Stratification of heart failure using biomarkers of myocardial fibrosis

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List of abbreviations

ACE	angiotensin converting enzyme
ADAMTS	a disintegrin and metalloproteinase with thrombospondin type I repeats
AMI	acute myocardial infarction
ATII	angiotensin II
BMI	body mass index
C	carboxy
CHF	congestive heart failure
CRT	cardiac resynchronisation therapy
CVF	collagen volume fraction
DCM	dilated cardiomyopathy
ECM	extracellular matrix
HCM	hypertrophic cardiomyopathy
HF	heart failure
ICD	implantable cardioverter defibrillator
ICTP	collagen type I carboxy-terminal telopeptide
IGF-1	insulin-like growth factor-1
LV	left ventricle
LVAD	left ventricular assist device
MI	myocardial infarction
MMP	matrix metalloproteinase
N	amino
NYHA	New York Heart Association
PICP	procollagen type I carboxy-terminal propeptide
PINP	procollagen type I amino-terminal propeptide
PIIICP	procollagen type III carboxy-terminal propeptide
PIIINP	procollagen type III amino-terminal propeptide
RAAS	renin-angiotensin-aldosterone system
RALES	Randomized Aldoctone Evaluation Study
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloproteinase
WHO/ISFC	World Health Organisation/ International Society and Federation of Cardiology

Summary

The shape and size of the heart is provided by the collagen network. Next to the cardiac geometry, the network plays an essential role in the tensile strength and the contractility of the myocardium. Two main fibrillar types of collagen are observed in the myocardium, collagen type I and type III. Both types are synthesised by fibroblasts. Prior to fibril formation, two propeptides of the collagen proteins have to be cleaved, the amino-terminal and carboxy-terminal propeptide. After cleavage, the collagen proteins can form a collagen fibre network in the myocardium. Degradation of collagen is provided by proteases called matrix metalloproteinases (MMPs). The activity of these MMPs can be inhibited by tissue inhibitors of metalloproteinases (TIMPs).
Alteration of the collagen turnover is a main characteristic of cardiac remodelling. Cardiac remodelling is a process involved in the onset and the progression of heart failure (HF). In HF, accumulation of collagen in the myocardium is often observed, called cardiac fibrosis. Cardiac fibrosis is responsible for an increased stiffness of the myocardium, loss of communication between myocytes during contraction, and alteration of the electrical activity of the heart. Finally, this can result in an increased risk in arrhythmias, a progression in HF, and in sudden cardiac death.
To improve diagnosis and prognosis of heart failure and the individual treatment of HF patients, it is essential to gain more knowledge about cardiac remodelling. With respect to the patient, but also to the clinics, it is required to use non-invasive, cheap, and easy methods in the clinics to acquire more knowledge about the diseased heart. Biomarkers should be an excellent tool for this acquirement. The aim of this thesis is to investigate which biomarkers, related to cardiac fibrosis, are currently known.
Several collagen synthesis and breakdown markers might be useful as a biomarker. To investigate the synthesis of collagen, the propeptides of both collagen type I and III, the amino-terminal propeptides PINP and PIIINP respectively, and the carboxy-terminal propeptide of collagen I, PICP, can be determined. To map out the breakdown of collagen, MMPs and TIMPs can be used as a biomarker. Also the carboxy-telopeptide of collagen I fibrils (ICTP), a peptide that is released in the blood during collagen I degradation, might be useful as a biomarker of cardiac remodelling.
In several studies is shown that in HF patients PIIINP and PICP levels, but also ICTP levels, are elevated compared to healthy patients. Studies investigating PINP levels in HF patients show either inconsistent results or the PINP levels are not altered in these patients. Unfortunately, it is thought that the amino-terminal propeptides are not always cleaved from the collagen fibrils. Therefore, PINP and PIIINP might be less reliable biomarkers. Whether the different MMP levels are changed in HF, is still contradictory. However, TIMP-1 is often shown to be increased in HF patients.
To use biomarkers of myocardial fibrosis in clinics, the biomarker level should reflect the amount of that collagen type in the myocardium. For a few biomarkers this correlation is shown, PIIINP seems to correlate with both collagen I and III in the myocardium, ICTP seems to correlate with myocardial collagen I, and PICP is thought to correlate with the collagen volume fraction in the myocardium. Unfortunately, this is based on only a few studies and not all biomarkers have yet been correlated to the myocardial collagen.
When biomarker levels in HF patients are different from healthy subjects, biomarkers may also have a predictive or prognostic effect. Based on current literature, PIIINP and ICTP seem to have a prognostic value; high levels of these biomarkers predict cardiac events or a worse outcome. However, until now, investigators determined the predictive effect of the biomarkers only in a retrospective way. To use these biomarkers in clinics, the exact cut off value for each biomarker has to be determined, which is applicable to various patients. Before reaching this level, more research is definitely required.

In conclusion, PICP, ICTP, PIIINP, and TIMP-1 seem to be very promising biomarkers as a diagnostic, prognostic, or predictive tool. However, more investigation is demanded to gain more knowledge in the specificity and sensitivity of these biomarkers, the cut off values of these biomarkers, and the correlation between these biomarkers and the amount of different collagen types in the myocardium. The influence of patient characteristics such as underlying disorders, stage of HF, BMI, age, and medication on the biomarker levels also have to be investigated more extensively.

Introduction

Cardiac remodelling in heart failure

Congestive heart failure (CHF) is a chronic and progressive condition of weak cardiac muscles. This condition results in a heart that is unable to maintain sufficient cardiac output to supply the body with oxygen. Next to this hemodynamic disorder, abnormalities of many other organs are involved in CHF, e.g. the lungs, kidneys, and the liver[1]. In 1964, the New York Heart Association (NYHA) defined stages of heart failure (HF) patients, called the NYHA functional classification. Since 1964, this classification has been revised several times, most recently in 1994[2]. The classification is as follows: class I) patients who have no symptoms and have no limitations of ordinary activities; class II) patients who have no symptoms in rest or in mild exercise, but have a slight and mild limitation of activity; class III) patients who have a marked limitation of activity, but no symptoms at rest; and class IV) patients who have symptoms already at rest and who are restricted to bed or chair. This classification is still widely used in clinical practice[2].

HF is not a disease itself, but it is the common end-stage condition of various disorders that have already damaged the heart. Prior to HF, other pathologies, such as hypertension, myocardial infarction (MI), genetic disorders, and idiopathic cardiomyopathies are present. In the onset of cardiac diseases, the body will try to compensate the dysfunction of the heart by increasing heart rate, contractility, circulating volume, and the development of hypertrophy. Although initially compensatory, these mechanisms will ultimately lead to increased cardiac workload and decompensation. A decreased cardiac output will be the consequence of this decompensation, hence the heart will fail: heart failure[1].

In this thesis, only the disorders hypertrophic and dilated cardiomyopathy and HF will be discussed. By the task force of the World Health Organisation and the International Society and Federation of Cardiology (WHO/ISFC), cardiomyopathies are defined as heart muscle diseases of an unknown cause. Dilated cardiomyopathy (DCM) is the most common form of cardiomyopathy[3]. In this disease mainly the left ventricle (LV), but sometimes also the right one, is dilated and the systolic ventricular function is disturbed. Hypertrophic cardiomyopathy (HCM) is characterised by a disproportionate hypertrophy of the ventricles, particularly the LV. The volume of the left ventricle in HCM is normal or reduced[4]. In HF, systolic and diastolic HF can be distinguished, however, often both contractility and filling of the heart are impaired[1].

The underlying mechanism of the impaired pump performance in HF is based on cardiac remodelling[5]. Cardiac remodelling is a predominant characteristic of HF; the diseased heart will try to compensate by remodelling of the tissue. This remodelling is accompanied by an increase in cardiac mass and a change in the shape of the heart. On cellular level, hypertrophy of both myocytes and non-muscular cells and hyperplasia of fibroblasts and endothelial cells will occur. In addition, also myocyte loss and cellular damage is observed in cardiac remodelling, especially after MI[5]. The process of remodelling is initiated by mechanical stress, ischemia, and certain hormones[5]. Essential in the cardiac remodelling is the change in the turnover of the interstitial or extracellular matrix (ECM). This turnover will be discussed in the sections below.

Extracellular matrix in the heart

The heart consists of muscular, vascular, and connective tissue compartments[6]. The contractile function of the heart is provided by cardiac myocytes[7]. However, next to the myocytes, also the ECM plays an important role in the contractility of the heart. Next to

the regulation of the contractility, the ECM regulates several cellular processes such as migration, differentiation, proliferation, and survival of cardiac cells[7].
The ECM, or interstitium, consists of interstitial fluid and several proteins that are produced by fibroblasts, such as proteoglycans and collagen. Collagen is the main part of the cardiac ECM. Collagen maintains the shape and the size of the heart, but also provides cardiac tensile strength[6, 8]; it actively transduces the force during systole and it is the main determinant of the diastolic stiffness[5]. The collagen network in the heart consists of three components: the epimysium, the perimysium, and the endomysium (figure 1). The epimysium consists of collagen fibres surrounding the epi- and endocardium. The perimysium, an extension of the epimysium, is situated between muscle bundles and between myocytes to form myofibres. The perimysium is important in sustaining the tension between different muscle bundles. The endomysium and endomysial weaves connect cells with each other. The endomysial fibres are thought to be connected with the cytoskeleton of the myocytes[6].

