as intact cTnT:I:C complex and cTnI:C complex. The hydrophobic cTnI may bind to other surfaces or proteins, so that epitopes necessary for the antibody binding in the immunoassay could be covered *(36)*.
- Circulating cTnI is susceptible to various biochemical modifications including phosphorylation, oxidation and proteolysis *(36, 156-158)*. cTnT may undergo proteolysis but is a more stable protein than cTnI *(158)*.

It has been suggested that the myopathy associated with ESRD may induce the expression of cTnT in replicating skeletal muscle. McLaurin et al *(129)* reported evidence of cTnT expression in skeletal muscle of dialysis patients. However they did not use the same antibodies (M11.7 and M7) that are used in the second and third generation cTnT assays. On the contrary, in another study there was no evidence of cTnT expression in skeletal muscle biopsies of ESRD patients *(159)*. Furthermore Ricchiuti et al *(160)* showed that cardiac TnT isoforms expressed in renal diseased skeletal muscle will not cause false-positive results by using the M11.7 and M7 antibodies. Therefore it is most likely that serum cTnT in ESRD patients, as measured by the second generation cTnT assay, originates from the heart.

Whether cTnT circulates in the free intact form *(161)* or as fragments *(162, 163)* in ESRD is a controversy *(164)*. The specific effects of the uraemic disease state on the chemistry of the cardiac troponins are unknown. Future studies characterizing the effects of uraemia on tissue content, compartmentation, release and clearance of different troponin subunits may provide helpful information to explain the discordance between cTnT and cTnI elevations in ESRD.

The ability of the assays to detect circulating forms of cardiac troponins in ESRD patients may be another explanation to the discordant results between the older cTnI assays and the cTnT assay. This hypothesis is supported by the results of a study on 143 ESRD patients showing that the newer cTnI assays Abbott Architect, Bayer Centaur and Beckman Accu-TnI were able to detect cTnI as effectively as the Roche assay detected cTnT with >75% of samples above the detection limits of the assays, while the Abbott AxSYM cTnI assay detected cTnI in only 35% of the samples and DPC Immulite in 16% of the samples *(165)*. However, using the 10% CV limits of the assays as discriminator, the cTnT assay had more samples elevated than did the newer cTnI assays.

In our study not only cTnI but also CKMB was less frequently elevated than cTnT. There are conflicting reports about the proportion of elevated CKMB in renal failure. Falsely elevated CKMB in up to 75% of ESRD patients have been demonstrated *(129, 142)*. In contrary, others found elevated CKMB in only 2% of 100 dialysis patients, where 7% had elevated cTnI *(99)*. Also CK have been reported in a high proportion of chronic dialysis patients *(166)*. However, none of our patients had elevated CK. This is in agreement with another study showing elevated CK levels in only 4.9% (3/61) and

elevated CKMB in 0% (0/61) of HD patients *(167)*. Furthermore we found that %CKMB was elevated (>5%) in 70% of HD patients and 42% of PD patients which makes it inapplicable for the diagnosis of AMI in these patients.

Associations between serum cTnT and other variables in HD and PD patients (paper I)

Significant correlation with all our pre-selected variables as shown in Table 2, strongly suggested that serum cTnT was not spuriously raised or an artefact in dialysis patients. One of the most important findings was the association between cTnT and LVH and our report was the first showing a positive correlation between cTnT and LVMI in HD patients. Median cTnT was approximately three times higher in HD patients with LVH than without. This finding has been confirmed in several larger studies. In a study on 258 chronic HD patients, predialysis levels of cTnT >0.10 µg/L were associated with LVH, and in multivariate analysis LVMI was independently associated with cTnT concentrations above the threshold *(168)*. In another study the cTnT level related directly to LVMI ($r=0.45$, $P<0.001$), interventricular septum ($r=0.36$, $P<0.001$) and posterior wall thickness ($r=0.40$, $P<0.001$), and in multivariate analysis, after age, LVMI was the second strongest independent predictor of cTnT level *(169)*. Using ROC curves, serum cTnT had a fair diagnostic value for the identification of LVH and of LV systolic dysfunction in patients on regular HD treatment, with the area under the corresponding ROC curve of 0.72 and 0.68 respectively *(170)*. The results suggest that cTnT may be used to screen for alterations in LV mass and function in clinically stable HD patients.

Not only in HD patients but also in PD patients, cTnT correlates positively with LVMI *(171, 172)*. Futhermore, it does not seem that the association between elevated cTnT and LVH is restricted to dialysis patients since in 176 stable outpatients with chronic kidney failure (GFR <60 mL/min), patients with cTnT concentrations >0.01 µg/L had a greater prevalence of LVH and congestive HF than patients with cTnT <0.01 µg/L *(173)*. It has also been shown that elevated cTnT is associated with LV dysfunction in both HD *(170)* and PD *(172)* patients. Also in non-dialysis patients with chronic kidney disease, slightly elevated cTnT is associated with LVH and several other known risk markers such as age, diabetes, previous HF episodes, anemia, greater pulse pressure, and advanced kidney disease *(173)*, and all these factors contribute to increased cardiovascular risk. However, although there is a correlation between cTnT and LVMI in CRF, the presence of LVH alone does not seem to explain troponin elevations in these patients *(174)*.

It is interesting to speculate that elevated cTnT in dialysis patients may be due to a leakage of this protein or its fragments from hypertrophic cardiomyocytes. It has been shown in experimental renal failure that microvascular heart disease is commonly associated with LVH *(175)*. In hypertrophy more ultrastructural components are synthesised with increased synthesis of enzymes and increased number and size of the cardiac muscle fibers. This may lead to an increased leakage of cTnT and other structural proteins from the cells. Furthermore the membranes of injured myocytes may lose their integrity and allow the exposure of intracellular proteins to the extracellular environment. It has also been shown experimentally that cTnT concentration increases in rat left ventricular myocardium after trophic stimuli and pressure overload *(176)*.

