Discussion

Cardiovascular disease (CVD) is the most common cause of mortality and morbidity in the Western world. Despite major progress with regard to therapy of CVD over the last 50 years, there is still much to learn about the progression of CVD, about the mechanisms of acute coronary syndromes, about the pathophysiology of unstable angina pectoris, and about the mechanisms of congestive heart failure (CHF). For practical reasons there is a need for novel assays to diagnose the various representations of CVD.

The present thesis describes the release kinetics of several cardiac proteins after reversible or irreversible myocardial damage and their role as cardiac biomarker. Topics studied in cell cultures and animals with cardiac overload were also investigated in clinical studies to translate results of basic research to diagnosis and therapy of human heart disease.

Troponin as a cardiac biomarker

In our experiments we used cultures of neonatal rat cardiomyocytes to characterize the release kinetics of troponin from cardiomyocytes undergoing reversible or irreversible cell injury. One of the advantages of this \textit{in vitro} model is the absence of protein clearance from this system. On the one hand we have demonstrated that the release of intact and degraded troponins from energy-depleted cardiomyocytes (due to severe or mild metabolic inhibition) did not occur before necrosis sets in, and was associated exclusively with extensive necrotic cardiomyocyte death. On the other hand we have demonstrated that troponin may be released from integrin-stimulated cardiomyocytes \textit{in vitro} in the absence of necrosis. The release mechanisms most likely differ between irreversibly damaged cardiomyocytes and integrin-stimulated cardiomyocytes.

Release of cardiac troponins from irreversibly damaged cardiomyocytes is a phenomenon that is known for more than 15 years. Due to a high cardiac sensitivity and specificity, cardiac troponins are nowadays the most frequently used biomarkers to diagnose acute myocardial infarction (AMI)\textsuperscript{1,2}. In serum of patients with AMI, cardiac troponins are detected as intact protein and as degradation products, and the release pattern of these degradation products changes in the days following onset of AMI\textsuperscript{3-6}. 
Similar results were found in our *in vitro* experiments. Both cardiac troponin I (cTnI) and cardiac troponin T (cTnT) were released from necrotic cardiomyocytes as an intact protein and as degradation products. However, the release of troponin degradation products has serious consequences for the detection of troponins by immunoassay. We found that the total quantity of both intact cTnI and cTnT per culture (cells+medium) at any time point was reduced compared with the total quantity at t=0 (100%), indicating a loss of intact cTnI and cTnT during metabolic inhibition. As protein clearance is absent in this closed system, post-translational modifications of troponin such as degradation, but possibly also phosphorylation, nitrosylation and oxidation may be responsible for this reduction of total troponin content per culture during metabolic inhibition. Post-translational modifications may result in impaired binding of the antibodies used in the immunoassays, leading to a reduced detection of intact troponin. By western blotting, using several antibodies, we observed a number of troponin degradation products that remained undetected by the immunoassay, and which may partially explain the reduction of total troponin content of the cultures during metabolic inhibition. However, even when using multiple antibodies at the same time, the troponin fragments observed did not completely clarify the reduction of total troponin content of the cultures during metabolic inhibition, indicating that the antibodies used do not detect all troponin degradation products formed during metabolic inhibition. Therefore, the results described in chapter 3 suggest that the detection of cardiac troponins depends on the immunoreactivity of the currently available antibodies for the troponin degradation products. Any undetected troponin degradation product in serum of patients with AMI may influence the result of the diagnostic assay. Therefore, we need more information about the troponin degradation products released by the myocardium and present in the circulation. More insight in troponin degradation would tell us whether we underestimate the extent of irreversible cell damage after AMI.

In a significant subset of patients with unstable angina pectoris (UAP), transient ischemia may lead to minor elevation of serum troponin. In our *in vitro* experiments we demonstrated that the release of troponins from energy-depleted cardiomyocytes (induced by mild or severe metabolic inhibition) was associated with necrotic cardiomyocyte death. These *in vitro* results support clinical studies suggesting that
elevated troponin levels in patients with UAP may reflect minor amounts of myocardial necrosis. These minor amounts of myocardial necrosis may explain why the presence of elevated troponin levels in patients with UAP has been associated with a substantial higher occurrence of major cardiac events over time\(^7,8\).