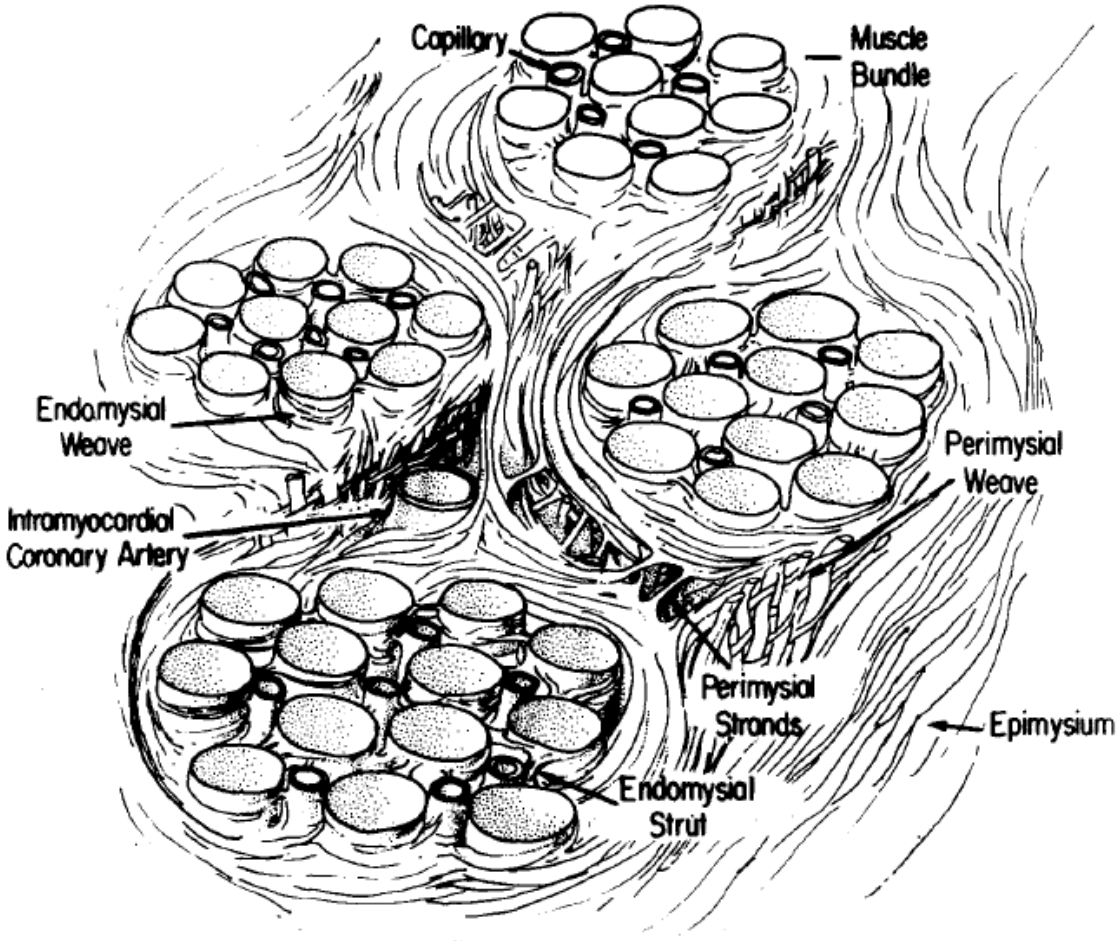

Figure 1. Schematic overview of the three components of the cardiac interstitial collagen network: the epimysium, the perimysium, and the endomysium. Figure adapted from Eghbali and Weber, 1990.

Collagen synthesis

Collagen can be divided in several classes: fibrillar, fibril associated, network forming, filamentous, short chain, and long chain collagen. At least 18 types of collagen are known until now[8]. Collagen type I, a fibrillar collagen type, is most abundantly expressed in adult human connective tissue. All knowledge about fibrillar collagen is mainly obtained from studies of this collagen type[9]. The cardiac ECM is predominantly composed of collagen type I. Next to type I, collagen type III, another collagen type belonging to the fibrillar class, is also abundantly present in the cardiac ECM. Several studies indicate that about 85% of the cardiac collagen consists of type I and 11% represents collagen III[10, 11]. Furthermore, small amounts of type IV and V are observed in the basement membrane of the myocytes, perivascular, and in the pericellular space[6]. Although collagen I and III both belong to the fibrillar type, they have different structures. Collagen I forms thicker fibres than type III and has a high degree of cross-linking between the fibres. This type is primarily responsible for the tensile strength of the heart. Collagen III fibres have a relatively small diameter, provide elasticity in the myocardium, and these fibres create a network providing the structural integrity of the collagen network and of the heart[12, 13].
Collagen I and III are synthesised by cardiac fibroblasts; only in these cardiac cells the mRNA of collagen type I and III is observed[14]. The fibrillar collagen is synthesised as a preprocollagen; a pro-α-collagen chain. In the endoplasmic reticulum, three pro-α-chains form a triple helix structure. This triple helix is named procollagen[15, 16]. All fibrillar procollagen types contain initially two propeptides: the amino (N)-propeptide and the carboxy (C)-propeptide (figure 2). Once the procollagen is localised to the ECM, these propeptides are cleaved by proteinases in a 1:1:1 stoichiometry[17]. Cleavage of the C-propeptides is prerequisite for the formation of I and III collagen fibres. However, at least the N-terminal domain of procollagen type III (PIIINP), but probably also the N-propeptide of procollagen type I (PINP), is not always removed completely. Moreover, fibril formation will still occur, together with incorporation of these propeptides[18].

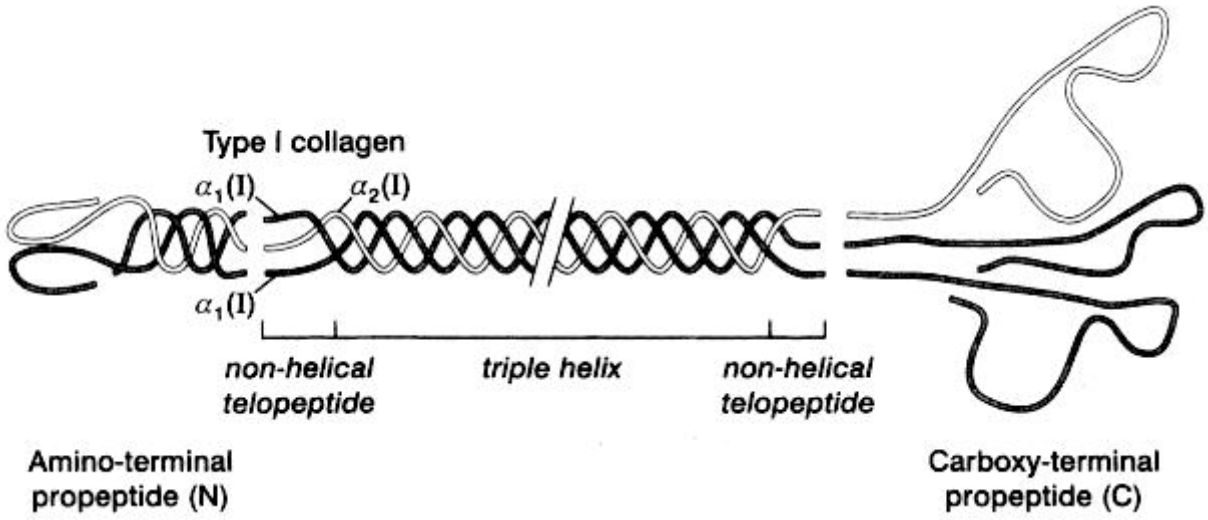

Figure 2. Collagen type I together with propeptides of the N- and C-terminal. Figure adapted from Weber, 1997.

After cleavage of the propeptides, the triple helix chain will form large collagen fibrils together with other collagen chains. The removal of the C-terminal propeptide from collagen I (PICP) and III (PIIICP) is accomplished by procollagen C-proteinases. The N-

terminal propeptide is cleaved by members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin type I repeats) family. *In vitro*, the N-terminal propeptide of collagen I (PINP) and III (PIIINP) is removed by ADAMTS-2, -3, and -14[19]. After cleavage of the propeptides, the propeptides are released into the blood, either directly or via the lymphatics[17]. Finally, they are degraded by the liver. Elimination of PICP occurs via endocytosis mediated by the mannose-receptor, while PINP and PIIINP are removed via scavenger receptors[17, 18]. However, elimination of the propeptides might also occur via the kidneys and via urine[17].