Our study showed that in HD + PD patients without clinical evidence of acute myocardial ischaemia, those with a history of IHD showed higher serum cTnT than patients without IHD, which was is in accordance with Haller et al who reported a correlation between the plasma cTnT concentration and indicators of coronary artery disease in ESRD patients *(159)*. This has been confirmed by more recent studies, and interestingly in multivariate analysis, elevated cTnT was associated more strongly with LV mass than with a history of IHD *(168, 169)*.

Table 2. Correlations between serum cTnT, plasma ET-1, plasma big ET-1 and other data in dialysis patients.

Patient group	Compared data		Correlation coefficient	P-value
HD[a] patients n=36	cTnT and:	Age	0.42	<0.05
		CKMB	0.53	<0.01
		big ET-1	0.45	<0.01
		%CKMB	0.48	<0.01
		LVMI[c] n=27	0.40	<0.05
	ET-1 and:	CK	-0.38	<0.05
		%CKMB	0.48	<0.01
	big ET-1 and:	%CKMB	0.55	<0.01
		LVMI n=27	0.45[†]	<0.05
PD[b] patients n=26	cTnT and:	Age	0.43	<0.05
		CKMB	0.66	<0.01
		Creatinine	-0.40	<0.05
		%CKMB	0.67	<0.01
HD + PD patients n=62	cTnT and:	Age	0.48	<0.001
		CKMB	0.57	<0.001
		CK	-0.33	<0.05
		Creatinine	-0.29	<0.05
		%CKMB	0.56	<0.001

[a]HD: haemodialysis, [b]PD: peritoneal dialysis, [c]LVMI: left ventricular mass index. [†]Correlation between big ET-1 post-dialysis and LVMI. ET-1 and big ET-1 was only analysed in HD patients.

Our findings of higher serum cTnT and CKMB concentrations in diabetic dialysis patients without IHD than in patients without DM and IHD, are consistent with Ooi et al, who reported a high percentage (58%) of increased cTnT in haemodialysed patients with DM *(96)*. The authors speculate that advanced glycosylation end-products could induce cTnT expression in noncardiac cells and affect membrane integrity. However, diabetic patients are at high risk of developing IHD, which may be present but undiagnosed in these patients. We found that not only serum cTnT but also serum CKMB were higher in patients with diabetes. Other studies have verified that diabetes and age are variables with strong association to elevated cTnT in HD patients *(168, 169)*.

The concept that cTnT is elevated secondary to myocardial pathologies is supported by associations between elevated cTnT and mitral annular calcification measured by transthoracic echocardiography *(177)*, and between elevated cTnT and severe coronary artery calcification (CAC score ≥400) measured by multirow spiral computed tomography *(178)*. In the latter study both cTnT and AxSYM cTnI were independently associated with severe CAC in multiple logistic regression analysis, after adjusting for age, duration of dialysis, diabetes and previous cardiac events. Even in the absence of diabetes and overt atherosclerotic disease, elevated cTnT seems to indicate subcellular myocellular damage. This was elegantly shown by Lipshultz et al who found that there was an inverse correlation between preload-dependent LV contractility and cTnT levels in 50 pediatric CRF patients without clinical heart disease *(179)*.

In our study there was a positive correlation between cTnT and CKMB concentrations, which is in agreement with other findings of an association between cTnT and CKMB in ESRD patients *(168, 180)*. The lack of correlation between cTnT and cTnI in the present study was probably due to the lack of sensitivity of the Opus assay to detect circulating cTnI molecules or fragments in ESRD patients. This is in agreement with a study on pediatric CRF patients, where there was a positive correlation between cTnT and CKMB, whereas cTnI did not correlate with any other variable *(179)*. In that study cTnT was elevated in 45% and Opus plus cTnI in 13% of the patients. Using another cTnI assay, positive correlations were evident between the serum concentrations of each of the three cardiac biomarkers cTnT , cTnI and CKMB in 38 patients undergoing chronic HD *(178)*. With the newer cTnI assays the concordance between cTnT and cTnI in ESRD is even more pronounced *(165)*.

A novel finding was the positive correlation between cTnT and plasma big ET-1. Although it does not prove causal relationship, it might strengthen the hypothesis that endothelin is associated with cardiac disease in dialysis patients. It also further underscore that cTnT is not spuriously rised in these patients, but more likely one of several important markers reflecting the altered biochemistry in uraemia. ET-1 is a potent vasoconstrictor with mitogenic, proinflammatory and antinatriuretic properties *(181)*, and the ET system is involved in numerous cardiovascular disease states, such as arterial hypertension, atherosclerosis, pulmonary hypertension, coronary artery disease and HF *(182, 183)*. In the present study, there was a correlation between big ET-1 levels post-dialysis and LVMI in HD patients, and HD patients with a history of IHD showed higher ET-1 concentrations than HD patients without IHD. The latter finding is in accordance with a study investigating 53 HD patients which showed that plasma

concentrations of ET-1 and big ET-1 were higher in HD patients with IHD than without *(184)*. The elevated plasma concentrations of ET-1, which were noted in IHD, may be due to episodes of ischaemia which is known to stimulate ET synthesis *(185)*. On the other hand, ET-1 may cause myocardial ischaemia since Pernow et al demonstrated that exogenous ET-1 elicited coronary vasoconstriction in healthy humans *(186)*. In addition, ET-1 has been associated with the development of LVH *(187)*, and Demuth et al have demonstrated a significant positive correlation between elevated plasma ET concentration and an increased LV mass in ESRD patients *(188)*. In a study on 59 HD patients cTnI and ET-1 correlated with LV mass and relative wall thickness, respectively, suggesting an association with LVH and/or myocardial ischaemia *(189)*. Furthermore, levels of ET-1 are higher in HD patients with type 2 diabetes than without *(190)*. In non-renal patients with chronic IHD, elevated Immulite cTnI levels showed a positive correlation with ET-1 levels *(191)*. Since ET has been implicated in the pathophysiology of CVD, it has been suggested that ET_A-receptor blockade may represent a potential target for the management of hypertension and cardiovascular protection in CRF *(192)*.

