Novel Heart Failure Biomarkers：

Physiological studies to understand their complexity

Weijie Du

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Chapter 1

Introduction

Heart failure (HF) is a complex clinical syndrome caused by impaired ability of the heart to pump sufficient blood to fulfill the body's metabolic demands. Major risk factors for HF are previous cardiovascular events, such as myocardial infarction, sustained hypertension and metabolic disorders including obesity and diabetes [1, 2]. HF remains a major health issue with a high morbidity and mortality and its prevalence is expected to increase as a result of the aging population and better treatment options for other cardiovascular diseases [3]. The clinical manifestations of HF involve dyspnea, fatigue, exercise intolerance and fluid retention, as well as subsequent pulmonary congestion and peripheral edema, amongst others. The clinical diagnosis of suspected HF patients is mainly based on the medical history of patients, physical examination and cardiac imaging. Since HF is a heterogeneous syndrome with different etiologies, individual HF patients will present a diverse set of signs and symptoms. Moreover, different co-morbidities are associated with HF and hence not all symptoms may be directly related to cardiac dysfunction. Therefore, additional parameters, providing better clinical stratification of HF patients, and delivering deeper insights in the underlying pathological processes, are eagerly awaited.

Heart failure with reduced and preserved ejection fraction

Currently, HF classification is rudimentary, and quite simply subdivided in HF with reduced Ejection Fraction, referred to as HFrEF, and HF with preserved Ejection Fraction, referred to as HFpEF. The two types of HF are equally distributed [4]. (Figure 1) Recently, a new category of HF with midrange EF (HFmrEF; 40%≤EF≤49%) was suggested and included in the 2016 ESC HF guidelines [5]. The major cause of HFrEF is ischemic heart disease that results in loss of cardiomyocytes followed by replacement fibrosis and concomitant cardiac remodeling. These events can lead to eccentric remodeling, resulting in left ventricular (LV) dilatation and reduced systolic function. HFpEF on the other hand is mostly observed in elderly patients, often with obesity and hypertension, and these patients have predominantly concentric cardiac remodeling. They are often characterized by diastolic dysfunction evidenced by prolonged LV relaxation, impaired LV filling and increased cardiac stiffness [6]. Considering these differences, it is remarkable that most HF patients receive similar HF treatment, typically consisting of beta-blockers, ACE-inhibitors, Angiotensin Receptor Blockers (ARBs), and Mineralocorticoid Receptor Antagonists (MRAs). Patients with more advanced disease may receive device therapy: intracardiac defibrillators (ICD), Cardiac Resynchronization Therapy (CRT), or, in end-stage refractory HF, Left Ventricular Assists Devices (LVAD). These therapeutic strategies have resulted in beneficial effects in improving clinical outcomes for HFrEF patients, but have not shown any beneficial effects for HFpEF patients in clinical trials [3]. We still lack sufficient insight in the complex HF syndrome to understand which therapies

Characteristic	HFrEF	HFpEF	HFmrEF
Dysfunction	Systolic, Diastolic	Diastolic	Mild systolic, Diastolic
LVEF	<40%	≥50%	40%-49%
Etiology	CAD, MI	Hypertension, AF, Diabetes	CAD (Primary cause), Hypertension, Diabetes
LV remodeling	Eccentric	Concentric	Eccentric or Concentric
Prognostication under Medical treatments	Improved	Not improved	Resemble HFrEF

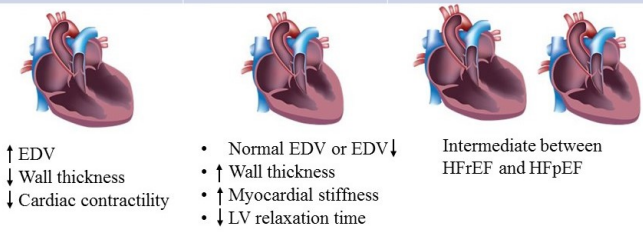

Figure 1

Pathophysiological characteristics of HFrEF, HFmrEF and HFpEF. HF can be sub-divided in HFrEF, HFpEF and HFmrEF. In response to coronary artery disease or myocardial infarction the heart undergoes eccentric remodeling resulting in HFrEF. Concentric remodeling, indicated by normal or reduced volume in diastole can result in HFpEF and is commonly observed in patients with hypertension, atrial fibrillation and/or diabetes. CAD=Coronary artery disease, MI=Myocardial infarction, AF=Atrial fibrillation, EDV=End-diastolic volume.