Besides release of troponin from irreversibly damaged cardiomyocytes, cTnI is released from viable cardiomyocytes \textit{in vitro} after stimulation of stretch-responsive integrins. cTnI from viable cardiomyocytes was released as an intact protein. Thus at least one difference with respect to the release mechanism of troponin is the absence of troponin degradation when studying integrin stimulated, viable cardiomyocytes and the presence of troponin degradation when studying necrotic cardiomyocytes during energy deprivation. As to the release of intact troponin from viable cardiomyocytes upon integrin stimulation, we favor the explanation that unbound intact cTnI is released from the cytosolic pool into the extracellular medium. In addition to the classical pathway for protein release involving the endoplasmatic reticulum and Golgi apparatus, there are other non-classical or alternative pathways\(^9\). Several studies have suggested that mechanically induced transient disruptions (“wounding”) of the sarcolemma is a constitutive event \textit{in vitro}\(^10\textsuperscript{-13}\). This mechanism is responsible for the release of proteins such as fibroblast growth factor-1 and -2, which are released despite the lack of a classic signal peptide sequence normally associated with exocytotic secretion\(^10\textsuperscript{;11}\). Transient membrane disruptions are repaired by a rapid resealing mechanism that requires extracellular Ca\(^{2+}\). Vesicle delivery, docking and fusion, similar to the exocytosis of neurotransmitters, may be involved in this resealing mechanism\(^14\). This mechanism delivers intracellular membrane to the site of plasma disruptions, where it is added via excocytosis\(^15\). Whether the leakage of free cytosolic troponin induced by integrin stimulation is identical to the release of fibroblast growth factor-1 and -2 via a transient increase in sarcolemmal permeability is not clear yet. If a transient increase in membrane permeability is responsible for the leakage of free cytosolic troponin, why is troponin release not accompanied by the release of lactate dehydrogenase (LDH) that is also a cytosolic enzyme? Therefore further investigation is needed to study this release mechanism of troponin. Cyclic stretch of cultured cardiomyocytes in a computerized...
stretch device\textsuperscript{16-18} can be used as a model to study the release of troponin from cardiomyocytes upon mechanical stretch. Transient increase of sarcolemmal permeability can be studied by uptake of exogenous fluorescence-labeled dextrans by stretched cardiomyocytes.

An alternative release mechanism that may be involved and that should be investigated is the release of troponin via secreted vesicles, or exosomes\textsuperscript{9}. Mild stress (brief hypoxia) in adult cardiac myocytes \textit{in vitro} has been demonstrated to triple the release of Heat Shock Protein 60 (HSP60) via exosomes into medium, in the absence of necrosis (no release of LDH)\textsuperscript{19}. The function of exosomes remains unknown, but these vesicles may have a significant role in intracellular signaling and are important mediators of the stress-induced release of proteins such as HSPs via a non-classical release mechanism\textsuperscript{20}. Isolated exosomes from integrin-stimulated, stretched and control cardiomyocytes can be studied for the presence of troponin by western blotting and inhibition of exosome formation by dimethyl amiloride can be used to study whether exosomes are involved in the release of troponin.

The experimental finding that troponin can be released from viable cardiomyocytes by a stretch-related mechanism in the absence of necrosis (chapter 4) is supported by a patient study (chapter 5). CHF patients with elevated troponin levels had higher serum NT-proBNP levels than CHF patients without elevated cTnT levels, whereas serum LDH levels did not differ between the two patient groups. This study indicates that patients with CHF who had elevated cTnT levels had myocardial cTnT release probably due to myocardial stretch associated with cardiac overload, instead of cTnT release due to necrosis. Elevated serum troponin levels in patients with CHF associated with elevated serum levels of NT-proBNP or BNP have been described in several clinical studies\textsuperscript{21-25}. However, many of these studies interpreted elevated troponin levels as an indication of myocardial injury. In contrast, we demonstrated that elevated troponin levels in patients with CHF were not explained by myocardial necrosis. We found a 15-fold increase of serum cTnT/LDH in CHF patients with elevated cTnT levels compared to cTnT/LDH ratio in CHF patients without serum cTnT elevations, indicating a preferential release of cTnT in the absence of LDH release. In patients with AMI serum LDH levels were strongly
correlated with serum cTnT levels, whereas this correlation was not observed in CHF patients with elevated cTnT levels.

The release of cardiac troponin in response to myocardial stretch in the absence of irreversible myocardial injury was also supported by a study of Neumayr et al.\textsuperscript{26} with healthy marathon cyclists. In all subjects, serum NT-pro-BNP levels increased immediately after the race, decreased again on the following day, and returned to baseline values within a week. Serum cTnT levels were negative in all subjects before the race and increased transiently immediately after the race in 45% of the athletes with levels ranging from 0.043 to 0.224 µg/L. One day after competition, cTnT had normalized in all athletes. NT-pro-BNP is considered to be the adequate volume regulatory response of a hemodynamically stressed heart to prolonged strenuous exercise. The observed kinetics of cTnT release substantiate a release from the free cytoplasmic pool due to the half-life of cytosolic cTnT. They concluded that in healthy cyclists, transient increases of NT-pro-BNP and cTnT are more likely to reflect cardiac fatigue than injury. Neilan et al.\textsuperscript{27} have demonstrated that increased serum levels of cTnT and NT-proBNP in marathon runners immediately after the race were inversely related with training mileage in the weeks before the marathon.