In accordance with previous reports, we did not find any positive correlation between serum cTnT and serum creatinine. On the contrary we found a negative correlation between these two analytes. This could be explained by a lower muscle mass in the older and severely ill patients in our study population. This was indicated by a significant negative correlation between age and creatinine (data not shown). A similar picture was found in 258 chronic HD patients, where patients with cTnT >0.1 µg/L were older and had a lower mean creatinine than patients with cTnT ≤0.1 µg/L *(168)*. However, choosing the cut-off limit 0.035 µg/L mean creatinine was equal in PD patients with and without elevated cTnT *(171)*. Although kidney function may contribute to the elimination of troponins *(193)*, most studies argue against the hypothesis that impaired renal function per se influence the clearance of cardiac troponins *(194-196)*.

Elevated cTnT is a marker of poor prognosis in PD patients (paper II)

The main finding of the present study was a significant relationship between poor long-term outcome and elevated serum concentrations of cTnT in a single sample from continuous ambulatory PD patients. Patients with cTnT values ≥0.04 µg/L at inclusion had approximately six times higher risk for all-cause death during the 48 months' follow-up (Figure 5).

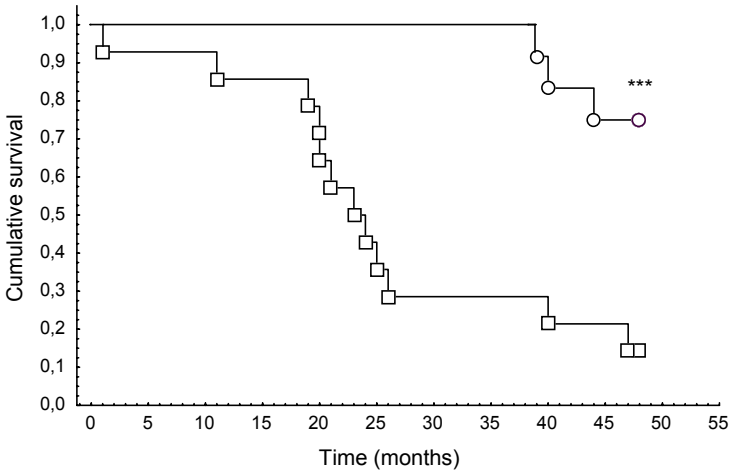

Figure 5. Kaplan-Meier survival analysis curves according to serum concentrations of cardiac troponin T (cTnT) in samples collected from peritoneal dialysis patients at time zero. ○: cTnT <0.04 µg/L (n=12). □: cTnT ≥0.04 µg/L (n=14). ***P<0.001 compared with the other survival curve.

At the time of publication of paper II, the relationship between elevated cTnT and increased mortality had been shown for HD patients *(97, 98, 102, 132)* but had not been reported using samples from PD patients. We also found a strong association between cTnT and the inflammatory marker CRP. In multivariate analysis together with albumin and age, both cTnT ≥0.04 µg/L and CRP ≥10 mg/L were independent risk markers for death. When co-morbidity was entered into the regression analysis, elevated cTnT was still an independent predictor of all-cause mortality, in contrast to congestive HF, atrial fibrillation and IHD. The survival was not influenced by cTnI, CKMB, CK, haemoglobin, parathyroid hormone, duration of renal disease or duration of PD.

Our findings were first confirmed by Duman et al, who showed that elevated cTnT but not cTnI (using the Immulite assay) predicted total and cardiovascular mortality in a group of 65 PD patients *(171)*. In that study cTnT ≥0.035 µg/L independently predicted total mortality with an odds ratio (OR) of 4.31 and cardiovascular mortality with an OR of 8.94. Furthermore they also found a positive correlation between cTnT and LVMI. In the second confirmatory study, on 238 chronic PD patients, a single random cTnT was predictive of all-cause mortality, cardiovascular death, noncardiovascular death, and fatal and nonfatal cardiovascular events independent of other possible risk factors including coronary artery disease, CRP, residual renal function, LVH, and systolic function *(196)*. In another article the same research group showed that cTnT, LV mass, and LV function are excellent predictors of cardiovascular congestion in PD patients, and that cTnT emerged as an independent predictor of cardiovascular congestion in a multivariate model including also LVMI and ejection fraction *(172)*. Our data and the combined data from these studies suggest that a single random serum cTnT is useful for risk stratification in clinically stable PD patients.

In a meta-analysis using data from 28 studies (including paper II and III in this thesis) published between 1999 and 2004, it was concluded that elevated cTnT identifies a subgroup of ESRD patients who have poor survival and a high risk of cardiac death despite being asymptomatic, whereas the clinical interpretation of elevated cTnI levels in ESRD remain unclear *(197)*. Accordingly, the US Food and Drug Administration has only recommended the use of cTnT for risk stratification in ESRD patients.

To our knowledge, an association between serum cTnT and the inflammatory marker CRP had not been shown before in PD patients, although an association between cTnT and CRP had been reported in HD patients *(102)*. It is known that loss of renal function leads to changes in serum/plasma composition that are associated with vascular disease, i.e. increase in lipids and inflammatory markers. Chronic inflammation is believed to cause accelerated arteriosclerosis resulting in CVD and increased risk for death *(198)*. The correlation between CRP and cTnT in the present study is logical since inflammation induces vascular injury reflected in the heart by cTnT release. The association between serum cTnT and markers of inflammation in ESRD is further discussed on page 29-30.