could be successful and we need better stratification possibilities, beyond classification on EF, to provide patients tailored therapies. Therefore, new methods are urgently needed to provide better diagnosis, risk stratification and therapeutic options for HF patients.

HF plasma biomarkers

In the past decades plasma biomarkers have gained great interest for their usefulness in HF diagnosis, prognosis and management. Typically, circulating biomarkers are released into the bloodstream in response to myocardial damage, myocardial stretch, or rather in response to a more generalized (systemic) reaction, such as inflammation or oxidative stress. As such, biomarkers have the potential to directly reflect HF pathological processes including myocardial stress, cell loss, impaired hemodynamics, neurohormonal activation, inflammation and extracellular matrix turnover (Figure 2). These properties could make these biomarkers interesting adjuvants to currently available diagnostic methods, and in the prognosis and treatment of HF. Natriuretic peptides (NPs), including atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are exclusively synthesized and secreted from the heart (atria and ventricles) in response to hemodynamic stress [7]. These peptides have emerged as established cardiac-specific biomarkers that provide incremental information to routine clinical evaluations for HF and are also included in the HF guidelines for diagnostic purposes [5, 8]. In

particular, the measurements of BNP, the N-terminal fragment of prohormone of brain natriuretic peptide (NT-proBNP), and the mid-regional fragment of prohormone of atrial natriuretic peptide (MR-proANP) are commonly used as the gold standard for diagnosis and exclusion of HF [9]. In addition, they are also used to predict adverse outcomes after acute and chronic HF [9]. However, clinicians should be aware that the values could be affected by a wide range of cardiac or extra-cardiac causes. Various risk factors of HF including age, pulmonary hypertension, renal disease and obesity could affect plasma NP levels [10-13]. In addition to NPs, cardiac troponins, which are specific markers of cardiomyocyte necrosis, have also been extensively studied and are used in clinical practice. The measurement of plasma troponin levels with high sensitivity troponin assays has been given a class I recommendation in acute HF and a class IIb recommendation in chronic HF in recent updated guidelines [14]. The incremental clinical value of these biomarkers has resulted in a strong drive to identify novel HF biomarkers that may have additional value in diagnosis, prognosis and disease management. Plasma proteins including Galectin-3 (Gal-3), Growth Differentiation Factor-15 (GDF-15) and soluble suppression of tumorigenicity 2 (sST2) have emerged as novel promising HF biomarkers [15, 16]. The levels of these biomarkers can represent specific pathophysiological processes and may provide additional information beyond the current clinical indicators and established NPs [17-19]. Besides plasma proteins, circulating microRNAs (miRNAs) have been suggested as biomarkers and have been investigated in cardiovascular diseases including myocardial infarction [20], hypertension [21], diabetes [22] and HF [23]. Although the potential of miRNAs as HF biomarkers is acknowledged, there are still many unresolved aspects requiring further investigation [24]. For example, some circulating miRNAs, like miRNA-328, showed complex changes in plasma levels. This circulating miRNA is strongly diminished in HFrEF and moderately decreased in HFpEF [25], whereas it is strongly elevated in patients with atrial fibrillation and after myocardial infarction [26, 27]. Like most circulating miRNAs, this miRNA is not cardiac specific and is also altered in other diseases, probably contributing to the complexity [28]. Although several cardiac enriched miRNAs have been demonstrated to be associated with HF severity, there are issues with reproducibility and we do not understand their biology, and as a result, none of them have so far been included in AHA or ESC HF guidelines. Additional confirmation of the robustness and usefulness of these biomarkers will be needed before they can be considered for clinical usage.