Collagen breakdown

Compared to other proteins, collagens have a relatively long half life, about 90–120 days. Breakdown of the ECM occurs with certain proteases, amongst which matrix metalloproteinases (MMPs)[8]. Figure 3 shows a schematic overview of the collagen turnover. MMPs are zinc-dependent enzymes[20] and can be synthesised by fibroblasts but also by other cells, such as leukocytes[21]. At current, the MMP family contains more than 20 different MMPs[22] with molecular masses ranging from 28-92 kDa[23]. MMPs can be divided into two types, membrane bound MMPs and secreted MMPs[20]. In the myocardium, different MMPs are expressed. MMP-1, -8, and -13, also named collagenases, are highly specific for collagen I and III and cleave the collagen into two fragments[24]. These fragments will denature at physiological conditions. The denatured collagen fragments can be further digested by MMPs named gelatinases, particularly MMP-2, but also MMP-9[25, 26]. Although the level of activity of MMP-9 is much lower, MMP-9 may influence the collagen degradation by inducing profibrotic pathways; MMP-9 is able to enhance the activity of profibrotic proteins such as transforming growth factor-β (TGF-β) and herewith increase the accumulation of the ECM[26]. MMP-2 can also degrade basement membrane proteins and newly synthesised collagen fibres[26]. Beside these collagenases and gelatinases, also MMP-3 (stromelysin) and MMP-7 are expressed in the human myocardium. Stromelysin is able to cleave basement membranes, proteoglycans, and elastin. MMP-7 has many substrates, e.g. collagen fibrils, proteoglycans, and fibronectin, since this MMP lacks specific substrate recognition sites[27].

Secreted MMPs are expressed as proMMPs. These proMMPs first have to be activated to get their catalytic activity. The proMMPs can be activated either by enzymatic cleavage of the prodomain of the MMP by proteases and by other MMPs, or by chemical modification of the proMMP[25]. The secreted proMMPs in the ECM may dock already on their ECM protein; once activated, a rapid proteolytic activity is provided[20, 25]. The exact breakdown and elimination of MMPs remains unknown until now; one assumes that the MMPs leak into the blood stream[28]. Another possible elimination pathway of the MMPs might be via the lymphatics[29].

MMPs can be regulated both at transcriptional and activation level. Mechanical stress, growth factors, and cytokines can regulate the transcription of MMPs via intracellular pathways[27]. Endogenous inhibitors named tissue inhibitors of metalloproteinases (TIMPs) can regulate the MMPs at activation level. To our knowledge, four types of TIMPs have been identified so far: TIMP-1-4[25]. TIMPs bind MMPs in a 1:1 stoichiometric way; they bind to the catalytic site of the MMPs, hereby preventing the enzymatic cleavage activity[25, 27]. All four types can inhibit every type of MMP, however, the efficiency differs[30]. Next to the natural inhibition of MMPs, TIMPs are thought to have other functions. For example, TIMPs have some effects on cardiac fibroblasts; they can increase cell proliferation, apoptosis, and collagen synthesis of the fibroblasts. Next to these effects, TIMPs can also stimulate the differentiation of fibroblasts into myofibroblasts, especially at the site of tissue injury[30]. In human myocardial tissue,

TIMP-1-4 are all expressed[30]. In HF, TIMP-1 is the most abundantly expressed type of TIMP[31].
The equilibrium between MMP and TIMP expression and activation plays an important role in multiple pathological processes, such as tumour metastasis, arthritis, but also in cardiac remodelling. Therefore, there is a growing interest for these proteins as possible therapeutic targets.

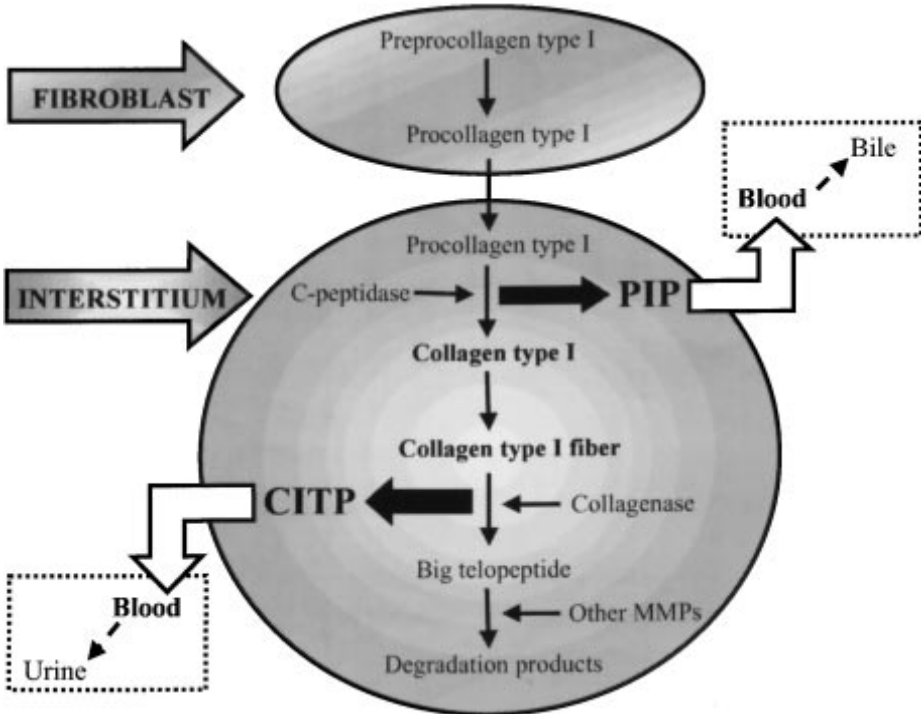

Figure 3. Schematic overview of the turnover of collagen type I. Figure adapted from López *et al*., 2001.

Fibrosis
Essential in cardiac remodelling is the turnover of the ECM, and herewith the accumulation of collagen in the myocardium. Accumulation of the collagen amount is called fibrosis. The main collagen type in cardiac fibrosis is type I, comparable to the healthy myocardium[5, 32, 33]. In several cardiac diseases the collagen type I/ type III ratio is increased. However, changes of this ratio and the consequences of these changes are not entirely known yet[13].
The formation of fibrosis plays a critical role in cardiac remodelling in cardiac diseases such as hypertension, HCM, heart failure, and after MI[5, 34]. The cardiac remodelling occurs mainly in the perimysial and endomysial components and leads to thickening of the collagen fibrils and fibres, an increase in the density of the endomysial weaves around the cardiomyocytes, and an increase in newly formed collagen fibres[35].
Fibrosis finally leads to dysfunction of the heart; elevated collagen content and increased cross-linking increases the myocardial stiffness and herewith diastolic dysfunction. This is called diastolic heart failure, which means that the filling of the ventricles during diastole is impaired[35, 36]. However, also in impaired systolic function (systolic heart failure), the collagen content of the heart is increased. This is mainly scar-related collagen deposition, but also perivascular fibrosis. In contrary, the mysial collagen network is decreased in systolic dysfunction[37]. Loss of the mysial collagen network might lead to an altered geometry, reduced coordination of the myocyte contraction, and