The release of troponin by a stretch-related mechanism may explain why in several pathological conditions plasma troponin levels are elevated in patients in whom myocardial necrosis is not a prominent aspect\textsuperscript{28-32} (Table). Nevertheless, this finding has some clinical consequences. Nowadays cardiac troponin is used as a biomarker of irreversible cell death. Elevated troponin levels in serum may not only reflect irreversible myocardial injury upon ischemia but may also reflect myocardial overload as a result of myocardial strain. Although serum troponin levels after irreversible myocardial damage are generally higher compared to the serum levels of troponin after myocardial stretch (Table, chapter 5), these results make the standpoint of the joint Committee highly disputable\textsuperscript{33}. This standpoint that the presence of a serum troponin concentration exceeding the 95\textsuperscript{th} or 99\textsuperscript{th} percentile of the reference distribution is considered to confirm the diagnosis “myocardial infarction”, even in case of microscopic zones of myocardial necrosis, cannot be correct. Both experimental and clinical studies in this thesis indicate
that troponin is not only a biomarker of necrosis but may also act as a biomarker of mechanical strain in the absence of necrotic cell death.

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<th>Table. Elevated troponin levels in ischemic cardiac diseases, non-ischemic cardiac diseases, and non-cardiac diseases</th>
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Abbreviations: RV, right ventricle; LV, left ventricle; AMI, acute myocardial infarction; UAP, unstable angina pectoris; CK, creatine kinase; Nt-proBNP, amino-terminal propeptide of brain natriuretic peptide.
Matrix Metalloproteinase as a cardiac marker
Matrix metalloproteinase (MMP) activities within the myocardium are strictly regulated at three levels being (i) transcription, (ii) activation and (iii) inhibition/deactivation, indicating a complex and dynamic system. MMPs are synthesized as a proform within several cell types, such as fibroblasts and cardiomyocytes, and can be secreted into the myocardial interstitium and finally into the circulation. ProMMPs can be activated intracellularly or extracellularly and are inhibited in the myocardial interstitium and/or in the circulation by tissue inhibitors of metalloproteinases (TIMPs)\(^48\). Therefore evaluation of circulating MMP levels as a marker of remodeling has some limitations. In animals and patients with left ventricular dilatation and CHF, enhanced expression and increased activation of MMPs have been identified\(^49:50\). In addition, Spinale et al.\(^51\) have demonstrated that MMP activity directly contributes to ventricular remodeling in CHF, since administration of an inhibitor of MMP activity during the development of CHF resulted in limited left ventricular dilatation and reduced wall strain. Other studies have demonstrated that circulating levels of MMPs in patients with CHF are positively correlated with left ventricular volumes and negatively correlated with left ventricular ejection fraction\(^52:53\). Taken together, these studies indicate that circulating MMP levels in patients with CHF may reflect the actions of MMP within the myocardium.

In this thesis we quantified MMP9 and/or MMP2 levels in (1) cultured cardiomyocytes after stimulation of stretch-responsive integrins, (2) monocrotaline-treated animals with adverse right ventricular remodeling and, (3) CHF patients with reverse left ventricular remodeling during cardiac resynchronization therapy (CRT). Stimulation of stretch-responsive integrins of cultured cardiomyocytes resulted in an increased activity of intracellular proMMP2 and active MMP2. Integrin stimulation was also associated with an increased release of active MMP2, whereas the release of proMMP2 was unchanged compared to control cardiomyocytes. These results indicate an important role for integrins in the regulation of MMP2 activity, which has also been described previously\(^54-56\).

In monocrotaline-treated rats with adverse right ventricular remodeling, myocardial proMMP2 levels (representing the sum of intracellular and interstitial proMMP2) were reduced, and plasma proMMP2 levels were unchanged. Further research should
elucidate whether myocardial proMMP2 is downregulated or converted to active MMP2. Although stimulation of stretch-responsive integrins in cardiomyocytes in vitro leads to increased levels of intracellular proMMP2, monocrotaline-induced adverse right ventricular remodeling resulted in decreased levels of myocardial proMMP2. An explanation of these opposite results is lacking, but could be found in the acute, adaptive response of integrin-stimulated cardiomyocytes and the chronic, failing state of the rats with monocrotaline-induced pulmonary hypertension.