Plasma biomarkers as potential therapeutic targets

The utility of plasma HF biomarkers is not necessarily limited to diagnostic and prognostic purposes, but these biomarkers could also constitute potential therapeutic targets. The

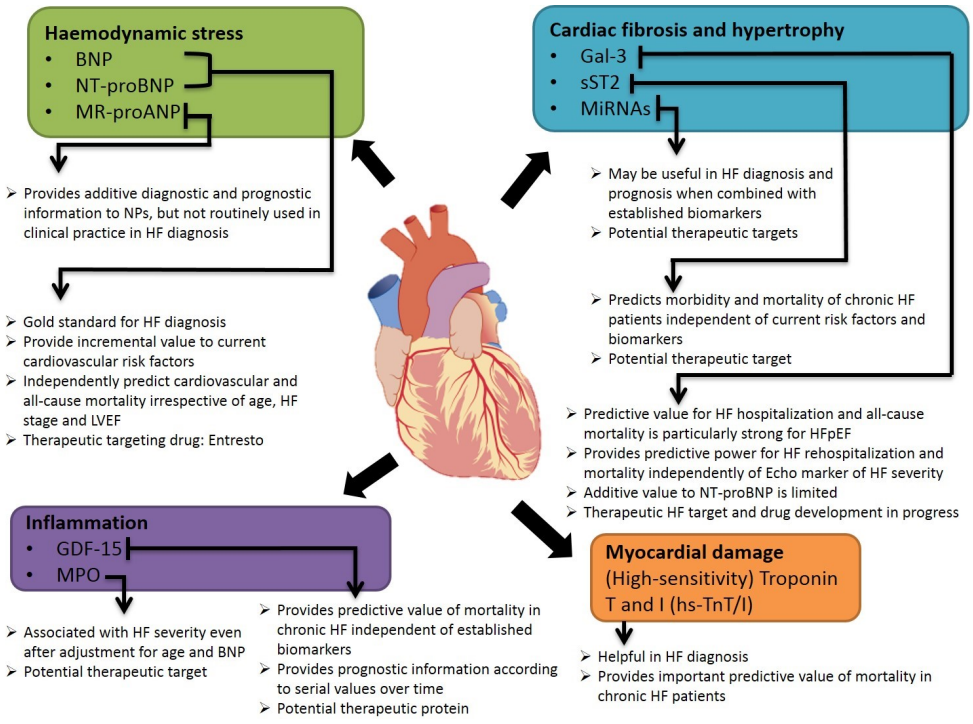

Figure 2

Functional roles and therapeutic potentials of biomarkers in HF. HF biomarkers can be classified according to their specific pathophysiological role in the progression of HF, respectively. BNP=brain natriuretic peptide; NT-proBNP=N-terminal fragment of prohormone of brain natriuretic peptide, MR-proANP=Mid-regional fragment of prohormone of atrial natriuretic peptide, GDF-15=Growth Differentiation Factor-15, MPO=Myeloperoxidase, Gal-3=Galectin-3, sST2=soluble suppression of tumorigenicity 2, MiRNAs= MicroRNAs.

beneficial effects of natriuretic peptides on vasodilatation and cardiac unloading has, for example, resulted in the generation of drugs that limit their degradation by inhibiting the peptidase neprilysin. One of the exciting new HF drugs, Entresto, consists of a neprilysin inhibitor prodrug, sacubitril and the angiotensin-receptor blocker (ARB), valsartan. Another potentially interesting drug target could be the HF biomarker Gal-3. Genetic and pharmacological inhibition of Gal-3 in mice has been shown to suppress cardiac fibrosis, cardiac remodeling and subsequent HF development [29, 30]. Myeloperoxidase (MPO), a protein released by activated neutrophils, has demonstrated to be increased in plasma levels in HF patients and is positively correlated with HF severity [31, 32]. Several studies have shown that MPO contributes to cardiac electrical and structural remodeling in post-MI or AngII infused mouse [33-35]. These results indicate that in addition to their potential as HF biomarker,

these molecules could also be therapeutic targets for treatment of HF. Again, besides protein we should also include miRNAs as potential HF targets due to their critical involvements in multiple pathological processes of HF [24, 36]. On one hand these can be easily targeted by antagomirs, on the other hand their levels can also be restored by mimics [24].