Correlations between cTnT and inflammatory markers before the start of dialysis treatment (paper III)

In predialysis patients significant correlations were found between cTnT and CKMB, IL-6, CRP and serum albumin, whereas the correlation between cTnT and fibrinogen was borderline significant (Table 3). As expected, the inflammatory markers were correlated to each other (Table 3). The cTnT levels were positively correlated to age whereas CKMB was not. Also IL-6, CRP, and serum albumin levels correlated to age.

Table 3: Correlations between serum cTnT, plasma IL-6 and other biochemical variables in ESRD patients shortly before the onset of dialysis therapy.

Compared variables		Rho[a]	P-value
cTnT[b] and:	IL-6[c]	0.23	<0.05
	CRP	0.30	<0.05
	fibrinogen	0.18	0.06
	albumin	-0.31	<0.001
	CKMB	0.52	<0.0001
IL-6[c] and:	CRP	0.61	<0.0001
	fibrinogen	0.42	<0.0001
	albumin	-0.35	<0.0001
CRP[d] and:	albumin	-0.19	<0.0001

[a]Rho, Spearman rank correlation coefficient. [b,d]n=115, [c]n=109.

Correlations between cTnT and markers of inflammation in predialysis patients had not been demonstrated previously, although an association between cTnT and CRP was reported in HD patients *(102)*. It is recognised that acute phase proteins and some cytokines are markers for cardiovascular risk both in the general population *(199, 200)* and in ESRD *(104, 201)* and in predialysis patients elevated CRP is a marker of atherosclerotic CVD *(202)*. Furthermore, CRP per se may be a mediator of myocardial injury *(203)*, and therefore the association of both markers to the presence of cardiac disease could explain the correlation between CRP and cTnT.

The association between cTnT and inflammation in the present study was further underscored by the correlations with other markers of inflammation, namely fibrinogen and serum albumin. Fibrinogen is an acute phase protein, which is associated with vascular disease in HD patients *(204)*, whereas serum albumin may decrease in malnutrition but is to a large extent lowered as a function of the acute phase response. Accordingly, predialysis patients with the lowest concentrations of albumin are those with both vascular disease and inflammation *(202)*.

The IL-6 system activity, which is the major mediator of the acute phase response, is often markedly up regulated in uraemic patients. A number of factors, such as hypertension, adiposity, infections, chronic HF, and the dialysis procedure may all contribute to increased IL-6 production and elevated plasma concentrations, and it has been hypothesised that IL-6 may play a central role in the genesis of inflammatory-driven malnutrition and that it may be regarded as a significant proatherogenic cytokine *(205)*. Our findings of a correlation between cTnT and IL-6 in predialysis patients was confirmed in a study by Wong et al, who also found a positive correlation between cTnT and IL-6 in dialysis and predialysis patients *(206)*. They also found elevated concentrations of IL-18 and tumor necrosis factor-α (TNF-α), although these cytokines did not correlate with cTnT. Potentially, IL-6, IL-18 and TNF-α may affect endothelial function and induce endothelial expression of chemokines and adhesion molecules promoting a pro-coagulant state with increased risk of myocardial injury *(207)*. Inflammatory markers may not only be predictors of coronary artery disease and cardiovascular death. In HD patients, TNF-α was a significant independent predictor of LVH *(208)*, and in another study HD-induced elevated CRP concentrations were associated with LVH and correlated with LVMI *(209)*. Thus, the combined use of cTnT, CRP and proinflammatory cytokines could provide useful diagnostic and prognostic applications in the management of ESRD patients.

The predictive value of cTnT in samples from predialysis patients (paper III)
In this follow-up study on 115 patients with chronic kidney disease, we showed for the first time that a single cTnT concentration measured shortly before the onset of dialysis therapy was a significant predictor of mortality. Thirty per cent of the patients had cTnT ≥0.10 µg/L which is comparable to a prevalence of 20 to 50%, as reported in HD patients *(66, 96, 139)*. Thus elevated cTnT levels seem to be associated to the uraemic syndrome rather than the dialysis treatment per se. This has been confirmed in a study on 222 non-dialysis patients with chronic kidney disease, showing an elevation above the 99th percentile reference limit (<0.01 µg/L) in 43% of the cases for cTnT, compared with 18% for ADVIA Centaur cTnI *(174)*. In that study increase in cTnT, but not cTnI, was associated with poorer survival. In a study on non-dialysis-dependent stable patients with moderate chronic kidney disease, cTnT was elevated in 16% of the patients and cTnT level showed a hazard ratio of 12 for cardiovascular morbid events during follow-up *(173)*.

The Kaplan-Meier survival analysis for the overall population (HD + PD) in the present study is shown in Figure 6. A highly significant difference in survival was observed for patient groups divided according to cTnT levels < or ≥0.10 µg/L.

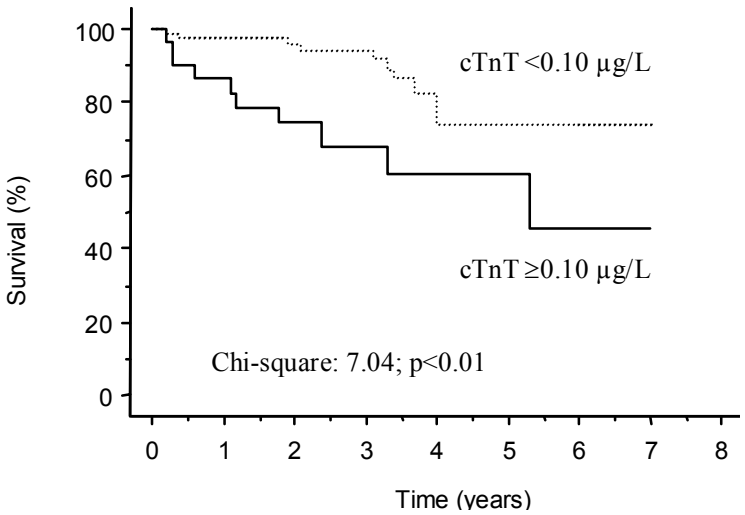

Figure 6. Kaplan-Meier survival analysis curves according to serum concentrations of cardiac troponin T (cTnT) in samples collected from 115 predialysis patients at time zero. Dotted line: cTnT <0.10 µg/L (n=81). Closed line: cTnT ≥0.10 µg/L (n=34).