slippage of cardiomyocytes. Although the collagen network is decreased in systolic HF, the total amount of collagen is increased. This implies that the formation of scar-related collagen and perivascular collagen is more increased than the breakdown of the mysial collagen. Because reparative fibrosis, e.g. as a result of myocyte loss, and perivascular fibrosis are not major determinants of increased myocardial compliance and ventricular structure remodelling, systolic failure can still occur, despite the collagen increase[35].
Cardiac fibrosis also alters the electrical activation of the myocardium. Increased activation delay due to electrical load mismatch by patchy fibrosis[38, 39] and conduction slowing enhance the vulnerability to arrhythmias[40]. Boulaksil *et al*. (submitted) reported that excessive interstitial fibrosis may provide electrical heterogeneity and conduction blocks[41]. Thus, fibrosis largely increases the risk of arrhythmias[5, 38-40, 42], accompanied with an increased risk in progression in cardiac failure and sudden cardiac death[5, 38, 42]. This might imply that the amount of cardiac fibrosis is correlated with the severity of HF. Remarkably, in two other studies is claimed that the severity of heart failure, in terms of the NYHA classification, does not seem to be related to markers of collagen I and III[43, 44]. However, there is still scarce knowledge about the exact correlation between these markers and myocardial fibrosis (as discussed later on). Therefore, although the functional characteristics described by the NYHA classification may not be directly related to the amount of fibrosis, in this thesis we still support the hypothesis that cardiac fibrosis affects the prognosis of HF.
Two types of fibrosis can be distinguished in the myocardium. One type is the reparative fibrosis. Since myocytes are thought to be terminally differentiated, lost myocytes due to necrosis or apoptosis, e.g. after a myocardial infarction, are replaced by collagen. This collagen deposit is necessary to preserve the cardiac structure and morphology after myocyte loss. Therefore, this fibrosis is named reparative or replacement fibrosis[45]. However, fibrous tissue formation can also occur without loss of cells. For example, collagen formation in the perivascular arterioles, endocardial and pericardial collagen deposition, increased amount of collagen between viable myocytes, and thickening of existing collagen fibres. This type of fibrosis is named reactive or interstitial fibrosis[45]. The fibrosis can be present in patchy, diffuse, or stringy pattern. Patchy fibrosis is fibrosis with long compact groups of strands, diffuse fibrosis is short-stranded fibrosis that is more distributed in the tissue, and a stringy fibrosis pattern reflects homogenously distributed fibrosis with long single strands[38]. In hypertensive hearts both patchy and diffuse fibrosis is observed[33]; in DCM all three different patterns are observed[38].
Fibrosis can be triggered by tissue injuries such as myocardial ischemia (hypoxia), inflammation, and hypertensive overload[43]. After exposure to these injuries, fibroblasts will proliferate and differentiate into myofibroblasts[5]. These cells produce more collagen and other ECM proteins. Possible triggers for this excessive synthesis of ECM components are certain growth factors such as TGF-β and insulin-like growth factor-1 (IGF-1)[46]. Also hormones of the renin-angiotensin-aldosterone system (RAAS), such as angiotensin II (ATII) and aldosterone, may affect the synthesis of collagen[32, 47], especially type I[48], *in vitro*.

Treatment and fibrosis
Until now, CHF patients with a reduced systolic function are mainly treated with pharmaceutical interventions. First of all, almost every patient with symptomatic HF needs diuretics to avoid renal dysfunction and electrolyte disorders. In addition to diuretics, patients are treated with other drugs that intervene in autonomous reactions that are involved in HF and its progression, such as glycosides, angiotensin converting enzyme (ACE) inhibitors or ATII receptor blockers, β-blockers, and aldosterone receptor antagonists. Next to this standard pharmaceutical therapy, surgical treatment should be

considered[49]. There is a growing interest in implantable devices and surgery, such as cardiac resynchronisation therapy (CRT), implantable cardioverter defibrillator (ICD), LV assist device (LVAD), or finally heart transplantation[50]. It is beyond the scope of this thesis to get into detail about these therapies, but McMurray and Pfeffer nicely reviewed the treatments of HF[50].

Because cardiac remodelling and fibrosis is thought to play an important role in the progression of HF, it is of great interest to focus on therapies that intervene in the formation of fibrosis. Several drugs intend to have a direct effect on the ECM turnover and herewith improve the prognosis of HF patients, e.g. drugs that affect the RAAS. As shown in both animal models and human studies, aldosterone antagonists, ATII receptor blockers, and ACE inhibitors have an effect on the cardiac ECM[43]. One large clinical study, the Randomized Aldoctone Evaluation Study (RALES), investigated the effects of the aldosterone receptor inhibitor spironolactone in patients with severe HF. This study has shown that spironolactone provides a significant reduction of cardiac events in patients with severe HF. The positive effects of this drug are thought to be provided by a decrease in heart failure progression and fibrosis formation in these patients[51].

Torasemide, a loop diuretic, is also thought to diminish the amount of collagen in HF. The decrease in collagen is observed both histological and in PICP levels of patients with HF[52]. Since torasemide is known to interfere with the secretion of aldosterone, the collagen decrease after torasemide usage might secondary be due to the effects of torasemide on aldosterone secretion[52]. This hypothesis is supported by the result that the diuretic furosemide, which does not interfere with the aldosterone secretion, does not decrease the collagen amount in the diseased heart[52].

Aim of the thesis

Since cardiac remodelling is such an important process in the onset and progression of HF, in cardiac sudden death due to arrhythmias, and hospitalisation due to cardiac events, it is relevant to gain more knowledge about this process. Biomarkers of cardiac remodelling, hence fibrosis, might be useful for non-invasive risk stratification for progression of HF and cardiac death. Herewith, patients at risk can be detected in an early stage, with a very cheap, quick, and easy method. Additional information of the pathophysiological condition of the heart may lead to improvement of therapy for each individual person (figure 4). The aim of this thesis is to investigate which biomarkers, related to cardiac remodelling, are known until now. In addition, it will be investigated whether these biomarkers can be used for prognostic and diagnostic information in HF patients. In this thesis, only studies are involved that investigated fibrosis in HF, DCM, and HCM patients. Since in ischemic HF reparative fibrosis occurs in the infarcted area, this type of structural heart disease is excluded for this thesis.

Biomarkers

Because cardiac fibrosis is associated with cardiac dysfunction and arrhythmogeneity, we hypothesise that early quantitative detection of fibrosis may play an important role in the treatment and prognosis of patients suffering from HF (figure 4). Therefore, it is important to examine the degree of the cardiac fibrosis in each patient. Although cardiac biopsies are very reliable for this investigation, non-invasive techniques such as biomarkers might be much more beneficial.
According to Morrow and De Lemos (2007), a cardiovascular biomarker has to meet three criteria: 1) the measurement has to be accurate, reducible, relatively cheap, and clinicians have to have an easy accessibility; 2) the biomarker has to add new information next to already existing tests; and 3) the biomarker helps to manage patients in clinic[53].

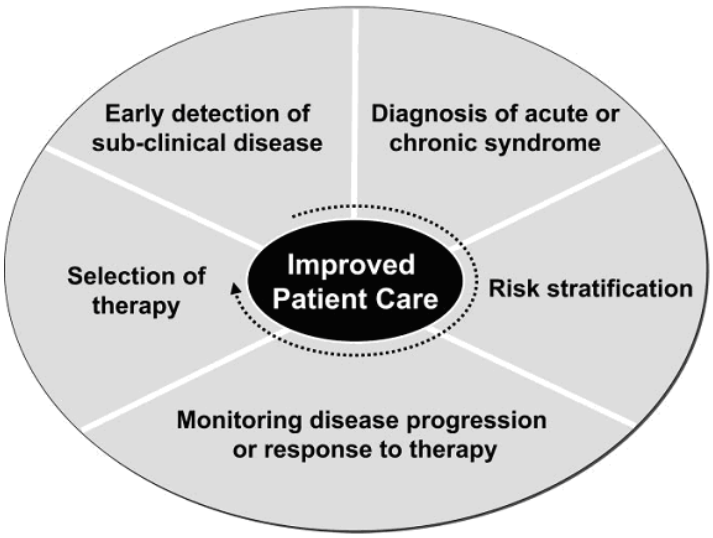

Figure 4. The clinical applications of cardiovascular biomarkers. Figure adapted from Morrow and de Lemos, 2007.

In addition to Morrow and de Lemos[53], Gonzalez and co-workers[54] require some more features of a biomarker of myocardial remodelling: a good sensitivity and specificity of the biomarker; the biomarker has to associate with structural or functional parameters that reflect the hallmarks of fibrosis and variation of the biomarker levels should be in parallel with these parameters; and there has to be a positive gradient from its concentration in coronary sinus blood towards its concentration in peripheral vein blood[54].
Braunwald divides the biomarkers related to heart failure into six classes: inflammation, oxidative stress, ECM remodelling, neurohormones, myocyte injury, and myocyte stress biomarkers[55]. Only the biomarkers that are related to the remodelling of the ECM, in other words fibrosis, will be discussed in this thesis.
Biomarkers related to cardiac remodelling are peptides that are implied in collagen synthesis, e.g. the propeptides PIIINP, PINP, and PICP, and degradation, such as ICTP. Biomarkers for the general degradation of the cardiac ECM are MMP-1, MMP-2, MMP-3, MMP-9, and TIMPs. In many studies, the levels of these biomarkers are used to evaluate the collagen turnover in the heart. In the following sections several studies are discussed that investigated these biomarkers in HF, DCM, or HCM. Table 1 gives an

overview of the studies that investigated biomarker levels in the different cardiac diseases and which are discussed in this thesis.

PIIINP

PIIINP is the N-terminal propeptide of procollagen type III (42 kDa). The propeptide is cleaved stoichiometrically once the procollagen is translocated to the ECM, prior to fibril formation. Therefore, the amount of PIIINP is a marker for the synthesis of collagen type III fibrils. However, as mentioned before, the N-terminal domain of collagen III is sometimes removed incompletely, resulting in incorporation of PIIINP in the collagen fibres[18]. Unfortunately, the percentage of incorporated N-propeptides in collagen fibres is currently not known. Levels of PIIINP might therefore underestimate the synthesis of collagen type III. Despite this limitation, PIIINP levels are still widely used as a marker for collagen III synthesis. In several human studies on patients with different types of heart failure, PIIINP was determined. Patients with DCM[56], HCM[36], and CHF[57], have higher serum PIIINP levels than healthy controls. This elevated level of serum PIIINP was also shown in hypertensive patients with diastolic HF, compared to hypertensive patients without diastolic heart failure[58].