AIMS OF THIS THESIS

The main aims of this thesis are:

1) Review current HF biomarker literatures
2) Investigate miR-328 as a potential anti-fibrotic target
3) Investigate MPO inhibition as a therapeutic option for HF
4) Investigate the tissue origin and cardiac specificity of novel HF biomarkers

As discussed above, plasma biomarkers can have great value in diagnosis, prognosis and HF management, but could also act as potential drug targets. Nevertheless, there are still many uncertainties and the value of many suggested HF biomarkers is still vague. Of note, the experimental and translational research in this field is limited and this may explain in part why there is slow progression. We therefore decided to conduct translational studies using animal models to investigate the therapeutic potential of certain cardiac biomarkers and to investigate the dynamic expression of these biomarkers in the heart and other tissues to explain their plasma level fluctuations.

In **chapter 2**, we review current clinical and experimental studies regarding the diagnostic and prognostic role of the most relevant and potential new HF biomarkers, and also indicate the deficiencies of these biomarkers in the utility for HF patient identification. We address some of the common issues and propose to investigate these elusive biomarkers more comprehensively in HF animal models.

In **chapter 3**, we investigated the role of miR-328 in cardiac fibrosis post-MI. Plasma levels of miR-328 are known to be elevated after myocardial infarction, and in transgenic mice, miR-328 overexpression was shown to induce cardiac fibrosis. In this study, we showed that miR-328 was strongly induced in cardiac tissue post-MI concomitantly with cardiac fibrosis. We showed that miR-328 specific antagomirs could act as a potential anti-fibrotic target both in vitro and in vivo. In **chapter 4**, we aimed to determine whether a novel myeloperoxidase (MPO) inhibitor, AZM198, could reverse cardiac adverse remodeling in an in vivo mouse model of pressure overload after 4 and 8 weeks. Plasma MPO has been shown to be elevated in patients with HF and using mouse studies a role for MPO in cardiac fibrosis was suggested. Although

we observed a temporal delay in cardiac hypertrophy, we did not observe anti-fibrotic effects with this inhibitor. Importantly, MPO plasma levels were not increased in these mice, despite strongly reduced cardiac function, challenging its potential function as a cardiac biomarker and target. In **chapter 5**, we describe an elaborate mouse plasma biomarker study involving three different mouse HF models. In particular, we included two models of HF with reduced ejection fraction (HFrEF), namely a transverse aortic constriction and a myocardial infarction model (TAC and MI) and one model with HFpEF characteristics generated by high fat diet (HFD) and angiotensin II (AngII) infusion (obesity/hypertension). We subsequently investigated HF biomarkers ANP, Gal-3, GDF-15 and TIMP-1 at three different levels: i) organ gene expression, ii) organ protein quantities and iii) plasma protein levels, all in relation to cardiac function and structure. Surprisingly, in contrast to the established HF biomarker ANP, plasma levels of HF biomarkers (Gal-3, GDF-15, TIMP-1) did not show a direct association with cardiac function. All biomarkers were elevated in cardiac tissue in diseased hearts, but this did not affect plasma pools. In contrast, high fat diet strongly elevated plasma levels of these biomarkers, most likely as a result of elevated production in adipose tissue. In **chapter 6**, we extend these observations by investigating cardiac expression and plasma levels of these biomarkers in a transgenic rat model with hypertension (Ren2). Finally, in **chapter 7**, we discuss these new findings and view them in a broader perspective and provide recommendations for future cardiac research in this field.

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Chapter 2

Novel heart failure biomarkers: Why do we fail to exploit their potential?