The survival analyses of the patient groups starting PD and HD separately, divided according to cTnT levels < or ≥0.10 µg/L showed a similar pattern. The Cox proportional hazard model was applied to the overall study population to adjust event-free times for cTnT, IL-6, age, CVD, malnutrition, DM, and male gender, respectively. While age, CVD, malnutrition, DM, IL-6, cTnT and male gender were associated with

mortality in the univariate analysis, only DM and cTnT were independently associated with mortality in a multivariate analysis. This underscores the strong predictive power of cTnT compared with other traditional risk factors for increased cardiovascular mortality in CRF. Although patients with a history of CVD had higher median level of cTnT, CVD did not fully explain the predictive power of cTnT. Similar results were found in a large study on 733 HD patients where risk of death remained increased 2- to 4-fold with elevated cTnT and 2-fold with elevated Dimension RxL cTnI after adjustment for other risk factors including a history of coronary artery disease *(66)*.

Our findings of higher serum cTnT in diabetic predialysis and dialysis patients are consistent with data from studies on both dialysis patients *(66, 96)*, and non-dialysis chronic kidney disease patients *(173, 174)*. It has been speculated that advanced glycosylation end-products could induce cTnT expression in non-cardiac cells and affect membrane integrity *(96)*. However, diabetic patients are also at high risk of developing IHD, which may be present but undiagnosed in this patient group.

The interplay between the heart and the kidney is complex and multifactorial with basic parameters like cardiac output, extracellular volume and blood pressure partly regulated by mediators (renin-angiotensin system, endothelin) and antagonists (natriuretic peptides) *(210)*. Chronic inflammation, vascular calcification, and oxidative stress seem to be important factors that, together with traditional risk factors, contribute to accelerated atherosclerosis and markedly increased mortality in ESRD *(211)*. Future studies should focus on the underlying molecular mechanisms and complex causes of cTnT elevation in the uraemic milieu. DNA polymorphisms in candidate genes related to inflammation signaling, vascular ossification-calcification, and oxidative stress response *(212)* may be helpful tools to test causality in such studies. In basic research, a signal pattern of microRNA expression associated with myocyte hypertrophy and heart failure in both mice and humans, has recently been described *(213, 214)*. Whether this is an important mechanism behind ventricular hypertrophy in renal failure patients remains to be studied.

4.2 EXPERIMENTAL STUDIES

Post-ischaemic cTnT release compared with haemodynamic function in the isolated rat heart (paper IV)

This was the first study comparing haemodynamics and cTnT release measured with the second generation cTnT immunoassay in isolated rat hearts. After stabilization, isolated hearts were exposed to 30 minutes of global ischaemia and 30 minutes of reperfusion. The kinetics of cTnT release in coronary effluent before ischaemia and during 30 minutes of reperfusion is shown in Figure 7. After 30 seconds of reperfusion, a short-lasting peak of cTnT release appeared which rapidly subsided. Thereafter cTnT gradually increased with a maximum after 20 minutes of reperfusion. This time point was used to study the correlation between cTnT and heart function.

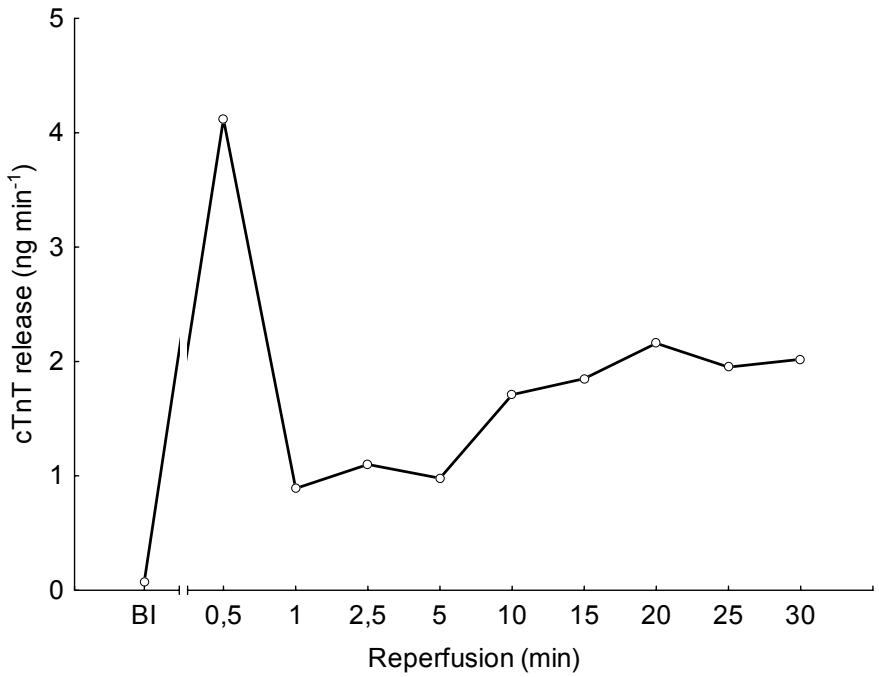

Figure 7. Mean cTnT release before 30 min of global ischaemia (BI) and during 30 min of reperfusion in isolated perfused rat hearts (n=7).

The release pattern of cTnT that we found in our model, with cTnT release reaching a plateau after about 20-30 minutes, has also been shown in other studies using isolated rat hearts *(111, 116)*. It seems that membrane damage leads most exclusively to leakage of an unbound cTnT pool; whereas prolonged ischaemia induces a continuous liberation of cTnT *(111)*. Theoretically the 6% of cTnT existing in the cytoplasm may be released early due to a rapid loss of membrane integrity, explaining the early cTnT peak in our study. There may also be an initial wash-out of cTnT accumulated in the extracellular fluid during the period of global ischaemia.