Unfortunately, only one study has related elevated PIIINP levels to the myocardial levels of collagen III, using human cardiac biopsies. Despite the inclusion of ischemic DCM patients in this study, no significant difference was observed between serum PIIINP levels of idiopathic DCM and ischemic DCM. This study has shown a correlation of 0.784 ($p=0.0013$) between serum PIIINP levels and the myocardial PIIINP fraction[56]. However, the significance of this correlation seems to be based on predominantly one aberrant sample. Next to this correlation, this study also shows a correlation between PIIINP levels and the collagen I fraction of the myocardium ($r=0.71$, $p=0.0104$). More research is definitely required to support the correlation between PIIINP and myocardial collagen III.

To our knowledge, Sato *et al.* were the first to investigate the relation between PIIINP levels and cardiac event prognosis. They observed a positive correlation between high PIIINP serum concentrations (>0.8 U/mL) and cardiac events after a follow up of 500 days in patients with DCM[59]. In this study, the serum concentration of PIIINP is expressed in units per mL; a unit associated with the activity of an enzyme. Therefore, this is an unusual unit for measuring serum concentrations of a biomarker. Unfortunately, the investigators do not provide any information about the used method and their choice for this method. Due to this unit, it is impossible to compare the results of this study with other studies in which units such as ng/mL were used.

Subsequently, three additional studies have been performed to investigate the relation between PIIINP and mortality. In a substudy of the previous mentioned Randomized Aldoctone Evaluation Study (RALES), Zannad and co-workers have shown that PIIINP levels can be used to predict the risk of hospitalisation and mortality of severe CHF patients. Serum PIIINP levels >3.85 µg/L have a significant negative correlation with survival and hospitalisation-free survival. The relative risk for patients with PIIINP levels >3.85 µg/L is 2.36 and 1.83 times higher for death and for death or hospitalisation respectively[60]. However, when patients are treated with spironolactone, no correlation is observed between PIIINP levels and death and/or hospitalisation. When the median value is considered as a cut off point, the relative risk of death of all causes is decreased in the spironolactone group with PIIINP >median value, compared to the placebo group with PIIINP >median value. Spironolactone treated patients with PINP and PICP levels higher than the median value also showed the decreased relative risk of death[60]. After treatment of spironolactone, levels of PIIINP and PINP were also largely decreased. All these date together suggest that spironolactone may decrease collagen formation and

the mortality and morbidity in CHF, predominantly in patients with high biomarker levels[60].

Cicoira *et al*. confirmed the increased mortality risk of high PIIINP levels in patients with mild to moderate CHF. Patients with plasma PIIINP levels >4.7 µg/L had a worse outcome regarding to survival[61]. Also Klappacher *et al*. observed an increased mortality risk in CHF patients with PIIINP levels ≥7 µg/L[56]. These studies suggest that PIIINP, thus indirectly the collagen III synthesis and amount of fibrosis, may have a predictive value for CHF patients regarding to death and hospitalisation.

Unfortunately, in all studies described previously, the cut off points are determined retrospectively and therefore differ from each other. Especially the cut off point in the study of Klappacher *et al.* is higher than the cut off points of the other two studies. One possible explanation for this remarkable difference is the amount of patients included in the studies; Klappacher *et al*. included only 41 patients, while Cicoira *et al*. and Zannad *et al*. included more CHF patients, respectively 101 and 261 patients. However, to use PIIINP as a predictive marker in clinics, one cut off point should be established.

Lombardi *et al*. investigated whether there is a relation between PIIINP levels and left ventricular function. They have shown that HCM patients have elevated serum PIIINP levels compared to controls. These PIIINP levels are inversely related to the end-diastolic diameter of the LV. Since the end diastolic diameter of the LV represents the diastolic dysfunction, this study suggests that PIIINP is a marker for passive diastolic dysfunction in HCM[36].

All previous mentioned studies are based on patients with cardiac diseases. In addition, Wang *et al*. investigated whether PIIINP levels are also related to echocardiographic determinants in ambulatory participants of the Framingham Study. This study has shown that in an adjusted multivariate model, plasma PIIINP levels are only strongly correlated with body mass index (BMI) and also marginally associated with age, but not with LV structure or function[62]. The results of this study suggest that in studying levels of PIIINP, also other factors such as BMI and age should be taken into account. The association between BMI and PIIINP levels might be related to insulin resistance[63], however, the origin of the elevated PIIINP levels in obese persons are currently unknown. In contradiction to this study, Alla *et al*. observed no relation between the biomarker concentration and BMI in the control patients[57]. A possible explanation for these conflicting results might be the difference between the statistical analyses used in both studies. Another remark to take into account is that underlying pathologies such as osteoporosis or hepatic fibrosis might also influence fibrotic biomarker levels. This flaw will be more extensively discussed in a later chapter.

PINP

PINP is the 70 kDa N-terminal peptide of collagen I. This marker can be used as a marker for collagen I synthesis. Since collagen I is mainly responsible for the cardiac fibrosis[32], this biomarker is used in studies investigating the cardiac matrix turnover, next to PIIINP. Although excessive collagen I deposition is characteristic for cardiac remodelling, Lombardi *et al*. did not find a significant difference in serum PINP levels between controls and HCM patients[36]. Also CHF patients exhibited no significant altered PINP levels compared to healthy controls[57], just like hypertensive patients with or without diastolic heart failure[58]. However, Lombardi *et al*. did observe a negative correlation between PINP levels and the passive diastolic function in HCM[36], suggesting that increased collagen I synthesis is related to a decreased passive diastolic function. Unfortunately, no other studies have investigated whether PINP levels may reflect the cardiac remodelling in HF and the usage of this biomarker as an additional diagnostic or prognostic tool.

Author (year)	Studied condition	*N* patients	*N* controls	Read out / end point	Studied biomarkers	General result	Main findings
Alla F (2006)	CHF (EF<35%)	239	92	Systolic dysfunction	PIIINP, PINP, PICP, MMP-1, TIMP-1	c	• PIIINP ↑ • PINP = • PICP = • MMP-1 ↓ • TIMP-1 ↓
Barton PJR (2006)	HF (deteriorating vs. end-stage)	27	28		MMP-1 and TIMP-1	e	• MMP-1 ↑ in deteriorating HF • TIMP-1 ↑ in deteriorating HF
Cicoira M (2004)	CHF (EF<45%)	101		Mortality Hospitalisation	PIIINP	a	• Survival rate ↓ when PIIINP >4.7 µg/L
Frantz S (2008)	HF	250	75	All cause death	MMP-9 and TIMP-1	a, c	• MMP-9 = • TIMP-1 ↑ • Survival rate ↓ when TIMP-1 >1917 ng/mL
George J (2005)	HF	88	30	Mortality Hospitalisation	MMP-2, MMP-3, MMP-9, TIMP-1	a, e	• MMP-2 ↑ • MMP-3 = • MMP-9 ↑ • TIMP-1 ↑ • MMP-2 correlates with NYHA classification • Cardiac event risk ↑ when MMP-2 >352 ng/mL
Jordan A (2007)	HF	50	53	Clinical endpoints	MMP-1 and TIMP-1	c	• MMP-1 ↓ • TIMP-1 ↑
Kitahara T (2007)	CHF (+/- preserved LV systolic function)	156		Mortality	ICTP	a	• Event-free rate ↓ when ICTP >7.3 ng/mL • ICTP levels not predictive in CHF with reduced LV function • Event-free rate ↓ when ICTP >4.9 ng/mL and preserved LV function
Klappacher G (1995)	DCM (EF<40%)	41	30	Mortality Histology	PIIINP, ICTP	a, b, e	• PIIINP ↑ • ICTP ↑ • Survival rate ↓ when PIIINP ≥7 µg/L • Survival rate ↓ when ICTP >7.6 µg/L • Correlation myocardial collagen III and PIIINP = 0.784 • Correlation myocardial collagen I and ICTP = 0.603 • Correlation myocardial collagen I and PIIINP = 0.71
Kramer F (2008)	HF in rats	10	10	Biomarker levels LV contractility	MMP-2 and TIMP-1	d	• MMP-2 ↑ • TIMP-1 ↑ • TIMP-1 correlates with LV contractility

Table 1 part I. Overview of studies investigating cardiac remodelling biomarkers in patients with heart failure or diseases prior to heart failure. CHF=congestive heart failure, EF=ejection fraction, HF=heart failure, LV=left ventricle, DCM=dilated cardiomyopathy, HCM=hypertrophic cardiomyopathy. All studies are discussed in the thesis, the main message of each study is shown in this table; a=study shows a predictive effect of a biomarker, b=study shows a correlation between a biomarker and the myocardial collagen fraction, c=results of the study support formation of collagen, d=results of the study support degradation of collagen, e=results of the study are contradictory concerning the collagen turnover.