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Abstract

Plasma biomarkers are useful tools in the diagnosis and prognosis of heart failure (HF). In the last decade, numerous studies have aimed to identify novel HF biomarkers that would provide superior and/or additional diagnostic, prognostic, or stratification utility. Although numerous biomarkers have been identified, their implementation in clinical practice has so far remained largely unsuccessful. Whereas cardiac-specific biomarkers, including natriuretic peptides (ANP and BNP) and high sensitivity troponins (hsTn), are widely used in clinical practice, other biomarkers have not yet proven their utility. Galectin-3 (Gal-3) and soluble suppression of tumorigenicity 2 (sST2) are the only novel HF biomarkers that are included in the ACC/AHA HF guidelines, but their clinical utility still needs to be demonstrated. In this review, we will describe natriuretic peptides, hsTn, and novel HF biomarkers, including Gal-3, sST2, human epididymis protein 4 (HE4), insulinlike growth factor-binding protein 7 (IGFBP-7), heart fatty acid-binding protein (H-FABP), soluble CD146 (sCD146), interleukin-6 (IL-6), growth differentiation factor 15 (GDF-15), procalcitonin (PCT), adrenomedullin (ADM), microRNAs (miRNAs), and metabolites like 5-oxoproline. We will discuss the biology of these HF biomarkers and conclude that most of them are markers of general pathological processes like fibrosis, cell death, and inflammation, and are not cardiac- or HF-specific. These characteristics explain to a large degree why it has been difficult to relate these biomarkers to a single disease. We propose that, in addition to clinical investigations, it will be pivotal to perform comprehensive preclinical biomarker investigations in animal models of HF in order to fully reveal the potential of these novel HF biomarkers.

Heart failure: A complex syndrome

Heart failure (HF) is a complex syndrome that is characterized by reduced cardiac function and results in insufficient cardiac output to meet peripheral tissue metabolic demands [1,2]. It is prevalent in Western society, with more than 8% of the population aged 75 and older being diagnosed with HF [1, 3, 4]. Reduced cardiac output leads to the accumulation of fluid in lungs and other tissues, resulting in breathlessness, peripheral edema, and fatigue [1]. Thus, HF is not limited to cardiac dysfunction but also affects extra-cardiac organs and tissues. Due to different etiologies and underlying pathophysiological processes, HF is a heterogeneous disease, and plasma biomarkers could potentially contribute to the improvement of patient stratification and to guide therapy. In clinical association studies, many potential HF biomarkers have been identified and investigated for their diagnostic and prognostic values. Despite these efforts, limited progress has been made in introducing these novel biomarkers into daily clinical practice. Because HF can affect multiple organs, and these novel biomarkers are not exclusively expressed in the heart, it is difficult to draw conclusions from their plasma levels and to directly associate the levels with specific indices of cardiac remodeling and function. This issue needs to be clarified, and most likely it will require preclinical investigations in animal models of HF in addition to clinical studies. Numerous excellent reviews that discuss HF biomarkers have been published [5-8], and this review is not meant to provide a complete overview of novel HF markers. Instead, we will briefly describe some novel (and established) HF biomarkers, and discuss them particularly in light of their (non-) cardiac nature and potential involvement in other diseases and conditions. We will outline challenges and pitfalls that we face and discuss why research should focus not only on clinical studies but also on preclinical studies using animal models.

Heart failure pathology

HF is the end-stage syndrome of most cardiovascular diseases, including myocardial infarction, hypertension, aortic stenosis, valve insufficiencies, and arrhythmias [1, 3, 9]. These diseases increase cardiac stress; to cope with this stress and to maintain cardiac function, morphological, structural, and functional alterations occur in the heart, a process termed cardiac remodeling [10]. Excessive extracellular matrix production (fibrosis) by fibroblasts and myofibroblasts, cardiomyocyte growth (hypertrophy), and infiltration of immune cells and elevated inflammation are the main processes that underlie cardiac remodeling [11-14]. Initially, these processes are beneficial and can be considered compensatory mechanisms, but with sustained cardiac stress, remodeling mechanisms eventually become pathological and reduce cardiac function. [10-14]. Ongoing cardiac fibrosis results in stiffening of the cardiac muscular wall, which affects cardiac relaxation and contraction, may limit oxygen and nutrient diffusion and

can disturb cardiac electrophysiology and induce rhythm disturbances [11]. Pathological cardiomyocyte hypertrophy limits cardiac function through alterations in Ca^{2+} handling, changes in excitation-contraction coupling, sarcomere dysfunction, increased oxidative stress, and metabolic and energetic remodelling [11-14]. A vicious cycle is set up in which further deterioration of cardiac function stimulates further remodeling, which eventually may result in decompensated HF [11, 15].