LVEDP and LVDP are the most important and widely used indices of cardiac contractile function in isolated perfused hearts from rat *(215, 216)* or mouse *(217)*. During the early reperfusion period we found no consistent correlation between cTnT and post-ischaemic dysfunction. After 20 minutes of reperfusion, there were neither correlation between cTnT release and LVEDP nor between cTnT release and LVDP. In a study by Yamahara et al, cTnT did correlate with LVDP after four hours of reperfusion in isolated rat hearts *(116)*. However, since it is difficult to maintain a good isolated heart preparation for such a long time, we wanted to evaluate cTnT in the early reperfusion. From the present study we concluded that studying both cardiac function and cTnT release provide supplementary information during early reperfusion in the isolated rat heart. When studying global ischaemia-reperfusion injury, both these end points should be included.

In subsequent studies we have shown that cTnT release into the coronary effluent correlates well with infarct size in both C57BL/6J (wild type) and apoE/LDLr KO mice *(123, 218, 219)*, suggesting that cTnT is a useful biochemical marker of cardiac damage in the Langendorff heart preparation.

Measurement of cTnT concentrations in coronary effluent with and without albumin (paper V)

To our knowledge this was the first published investigation on the stability of cTnT and the precision of cTnT assays in the buffer effluents from isolated hearts. The main findings in the present study were that cTnT concentrations in coronary effluent from isolated rat hearts perfused with Krebs-Henseleit buffer declined rapidly in room temperature with a half life for cTnT of about 1-2 hours; and this loss of cTnT was attenuated by adding bovine albumin to the samples. In albumin free effluent the loss of cTnT continued over time but with albumin added there was an initial, less pronounced cTnT decline that seemed to cease after about 160 minutes after withdrawal from freezer. By adding albumin it was possible, even after several hours in room temperature, to refreeze and thaw the samples a second time with a high recovery of cTnT. The cTnT loss could not be prevented by using polystyrene or siliconised glass tubes. The effect of time, freezing, thawing, and addition of albumin is shown in Figure 8.

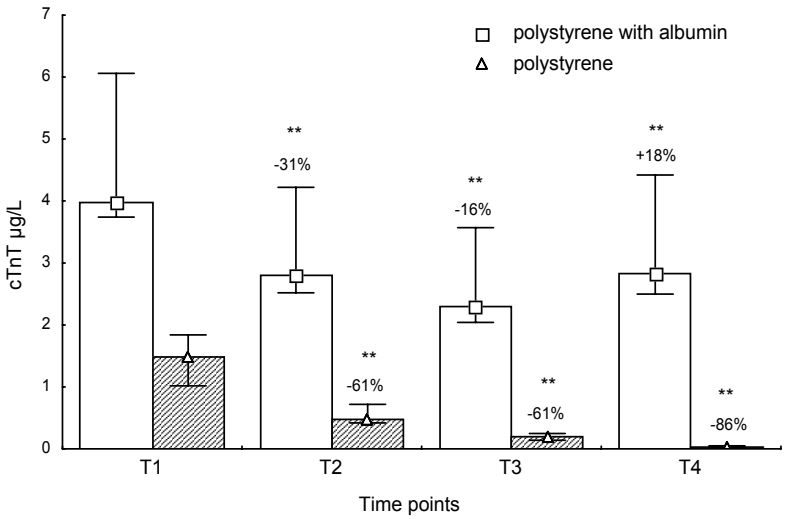

Figure 8. cTnT in coronary effluent from isolated rat hearts after 30 min of ischaemia followed by 20 min of reperfusion, collected in polystyrene tubes with and without albumin. Time points are minutes after withdrawal from freezer. T1: 60-70 min; T2: 150-160 min; T3: 240-250 min. At T4 samples had been freeze-thawed a second time and re-analysed the next day. Box: Median. Whisker: 25th-75th percentiles. **P<0.01 compared with previous time point. Per cent change of cTnT concentration compared with previous time point is shown. cTnT concentrations in tubes with albumin were higher than in tubes without albumin at all time points (P<0.001).

The reason for chosing bovine albumin with a final concentration of 40 g/L was twofold: first we wanted to prevent cTnT adsorption; secondly we wanted a solution similar to human serum and plasma, in which albumin is the most abundant protein with a normal concentration of 36-48 g/L. Since the cTnT assays employed are designed for serum and plasma, a very low albumin concentration might yield false results due to matrix effects. For instance, the cTnT concentration in albumin-free modified Krebs-Henseleit buffer was elevated when using the first generation cTnT ELISA *(117)*, probably due to matrix effects. In our studies, using the second and third generation assays, cTnT was <0.05 µg/L in troponin T free Krebs-Henseleit buffer with and without albumin.

In the present study CK and LD also showed higher activities in coronary effluent with albumin. This confirmed the findings of Dunphy et al, that the addition of albumin to Krebs-Henseleit perfusate of isolated rat hearts enhanced the storage stability of CK activity *(220)*. Furthermore, mitochondrial CK activity in glass tubes has previously been shown to be 10 times higher in Hepes buffer with albumin than without *(221)*. The present results and other data *(222-224)* indicate that adsorption may be a general problem when measuring low concentrations of proteins in dilute solutions like urine, cerebrospinal fluid and different buffers.

Adding albumin in the coronary effluent improved the precision of the cTnT assay markedly, lowering the within-assay CV from 20-30% to 5%. The improved precision when using effluent with albumin makes the assay reproducible, and useful for the detection of relatively small differences in cTnT release between groups. By using effluent with albumin it is possible to detect a mean difference of cTnT between groups of approximately 15% (2.8*CV%), whereas a difference of 70% is needed when effluent without albumin is used. Finally, as shown in the present study, by using effluents without albumin one can easily get false differences between groups by analysing samples at different time points. This risk may be minimised by using coronary effluent with albumin and by standardising the time point of analysis.