Author (year)	Studied condition	*N* patients	*N* controls	Read out / end point	Studied biomarkers	General result	Main findings
Lombardi R (2003)	HCM	36	14	Diastolic function	PICP, PINP, PIIINP, ICTP, MMP-1, MM-2, MMP-9, TIMP-1	e	• PIIINP ↑ • PINP = • PICP = • ICTP ↑ • MMP-1 = • active MMP-2 ↑ • active MMP-9 ↑ • TIMP-1 ↑ • PINP and PICP correlate with diastolic dysfunction • PIIINP inversely related to LV end diastolic diameter
Lopez B (2004)	CHF + loop diuretics (torasemide/ furosemide)	39		Histology	PICP, ICTP	b	• Correlation myocardial collagen and PICP = 0.88 • Collagen fraction ↓ in CHF + torasemide
Martos R (2007)	diastolic HF	32	54	Biomarker levels	PIIINP, PINP, PICP, ICTP, MMP-1, MMP-2, MMP-9, TIMP-1	e	• PIIINP ↑ • PINP = • PICP ↑ • ICTP ↑ • MMP-1 = • MMP-2 ↑ • MMP-9 ↑ • TIMP-1 =
Querejeta R (2000)	hypertension	26	10	Histology	PICP	b, c	• PICP ↑ • Correlation myocardial collagen and PICP = 0.471
Querejeta R (2004)	hypertension +/- HF	65	12	Histology	PICP	b, c	• PICP ↑ • PICP ↑ in hypertension + HF • Correlation myocardial collagen and serum PICP = 0.867 • Correlation myocardial collagen and coronary PICP = 0.759
Sato Y (1997)	DCM	21		Cardiac events	PIIINP	a	• Risk for cardiac event ↑ when PIIINP >0.8 U/mL
Schwartzkopff B (2002)	DCM (EF<45%)	43	47	Biomarker levels	PICP, ICTP, MMP-1, TIMP-1	e	• PICP = • ICTP ↑ • MMP-1 ↑ • TIMP-1 ↑
Spinale FG (2000)	DCM	37	13	Zymography Immunoblotting	MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1	d	• MMP-1 ↓ • MMP-2 ↑ in idiopathic DCM • MMP-3 ↑ in idiopathic DCM • MMP-9 ↑ • TIMP-1 =
Thomas CV (1998)	DCM	7	8	Zymography Immunoblotting	MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1	e	• MMP-1 ↓ • MMP-2 = • MMP-3 ↑ • MMP-9 ↑ • TIMP-1 ↑
Zannad F (2000)	CHF (EF<35%)	261		Mortality Hospitalisation	PIIINP, PINP, PICP, MMP-1, TIMP-1	a	• Survival rate ↓ when PIIINP >3.85 µg/L

Table 1 part II. For explanation and abbreviations of the table see part I.

In a review of Risteli and Risteli, some flaws about using PINP levels as a marker for collagen I synthesis are described. First, there is a delay between the release of PINP, compared to the release of the C-terminal procollagen I (PICP). Second, like PIIINP, PINP may not always be removed from the collagen, suggesting that PINP levels might give an unreliable value. Third, they suggested that uncleaved PINP could degrade into a monomer. This monomer might still react in some PINP assays, resulting in an overestimation of collagen I synthesis[17]. Based on these flaws, other collagen I synthesis markers, such as PICP, might be better to use as a biomarker.

PICP

The synthesis of collagen type I can also be measured by determination the levels of the 100 kDa C-terminal propeptide of collagen I, PICP. In contrast to PINP and PIIINP, PICP is cleaved of the procollagen without exception[18]. Querejeta and co-workers have observed a direct correlation between serum PICP levels and the amount of collagen in hearts of hypertensive[64] and CHF[52] patients. These results suggest that PICP levels might be representative for myocardial fibrosis. Furthermore, PICP levels can be used to discriminate between moderate and severe myocardial fibrosis in hypertensive patients, with 75% sensitivity and 78% specificity[64]. Since hypertension is a major risk factor for heart failure, in a subsequent study Querejeta *et al*. have determined myocardial fibrosis and PICP levels in hypertensive patients with and without HF. They have shown that both myocardial fibrosis and PICP concentrations were higher in hypertensive patients with HF[65]. However, in the study of Lombardi *et al*., no significant changes were found in serum PICP levels between HCM patients and healthy controls[36]. In patients with DCM, serum PICP levels had a tendency to increase, however, the increase was not significantly different[66]. Results of all these studies might suggest that the relation between PICP levels and state of disease is dependent on the type of the disorder; hypertensive patients are thought to have elevated levels of PICP, while the PICP levels in HCM and DCM patients are not increased compared to controls. Whether the elevated level of PICP is due to an increased amount of myocardial fibrosis is difficult to retrieve, while the studies on DCM and HCM patients did not perform myocardial biopsies. However, Querejeta and co-workers investigated in several studies the myocardial collagen fraction. The collagen volume fraction (CVF) in hypertensive patients was 5.23 ± 0.38 %, the CVF in CHF patients was 7.63 ± 0.80 %, and in CHF patients with hypertension the CVF was 7.91 ± 0.55 %[65]. In a normal heart, the CVF is 1.95 ± 0.07 %[64]. The corresponding PICP levels were 118 ± 6 µg/L, 138 ± 10 µg/L, and 139 ± 6 µg/L respectively[52, 64, 65]. These results suggest that an increase in PICP levels is associated with an increase in CVF.

Like PINP, PICP is also cleaved of the procollagen molecule in a 1:1 stoichiometric fashion. This would suggest that both biomarkers will give similar results regarding to cardiac remodelling in heart failure. Indeed, some studies observed no difference at all in both PINP and PICP levels of HF patients and healthy controls[36, 57]. However, Martos *et al.* did observe differences between PINP and PICP levels; PICP was elevated in HF, while PINP was not increased[58]. Regarding to the synthesis of collagen I fibres, this is a remarkable phenomenon, since both procollagens are cleaved of one fibre and are both released into the blood. However, as discussed in the section above, PINP as a biomarker has some limitations. Furthermore, the elimination of both propeptides differs from each other. PICP is eliminated via the mannose-receptor endocytosis pathway by the liver, while PINP is eliminated by the liver via scavenger receptors[17]. All these differences between the two biomarkers might be an explanation for the different results between the levels of PINP and PICP.

ICTP

The small C-terminal telopeptide of collagen type I (ICTP; 12 kDa) is cleaved by collagenase in a 1:1 stoichiometric way, during the breakdown process of collagen type I fibrils[67]. Subsequently, ICTP is released into the bloodstream[15]. Therefore, this peptide can be used as a biomarker reflecting the breakdown of collagen I. Because in HF the equilibrium between collagen synthesis and breakdown is thought to shift towards the collagen synthesis, a decrease in ICTP levels in CHF patients is expected. Moreover, in DCM patients the ICTP levels might be increased due to dilation of the ventricles. This hypothesis is supported by the study of Klappacher *et al*.; they observed increased serum ICTP levels in DCM patients, compared to healthy controls. The ICTP levels also correlated significantly with the myocardial levels of collagen I ($r=0.603$; $p=0.0527$)[56]. However, the correlation between collagen I and ICTP is a positive relation. Since ICTP is thought to reflect collagen I degradation, the positive correlation seems contradictory. Unfortunately, to our knowledge this is the only study concerning the correlation between collagen I and ICTP levels. Next to these results, Klappacher and co-workers also observed an increased risk of mortality when the ICTP levels were higher than the cut off value (serum ICTP>7.6 µg/L), suggesting that a rise in the breakdown of collagen I results in an increased risk of mortality in DCM patients[56]. However, a limitation of this study is that they did not investigate the synthesis of collagen I; determination of only collagen I breakdown could lead to a biased view of the collagen turnover. Another study concerning ICTP levels in DCM patients has also observed increased ICTP levels in these patients, compared to healthy controls[66]. Levels of other degradation biomarkers were also elevated in this study, while PICP levels were not changed significantly. The results of these studies would be in line with the ventricular dilation seen in DCM patients.

Martos *et al*. observed an elevation of ICTP levels in patients with diastolic HF, compared to hypertensive patients without diastolic HF, but also the PICP and PIIINP levels are increased in these patients. These results suggest an increase in collagen turnover. In more severe phases of diastolic dysfunction the increase in collagen turnover is even higher. However, these results do not show anything about the net accumulation or degradation of collagen in the myocardium. Unfortunately, this study did not investigate the relationship between biomarker levels and survival rate[58].