Different etiologies of HF result in different types of remodeling. For instance, myocardial infarction activates inflammatory pathways, stimulates replacement fibrosis and may drive eccentric hypertrophy, resulting in HF with reduced ejection fraction (HFrEF). Hypertension, on the other hand, may drive concentric hypertrophy and interstitial fibrosis, resulting in HF with preserved ejection fraction (HFpEF). Today HFpEF, which is often the result of hypertension, obesity, and aging, is becoming more prevalent [16]. A systemic proinflammatory state that causes coronary microvascular endothelial inflammation has been proposed as one of the main mechanisms that drives HFpEF development [9]. Coronary microvascular endothelial inflammation is believed to disturbe the nitric oxide balance and protein kinase G (PKG) activity in adjacent cardiomyocytes may drive sarcomeric alterations, and, together with enhanced interstitial fibrosis, promote diastolic dysfunction [9]. For HFrEF, therapeutic options that include β-blockers, angiotensin-converting-enzyme (ACE) inhibitors, and angiotensin receptor blockers (ARBs), which can slow down disease progression, are available; however, none of the current therapies have been shown to be successful in clinical trials with HFpEF. Together, this exemplifies that HF is not a single syndrome but a complex disorder, and we urgently need methods to distinguishthe different HF modalities and underlying processes.

In addition to the clinical investigation, echocardiography is an important tool to diagnose HF, and it can be used to distinguish certain types of HF and to monitor disease progression [1,17]. However, it does not provide insight in the underlying molecular and cellular processes. Plasma biomarkers have the potential to provide information about specific processes (e.g. interstitial/ replacement fibrosis, endothelial dysfunction, and pathological hypertrophy) that drive cardiac dysfunction and the transition from compensated to decompensated HF in the individual HF patient; they may also add prognostic value and help in guiding therapy.

Established heart failure biomarkers

Cardiac strain markers

Several biomarkers have been included in the guidelines for HF treatment of the European Heart Association (ESC) and American Heart Association (AHA) [1,2]. The scientific evidence

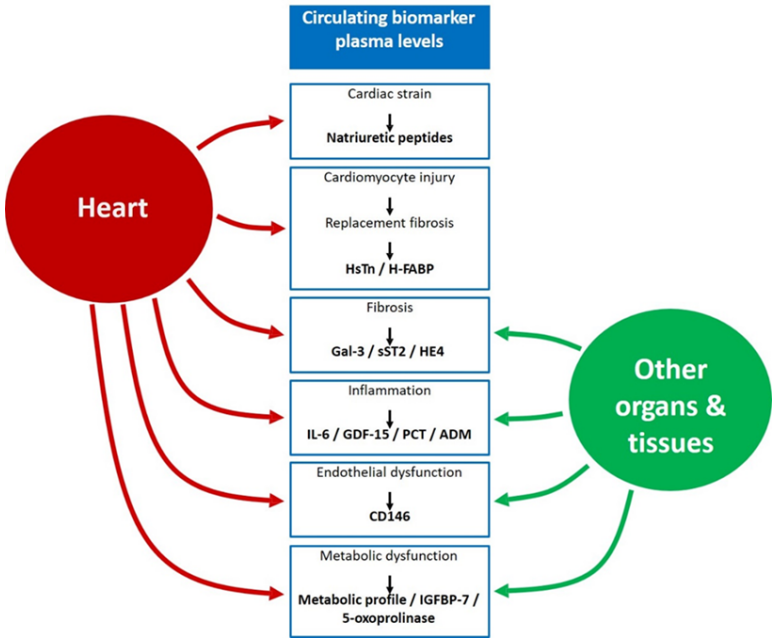

Figure 1
Novel heart failure biomarkers are not cardiac specific. A schematic depiction of the contribution of the heart and other organs and tissues to circulating plasma levels of several protein/peptide heart failure (HF) biomarkers. Only a selection of biomarkers is cardiac specific and many (novel) HF biomarkers are also produced in other organs and tissues. Within the boxes, the names of the biomarkers and associated processes are shown. Abbreviations are explained in the text.