Myocardial proMMP9 levels in monocrotaline-treated rats were increased, indicating a differential expression of myocardial proMMP2 and proMMP9 in rats with adverse right ventricular remodeling. We also observed a difference with regard to CRT-induced changes between plasma MMP2 and MMP9 activity in CHF patients. Plasma MMP9 activity in patients with reverse left ventricular remodeling was significantly reduced by CRT, whereas plasma MMP2 activity was unchanged. This finding has also been reported by Nishikawa et al. who showed that MMP9 rather than MMP2 plays an important role in ventricular dilatation.

In conclusion, the studies described in this thesis demonstrate that plasma levels of MMP2 are not useful as a marker of ventricular dilatation, because plasma MMP2 levels in animals with adverse ventricular remodeling and patients with reverse ventricular remodeling did not change upon treatment or therapy. However, reverse ventricular remodeling is associated with a reduction in plasma MMP9 levels, indicating that plasma MMP9 can be used as a marker of reverse ventricular remodeling which is useful to evaluate the response of CHF patients to CRT. Whether increased expression of myocardial proMMP9 in animals with adverse remodeling is also associated with increased plasma MMP9 levels, remains to be elucidated.

**Tenascin-C as a cardiac marker**

The pathological expression pattern of tenascin C (TNC) in myocardial tissue suggests that TNC may be a useful marker of myocardial disease activity. Previous studies have shown that serum TNC levels can be used as a marker of ventricular remodeling in patients after AMI and in patients with dilated cardiomyopathy. This thesis describes the role of TNC as a marker of adverse right ventricular remodeling in animals with heart
failure, and as a marker of reverse left ventricular remodeling in patients with CHF during CRT.

In rats with monocrotaline-induced pulmonary hypertension, upregulated TNC gene expression resulted in re-expression of TNC protein levels in myocardium and elevated plasma TNC levels. Right ventricular volumes and right ventricular ejection fraction correlated with TNC plasma levels, suggesting that plasma TNC levels reflect myocardial levels of TNC and that plasma TNC levels can be used as a marker of ventricular dilatation. However, TNC is not a cardiac-specific extracellular matrix protein and may also be expressed in other organs. We demonstrated the presence of three TNC isoforms (250, 210 and 80 kDa) in heart tissue of animals with right ventricular failure.

The mechanism by which TNC contributes to cardiomyocyte slippage and ventricular dilatation, leading to an impairment of cardiac pump function, is complex. We demonstrated that monocrotaline-induced right ventricular dilatation is associated with TNC re-expression, a significant downregulation of α6 integrin gene expression, and a tendency of downregulation of β1 integrin gene expression. Downregulation of integrin α6 gene expression in failing myocardium may lead to a reduction of α6 integrin protein expression and a reduction of functional costameric adhesion complexes, resulting in weakened binding of cardiomyocytes to extracellular matrix. Whether this downregulation of α6 integrin is due to an altered extracellular matrix composition or whether it is the direct interaction with TNC remains to be elucidated.

In CHF patients, CRT leads to reverse left ventricular remodeling in the majority of patients. In this thesis we demonstrated that reverse left ventricular remodeling was associated with a significant decrease in serum TNC levels. Non-responders to CRT have no reverse left ventricular remodeling, and in those patients plasma TNC levels did not change. This study indicates that serum TNC levels can be used as a marker of reverse left ventricular remodeling to evaluate the response to CRT in patients with CHF. The patient group in this study is relatively small and we did not detect a significant difference in serum TNC levels between responders or non-responders at baseline, nor at 6 months follow up. Although baseline serum TNC levels in patients with CHF can not predict the response to CRT, serial serum TNC levels can be used as a
marker of reverse left ventricular remodeling and to evaluate the response to CRT after 6 months.

Concluding remarks
The studies described in this thesis demonstrate the relevance of cardiac troponins, MMPs and TNC as cardiac markers of reversible and/or irreversible myocardial injury. Elevated serum troponin levels in patients are not only the result of troponin release from irreversibly damaged myocardium, but may also occur from viable or reversibly damaged myocardium as a response to myocardial strain. Plasma MMP9 levels rather than plasma MMP2 levels are a potential marker of ventricular dilatation in heart failure, but due to the complex regulation of MMPs further research is needed to strengthen this conclusion. Re-expression of myocardial TNC during the development of heart failure may contribute to cardiomyocyte slippage and ventricular dilatation. As serum TNC levels in patients with CHF declined during CRT, serum TNC levels can be used as a marker of reverse left ventricular remodeling.

References


General discussion