Kitahara *et al*. investigated the predictive value of ICTP for cardiac events in CHF patients with preserved LV function (ejection fraction (EF) ≥50%) (i.e. diastolic heart failure) and non-preserved LV function (EF ≤50%)[68]. In the total patient group, very high ICTP levels (>7.3 ng/mL) had a significant lower event-free rate. In patients with a reduced systolic function, there was no significant difference in the event-free rate between high (>4.8 ng/mL) and low levels (<4.8 ng/mL) of ICTP. However, in the group of patients with preserved systolic function, difference in event-free rate was observed between high and low ICTP levels; patients with high ICTP levels had a decreased event-free rate[68]. These results suggest that ICTP may predict cardiac events independently, especially in CHF patients with preserved LV systolic function[68]. However, since ICTP is a breakdown product of collagen I, elevation of ICTP would imply an increase in the breakdown of fibrosis. According to this thesis, this should be positive for the prognosis of HF patients; however, Kitahara and co-workers show the opposite. This may be explained by the fact that they only evaluated ICTP and no biomarkers of collagen synthesis. Based on this study we can not draw any conclusions about the collagen turnover. Another explanation might be that although the degradation of the collagen type I fibres is increased, collagen type III fibre formation is increased, or the remaining collagen type I fibres are thickened.

Lombardi *et al*. determined several biomarkers in HCM patients. They observed elevated serum ICTP levels, while the PINP and PICP levels did not alter significantly in HCM patients, suggesting a shift of the collagen equilibrium towards collagen type I breakdown[36]. In hypertrophic patients this is a remarkable observation, since hypertrophy is thought to be related to an increase in myocardial stiffness and an increase in collagen deposition. However, Lombardi *et al*. also divided the HCM patients into two groups: with and without diastolic dysfunction. Contradictory to the results of Martos *et al.*, patients with diastolic dysfunction showed a significant positive correlation between the PINP and PICP levels and the diastolic dysfunction, but also between the PINP-ICTP ratio and the diastolic dysfunction[36]. These results support the hypothesis that hypertrophy together with diastolic dysfunction is related to an increase in collagen deposition, while Martos *et al*. observed an increase in turnover of the collagen.

MMPs and TIMPs

As described previously, many MMPs are expressed in the myocardium. In heart failure, the expression of MMPs is altered; however, not all MMPs are involved[20]. Based on literature, at least MMP-1, -3, -9, and TIMP-1 are involved in HF (reviewed by Spinale[27]). Thomas *et al*.[69] en Spinale *et al*.[70] both studied MMP levels in the myocardial tissue of DCM patients. Thomas and co-workers investigated only idiopathic DCM, while Spinale and co-workers divided DCM into idiopathic and ischemic DCM. Both studies showed an increase in MMP-9 levels and a decrease in MMP-1 levels in DCM myocardium, compared to healthy hearts. However, the level of other MMPs is less evident. In idiopathic DCM patients, the level of MMP-3 seems to be increased[69, 70], while in ischemic DCM MMP-3 seems to be unchanged compared to healthy myocardium[70]. MMP-2 levels in idiopathic DCM remains contradictory; Spinale and co-workers observed an increase in MMP-2[70], while Thomas *et al*. did not find a significant alteration in MMP-2 levels[69]. In ischemic DCM, MMP-2 levels are not altered either[70]. Whether TIMP-1 levels are altered in DCM is also still contradictory, Spinale *et al*. did not observe a significant change in TIMP-1 in both idiopathic and ischemic DCM[70], while Thomas *et al*. showed a large increase in TIMP-1 levels (>500%) compared to healthy controls[69]. Although the results concerning the MMP and TIMP-1 levels in DCM tissue are diverge, the zymographic MMP activity in the myocardium is significantly increased in both studies, suggesting that the collagen breakdown is increased in DCM patients[69, 70].

However, studies investigating serum and plasma levels of MMPs and TIMPs of HF patients do not confirm these results completely. In CHF patients, serum levels of MMP-1 and TIMP-1 are described to be decreased[57], while another study observed an increase in TIMP-1 and a decrease in MMP-1[71]. In DCM patients an increase in serum levels of MMP-1 and TIMP-1 is shown[58, 66], in contradiction to the tissue levels of MMP-1 and TIMP-1 in the studies described above. Barton *et al*. discriminated stable end-stage heart failure and deteriorating heart failure. They observed an increase in MMP-1 and TIMP-1 expression in deteriorating HF, while the expression in stable end-stage HF was comparable to control hearts[31]. Since mechanical signals can stimulate MMP-expression, this might be an explanation of the increase in MMP-1 in deteriorating HF. This hypothesis is supported by the observation that an increase in MMP-1 is reported in systolic HF patients, while in diastolic HF patients no increase is observed[37, 58].

Although these studies are only a small amount of all studies performed on MMP and TIMP expression in heart failure, they show much variability in the levels of these ECM proteins. One of the explanations might be that every study determines the levels in patients with different underlying disorders. Whether the underlying cause of HF plays a role in the altered expression of MMPs and TIMPs remains contradictory; Herpel *et al*. and Barton *et al*. have shown that expression of the MMPs differs in different

cardiomyopathies[31, 72], while for TIMP-1 expression, the underlying cause seems not to be important[21]. Therefore, the results for TIMP-1 levels might be more useful to compare the different studies.
In addition to the limitation of different cardiomyopathies, one should also keep in mind that cardiac remodelling is a dynamic process. Especially during heart failure, there are different phases in this process. In these phases, the MMP activity is also fluctuating[73]. Therefore, it is important to take the different phases into account when determining MMP levels in heart failure. Another flaw in measuring MMPs and TIMPs is the method used in all aforementioned studies. They all determine the levels of different MMPs and TIMPs in HF patients by histology or immunohistochemistry (biopsy) or by enzyme-linked immunosorbent assay (plasma and serum). However, these methods do not discriminate between proMMPs, active MMPs, or MMP-TIMP complexes. Therefore, results provided by these methods have only limited value. To our knowledge, only Lombardi *et al*. discriminated between active and free MMP-1. In their study, they observed elevated levels of active MMP-2, active MMP-9, and total TIMP-1 in HCM patients, while both free MMP-1 and active MMP-1 was not altered[36]. Another way to determine the MMP activity is by measuring the MMP/TIMP ratio. Unfortunately, in many studies either they do not determine this ratio, or only the MMP-1/TIMP-1 ratio is measured, while the ratio of other MMPs to TIMPs is not investigated in these studies.
Despite all these gaps and contrary results about MMP and TIMP levels in HF, several studies have investigated whether MMP and TIMP levels might be useful as a prognostic or predictive tool in HF. Kramer *et al*. investigated whether there is a correlation between MMP-2 or TIMP-1 plasma levels and the left ventricular contractility (as a measure of heart failure) in HF induced rats. Both MMP-2 and TIMP-1 are increased in HF rats compared to controls, but only the TIMP-1 plasma concentration was related to the LV contractility[74]. George *et al*. studied the correlation between the NYHA functional classes and the serum MMP levels in CHF patients. They observed higher serum MMP-2, MMP-9, and TIMP-1 levels in CHF, but only the level of MMP-2 correlated with the NYHA functional classes. MMP-3 levels were also determined, but these levels did not alter significantly between CHF patients and healthy controls. In addition, in this study it was observed that high levels of MMP-2 (>352 ng/mL) increase independently the risk of hospitalisation for HF, death, or both[75]. Until now, this is the only study that has observed this predictive value of MMP-2. In contrast to the latter study, Frantz *et al*. suggested that TIMP-1 is a strong predictor of mortality. They observed a large increase in plasma TIMP-1 levels in CHF patients, compared to healthy controls (1640 ng/mL vs. 735 ng/mL respectively)[21]. Next to this result, they also observed that patients with high TIMP-1 levels (>1917 ng/mL) have a much higher mortality rate than patients with lower levels (<1390 ng/mL). However, a cautionary note on these results is that Frantz *et al*. used all-cause mortality to investigate the mortality rate, while George *et al*., who did not find a predictive value for TIMP-1, used mortality due to CHF as an endpoint[75]. Jordan *et al*. investigated in moderate CHF patients the association between MMP-1 and TIMP-1 serum levels and clinical endpoints (death due to any cause, readmission due to HF, and heart transplant). They observed a decrease in MMP-1 levels and an increase in TIMP-1 levels in CHF patients in comparison with healthy controls. Patients with an endpoint appeared to have also significant lower MMP-1 levels and higher TIMP-1 levels. These results suggest that a decrease in MMP-1 and increase in TIMP-1, hence less collagen degradation, have a poor prognosis in moderate CHF patients[71].
In conclusion, alteration in MMP and TIMP expression is definitely involved in HF. However, which MMPs or TIMPs are involved and the underlying mechanisms of these alterations are currently unclear. TIMP-1 seems to be the most promising marker, but

prior to the introduction of MMP and TIMP as a biomarker in the clinic, further investigations are recommended.