for the use of natriuretic peptide levels is overwhelming and their use in the clinic is widely established [18]. The two most important variants, atrial-type natriuretic peptide (ANP) and B-type natriuretic peptide (BNP), are mainly produced and secreted by the atria and ventricles, respectively [18]. Cardiac wall stress, generating mechanical strain in cardiomyocytes, enhances the production and secretion of these peptides [19-21]. ANP and BNP are synthesized as proANP and proBNP precursor proteins; upon secretion into the circulation, the N-terminal inactive domains (NT-proANP and NT-proBNP) are cleaved off, releasing the active ANP and BNP hormones [22, 23]. ANP and BNP reduce peripheral vascular resistance and blood pressure by inducing a shift in fluid from the intravascular to the extravascular compartment, by promoting natriuresis, and by reducing the sympathetic tonus in peripheral vessels [18, 24]. ANP and BNP are removed from the circulation by receptor-mediated internalization and metabolisation and via proteolytic degradation by neprilysin (also termed neutral endopeptidase). Due to faster clearance of ANP by both pathways, the circulating half-life of ANP is only 3–5 minutes as compared to 23 minutes for BNP. Because of its very short half-

life and its instability in plasma, ANP is not an attractive biomarker; thus either BNP or NTproBNP (half-life 60–120 min) are currently being used as biomarkers [23, 25]. The stable mid-region of NT-proANP, MR-proANP, is also mentioned in the ESC guidelines for diagnostic and prognostic purposes, particularly in acute HF [1, 26].

Plasma levels of natriuretic peptides are widely used in the diagnosis of patients who are suspected to have HF and are valuable in the evaluation of patients with both HFrEF and HFpEF [1, 27]. Normal levels of natriuretic peptides largely exclude the presence of HF, and therefore levels are particularly useful to rule out HF,especially in the acute setting [1, 27-30].

The levels of natriuretic peptides can be influenced by other syndromes and diseases, and kidney dysfunction is an important factor that may elevate natriuretic peptide levels [31]. In addition, obesity may be associated with lower natriuretic peptide concentrations and this may modestly reduce diagnostic sensitivity in morbidly obese patients [32]. Importantly, with the positive results of clinical HF trials with entresto (LCZ-696) [33, 34], the introduction of this drug in daily clinical HF practice will make the interpretation of BNP levels in such treated patients more difficult. Entresto is made of the angiotensin-receptor blocker (ARB) valsartan, and the neprilysin inhibitor prodrug sacubitril; the latter inhibits degradation of natriuretic peptides, thereby enhancing their beneficial effect during cardiac stress [35]. The concept that the lower the BNP levels in chronic HF patients, the better the prognosis during treatment monitoring will no longer hold true in these patients. Because NT-proBNP and MR-proANP are not subject to breakdown by neprilysin, these biomarkers can still be used for patient monitoring in this setting [36].

Cardiac injury markers

Troponin I and T are another pair of proteins that are mentioned in the HF guidelines [1, 2]. Troponins are released upon myocardial damage and elevated plasma levels of troponin point to acute coronary syndrome or pulmonary embolism as the cause of acute decompensation [1, 37]. Like natriuretic peptides, the advantage of troponins is the cardiac origin of these proteins; although skeletal muscle also contains troponins, these isoforms are not detected by the cardiac specific isoform assays [38, 39]. With the development of high sensitivity cardiac troponin (hsTn) tests, elevated levels of cardiac troponin can be measured in the absence of acute myocardial damage, in particular in patients with stable chronic HF [37]. It has been suggested that troponins are also released during chronic low- grade cardiac ischemia, necrosis, apoptosis and autophagy [1, 37]. Therefore, hsTn can be elevated because of ongoing myocardial damage, which is present in patients with non-acute chronic HF, in the absence of a clear episode of myocardial ischemia [1, 37]. The example of troponin shows that, although a marker can be