Serum cTnT in mice (paper VI)

This study was the first in vivo evaluation of blood sampling for murine cTnT measurement with the third generation cTnT assay. Blood sampling from the thoracic cavity may be convenient in connection with heart isolation, but the present study demonstrated that serum cTnT from the thoracic cavity was considerably increased compared with serum from the jugular vein (Figure 9). In fact, median serum cTnT from the thoracic cavity in C57BL/6J mice was about 20-90 times higher, and in ApoE/LDLr KO mice about 30 times higher than cTnT measured in serum from the external jugular vein in the same mouse. The difference was most likely explained by the larger surgical trauma, and thereby heart strain, associated with sampling from the thoracic cavity.

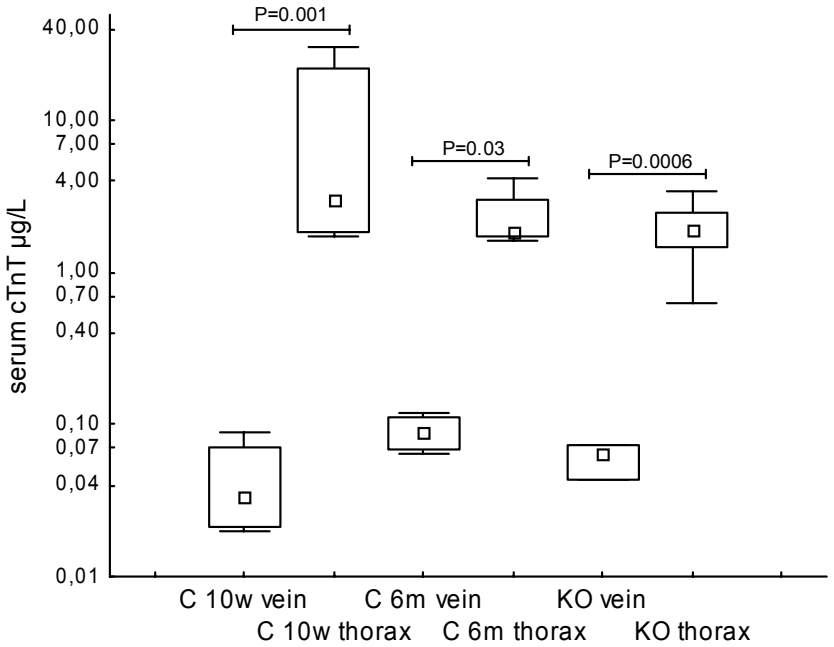

Figure 9. Serum cTnT in samples drawn from the external jugular vein of 10-week-old C57BL/6J mice (C 10w vein) (n=7), 6-month-old C57BL/6J mice (C 6m vein) (n=4) and apolipoprotein E and low density lipoprotein receptor double knockout mice (KO vein) (n=7); or from the thoracic cavity in the same mice (C 10w thorax, C 6m thorax and KO thorax). Mid point, median; box, interquartile range; whisker, range. Logarithmic scale on y-axis. The detection limit of the assay was 0.01 µg/L.

Still, it is notable that there were detectable cTnT concentrations (≥0.01 µg/L) also in the samples from the external jugular vein. The mouse is the only studied animal with substantially elevated baseline levels of cTnT *(112, 124)*. The reason for this is not known but it has been speculated that the slightly elevated cTnT levels in sham-operated mice may have been caused by cross-reactivity of the antibodies with skeletal muscle troponin T *(124)*. However, our finding of a median cTnT content in skeletal muscle less than 0.1% of the cTnT content in heart muscle argue against this (see page 38). The high cTnT concentrations found in blood from the thoracic cavity makes the

model very insensitive to detect small differences in myocardial injury between groups, and therefore sampling from the external jugular vein should be preferred.

cTnT content of heart and skeletal muscle in ApoE/LDLr KO mice and wild-type C57/BL/6J mice (paper VI)

The cTnT content per wet weight heart muscle from C57BL/6J 10 weeks, C57BL/6J 6 months and ApoE/LDLr KO mice are shown in Figure 10. There were no significant differences between groups. Also when C57BL/6J 10 weeks were taken together with C57BL/6J 6 months (n=11) and compared with ApoE/LDLr KO mice, there was no difference in cTnT content: 314 (295-322) vs 340 (308-356) µg/g, P=0.10.

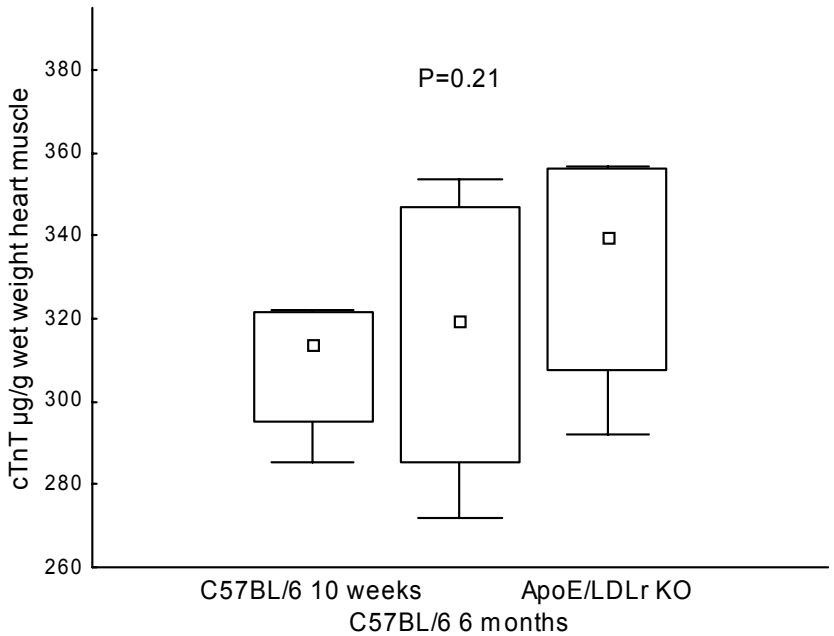

Figure 10. cTnT content in heart muscle from 10-week-old wild-type mice (C57BL/6J 10 weeks) (n=7), 6-month-old wild-type mice (C57BL/6J 6 months) (n=4) and apolipoprotein E and low density lipoprotein receptor double knockout mice (ApoE/LDLr KO) (n=7). P-value was calculated with the Kruskall-Wallis test. Mid point, median; box, interquartile range; whisker, range.