Discussion

As shown in the previous sections, biomarkers for cardiac remodelling are already intensively studied in the last recent years. Using biomarkers as a prognostic, predictive, or diagnostic tool seems very promising. To evaluate therapies and their effect on cardiac remodelling, biomarkers can also be useful. Based on literature especially PICP and ICTP seem to be useful biomarkers as a representative of cardiac fibrosis in HF patients (see also table 1). However, there is still much variety between the different studies; sometimes they are even contrary to each other. In this section, the use of biomarkers of cardiac remodelling as a predictive or diagnostic tool will be discussed.

First of all, in all studies described in this study, different disorders are observed and in every study other inclusion criteria were used. Of course, without an adequate therapy, every cardiac disorder will finally result in heart failure. However, the different original cardiac disorders might give conflicting results between studies. HCM goes hand in hand with hypertrophy of the myocytes and an increased stiffness of the ventricular wall, suggesting an increase in (interstitial) collagen fibres. HCM patients are therefore thought to have an increase in biomarkers reflecting the synthesis of collagen, e.g. PIIINP, PINP, and PICP. Dilation is partially explained by splitting and rupture of the collagen type I fibres[66]. Hereby the framework of the heart will change. This change reduces the tensile strength and the transmission of force during systole and diastole. This would suggest a decrease in collagen content. However, the opposite is shown in HF with dilated LV; accumulation of interstitial collagen is observed[35]. Possible explanations would be that only the cross-links between the fibres are reduced in dilated myocardium, or that the equilibrium between collagen I and III is shifted towards collagen type III in dilated HF, a less stiff collagen type than collagen I[35]. As discussed before, Lopez *et al*. also observed an increase in myocardial collagen content. While they observed a decrease in the mysial collagen network, which may lead to dilated HF, but an increase in perivascular and scar-related collagen[37]. This might be another explanation for the increase of the collagen amount in DCM, despite the dilation of the myocardium.

In studies on patients with the same underlying disorders, different levels of one biomarker are still observed. CHF patients of each NYHA class, i.e. with the same grade of CHF, are thought to have similar pathological characteristics, and herewith similar levels of cardiac remodelling and fibrosis. This would suggest that the biomarker levels of collagen turnover of these patients would correspond with each other. However, this is not the case; every study showed different levels of biomarkers in corresponding patients. This might at least partially be explained by different patient characteristics, such as HF treatment, other disorders of the patient, age, and BMI. First, medication of the HF patients may differ. However, almost every HF patient is treated at least with the standard pharmacotherapy. In all studies discussed in this thesis, treatment of the patients was investigated and implied in the exclusion criteria. Moreover, drugs may influence biomarker levels, e.g. the elimination of the peptides by the liver. Based on pharmacological properties, Diez and Laviades reported that at least the ACE inhibitors quinapril and lisinopril do not interfere with the renal or hepatic elimination of PIIINP, PINP, and PICP[67]. However, whether other frequently used drugs in CHF may interfere with the elimination of the biomarkers remains to be studied. Second, as previously mentioned, collagen and ECM turnover is not a tissue-specific phenomenon. Besides, fibrosis occurs in other organs too. Therefore, elevated biomarker levels might not be only of cardiac origin, but may also be caused by other diseased organs, such as bone, liver, and lungs[56]. To obviate this flaw, almost all studies discussed in this thesis did not include patients with other disorders in which fibrosis or excessive matrix turnover is

implied, such as liver fibrosis, rheumatoid arthritis or osteoporosis. Moreover, Klappacher *et al*. observed that after a multivariate regression analysis, PIIINP and ICTP still predicted mortality of DCM patients, independent of hepatic disorders[56]. Next to these disorders, also other patient characteristics may influence fibrotic markers. For instance, PIIINP levels are thought to be independently correlated with BMI and age[62]. This implies that in further investigations, cardiac remodelling biomarker levels should be adjusted for several patient characteristics, at least for other (profibrotic) diseases, BMI, and age.

Another aspect that may explain the differences between patients, is that HF and cardiac remodelling is a continuous process, while most studies are cross-sectional and the biomarkers represent the cardiac remodelling only at one time-point. To obviate this, biomarker levels should be determined at different time-points in this process, together with the myocardial tissue composition. As reported in table 1, the studies used in this thesis included a relatively small amount of patients. Larger studies might also eliminate differences between studies with comparable underlying pathologies.

In biomarker investigation, another question rises: is the biomarker level in the serum representative for the tissue levels? Collagen is present everywhere in the connective tissue, also collagen type I and III. One method to correlate the biomarker levels with the cardiac remodelling is by using immunohistochemistry or histology of heart biopsies. The level of PICP is directly related to the fibrillar collagen fraction of the myocardium[52, 65], suggesting that this biomarker is representative for cardiac collagen. Also circulating PIIINP seems to correlate with collagen type III, but also with collagen I[56], which makes PIIINP less useful for the representation of only collagen III synthesis. ICTP levels are thought to correlate with myocardial collagen type I[56]. As discussed before, this relation would be expected to be a negative correlation; however, the opposite is shown in this study. Despite the flaws of this study, this is the only research group that has used specific antibodies to detect collagen I and III. Many other studies which were performed to detect collagen in tissue, used picrosirius red staining. On a cautionary note, picrosirius red staining does not bind only to one specific collagen type, but to all collagen types[76]. Therefore, to compare the amount of collagen staining with biomarker levels, all biomarkers related to the collagen types should be implied. Unfortunately, the direct correlation between biomarkers and myocardial collagen is only investigated in a few studies and has not yet been studied for all biomarkers. Especially in human cross-sectional studies, it is difficult to obtain heart tissue. A non-invasive method to achieve information about heart tissue components is measuring the heart function by echocardiography. However, with this method the tissue composition cannot be exactly mapped out and the results can only be used as a comparison between patients within one study.

In one of the previous sections the characteristics of a biomarker are described. According to Gonzalez *et al*., one feature of a cardiac biomarker is that there should be a positive gradient of the biomarker from its concentration in coronary sinus blood towards its concentration in peripheral blood[54]. This gradient is indeed observed by Querejeta *et al.,* for the biomarker PICP in patients with hypertensive heart disease[65]. Next to PICP, this gradient is also observed for MMP-1 and TIMP-1[37]. Moreover, this gradient should in future also be determined for other peptides that are suggested to represent myocardial remodelling.

Another flaw in measuring biomarkers in general is elimination of the peptides. The removal of the peptides discussed in this thesis is regulated differently. The elimination occurs via different pathways and can be regulated by e.g. growth factors and hormones. Moreover, elimination of the peptides is a variable process, which might influence the levels of the biomarkers[17, 18]. There is also scarce knowledge about the

stability of the peptides. Uncleaved PINP for example could be degraded into a monomer which can still be detected by commercially available detection kits[17]. Currently, there is no information about the stability of the other biomarkers discussed in this thesis. To our opinion, it is important to acquire additional information about stability of the peptides, both in the body as during storage, to prevent unreliable values. Gerlach and Tanus-Santos reported that the levels of MMP-9 and TIMP-1 are different in serum compared to plasma[77]. They suggest that these biomarkers can also be released by platelets or leukocytes after activation or processing of these cells. Therefore they recommend not to use serum samples to measure biomarkers, at least MMP-9, for prognostic or diagnostic purposes[77]. The studies discussed in table 1 used either serum or plasma to evaluate the protein levels. Unfortunately, the authors did not explain the choice for either serum or plasma usage. Further research is recommended to investigate whether other peptides and biomarkers also have different levels in serum and plasma.

Altogether, usage of biomarkers in clinical situations seems to be very promising as an additional tool. Based on the investigations until now, PICP, ICTP, PIIINP, and TIMP-1 are likely the most promising biomarkers reflecting fibrosis in HF. Unfortunately, based on current knowledge, it is not possible to determine prognostic, diagnostic, or predictive cut off values for CHF patients. Although several studies claim to show cut off values for several biomarkers, they do not use their cut off values in a prospective way. The cut off points in these studies are determined retrospectively; with a receiver operating curve the investigators try to obtain the ideal cut off point with a significant prognostic value. However, to use the biomarkers in a prognostic or diagnostic way, more prospective research is definitely required. The correlation between biomarker levels and the cardiac fibrotic fraction is another important part that has to be studied more intensively. Since histological investigation of cardiac fibrosis in humans is difficult, usage of animal models might be good to require additional information of this correlation.

By expanding the knowledge about fibrotic biomarkers, diagnosis of HF and severity of cardiac remodelling may hopefully be determined by these biomarkers in future. This non-expensive method might give important additional information about the diseased heart. This will result in a more accurate and individual treatment with higher success of treatment for each patient. Hopefully this may also result in at least partially preventing the progression of heart failure or even hospitalisation and cardiac death.

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