Since the ApoE/LDLr KO mice have about 1.7 times higher heart weight than C57BL/6J mice *(123)*, comparison between these groups in isolated heart models may be difficult. One way to estimate the myocardial injury would be to calculate the release of cTnT in coronary effluent per gram wet weight heart, and thereby compensate for differences in heart size. The rational for doing so is based on the assumption that the cTnT content of the hearts is equal. In the present study, using the third generation cTnT assay, we showed that this is the case. In other studies we have proved the usefulness of calculating the cTnT release per heart weight to evaluate myocardial damage in studies comparing C57BL/6J and ApoE/LDLr KO mice in

isolated heart models *(218, 219)*.

The cTnT content per wet weight skeletal muscle from C57BL/6J 10 weeks (n=4), C57BL/6 6 months (n=4) and ApoE/LDLr KO mice (n=7) were 0.17 (0.08-0.22) µg/g, 0.20 (0.10-0.22) µg/g and 0.29 (0.03-0.43) µg/g, respectively, with no differences between groups (P=0.40). The median content of cTnT in skeletal muscle was 0.05% of the median content of cTnT in heart muscle in C57BL/6J 10 weeks, 0.06% in C57BL/6J 6 months, and 0.08% in ApoE/LDLr KO mice. The data suggest that there are only insignificant amounts of cTnT in skeletal muscle of mice, which are in agreement with findings in other species *(225)*. Further, western blot analysis revealed no evidence of cTnT expression in skeletal muscle samples of adult mdx mice, an animal model for Duchenne muscular dystrophy *(226)*. Thus, circulating cTnT in mice is most likely of cardiac origin.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

General conclusions
Elevated serum cTnT is associated with inflammation and poor outcome in ESRD. Circulating cTnT may reflect LVH and/or subclinical myocardial cell damage in dialysis patients, which suggests that cTnT in serum from these patients, originates from the heart. Serum cTnT is frequently elevated not only in PD and HD patient, but also in patients with severe chronic renal failure close to the start of dialysis treatment. Elevated cTnT levels are associated to the uraemic syndrome and not to the dialysis treatment per se.

In the rat Langendorff preparation cTnT concentration in coronary effluent is complementary to haemodynamics in the evaluation of cardiac damage. Albumin should be added to coronary effluent to prevent cTnT loss and to increase precision of the assay. Hearts of C57BL/6J and ApoE/LDLr KO mice have similar cTnT content. Elevated baseline levels of serum cTnT in mice are not caused by troponin T from skeletal muscle, making cTnT a specific marker of cardiac damage also in this species.

Specific conclusions
Clinical studies

- Serum cTnT is frequently elevated in patients with severe chronic renal failure without symptoms of acute myocardial ischaemia.
- There is an association between serum cTnT and LVH and between cTnT and IHD in dialysis patients without acute myocardial infarction.
- Serum cTnT correlates with plasma big ET-1 in HD patients.
- There is a significant relationship between poor long-term outcome and elevated serum concentrations of cTnT and CRP in a single blood sample from PD patients.
- An elevated concentration of cTnT $\geq$0.10 µg/L in ESRD patients close to the start of dialysis predicts outcome during dialysis treatment.
- cTnT correlates with IL-6, fibrinogen and CRP in severe chronic renal failure.
- Elevated cTnT levels seem to be related to the uraemic syndrome rather than the dialysis treatment per se.

Practical implications: cTnT is one of the most powerful risk markers in ESRD. Patients with chronic renal failure and elevated cTnT should be examined for cardiac disease. We suggest that cTnT is measured before the start of chronic dialysis treatment and then 1-2 times per year thereafter to obtain individual baseline levels.

Experimental studies

- After 30 minutes of global ischaemia in the isolated rat heart model, there is an early short-lasting peak of cTnT, followed by a gradual increases of cTnT release with a maximum after 20 minutes of reperfusion.

- Coronary effluent cTnT after 20 minutes of reperfusion is complementary to haemodynamics in the evaluation of cardiac damage in rat Langendorff preparation.

- Albumin should be added to coronary effluent to prevent cTnT loss and to increase precision of the assay.

- Although ApoE/LDLr KO mice are larger and have higher heart weights than C57BL/6J mice, the cTnT content per wet weight heart is similar.

- Serum cTnT in samples from the jugular vein of mice is only slightly elevated.

- Elevated baseline levels of cTnT in mice are not caused by skeletal muscle troponin T.

Practical implications: cTnT is a useful cardiac marker during early reperfusion in the isolated rat and mouse heart. Correct preanalytical sample handling is crucial. Measurement of effluent cTnT may be a convenient alternative to time consuming chemical staining methods for the estimation of cardiac damage.

Future perspectives

Future studies are needed to elucidate the specific pathogenic mechanisms causing elevated cTnT in chronic renal failure. Studies on the impact of potential therapeutic interventions on cTnT levels and mortality and morbidity in ESRD should be conducted. The cTnT immunoassay can be used in experimental studies of myocardial injury and necrosis caused by ischaemia. cTnT is probably useful in other models of myocardial damage and may be a useful tool in studies on mechanisms behind the development of troponin elevation and cardiac disease in renal failure and other disease states.

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