tissue specific, in this case, cardiac specific, it is not necessarily disease specific (e.g. elevated in both acute myocardial ischemia due to myocardial infarction and chronic low grade myocardial damage in HF). Because dead cardiac myocytes are not renewed but are replaced by fibrosis [11], it is tempting to suggest that cardiac troponins could be considered as plasma biomarkers of ongoing replacement fibrosis in HF (Figure 1).

Table 1. Overview of selected (novel) heart failure biomarkers and associated conditions besides heart failure.

Heart failure biomarker group	Biomarkers	Biomarker level also associated with	References
Biochemical strain	Natriuretic peptides (i.e. BNP, NT-proBNP)	Fluid overload Obesity (lowering of levels) Kidney dysfunction	[18,22-24,26,31,32,192]
Cardiomyocyte injury	hsTn	Myocardial infarction	[37-39]
	H-FABP	Myocardial infarction	[99-103,105,106]
Extracellular matrix turnover and remodeling	Gal-3	Kidney fibrosis Kidney dysfunction COPD Breast cancer Gastric cancer Obesity	[7,43-58]
	sST2	Breast cancer Gastric cancer Diabetic nephropathy Liver failure	[41,42,62-66,68-71]
	HE4	Ovarian cancer Kidney fibrosis Kidney dysfunction Colorectal cancer	[72-82]
Inflammation	IL-6	Infection Post surgery Stroke	[110,111,115-119,121]
	GDF-15	Pulmonary embolism Pulmonary arterial hypertension Pneumonia Renal disease Sepsis	[126-132]
	PCT	Bacterial infection Pneumonia Systemic inflammation Sepsis Kidney dysfunction Venous congestion	[134,135,137,138]
	ADM	Sepsis Diabetische retinopathie Pneumonia COPD	[139,141-143,193-195]
Metabolism	IGFBP-7	Hepatocellular carcinoma Insulin resistance Metabolic syndrome Kidney injury Endometriosis Diabetic hemodialysis Soft tissue sarcoma COPD	[87,91-98]
Endothelial dysfunction	CD146	Pulmonary edema Peripheral venous congestion Liver cirrhosis Kidney dysfunction Atherosclerosis COPD	[146,148-159]

ADM: adrenomedullin; BNP: brain natriuretic peptide; CD146: cluster of differentiation 146; COPD:chronic obstructive pulmonary disease; Gal-3: galectin-3;GDF-15: growth differentiation factor 15; HE4: human epididymis protein 4; H-FABP: heart fatty acid-binding protein; hsTn: high sensitivity cardiac troponin; IGFBP-7: insulin-like growth factor-binding protein 7; IL-6: interleukin 6; NT-proBNP: N-terminal prohormone of brain natriuretic peptide; PCT: procalcitonin; sST2: soluble suppression of tumorigenicity 2.

The impasse of novel HF biomarkers

Extracellular matrix turnover and remodelling markers

Both natriuretic peptides and troponin show that different biomarkers can provide different types of information. Therefore, a multi-marker approach has been suggested to combine the information provided by established and novel HF biomarkers to improve the current management and evaluation of HF patients. For this purpose, besides natriuretic peptides and troponin, the biomarkers, galectin-3 (Gal-3), and soluble suppression of tumorigenicity 2 (sST2), were included in the American College of Cardiology (ACC)/AHA HF guidelines as markers of myocardial fibrosis, with a class IIb recommendation, to be considered for additional risk stratification of HF patients [1, 2]. Gal-3 and sST2 and their relation to HF have been extensively investigated and reviewed [6, 7, 40-42]. The inclusion of these novel biomarkers in official guidelines supports their possible additive value, but despite numerous years of intense investigations, the potential of these biomarkers remains vague.

Fibrosis marker Galectin-3

Galectin-3 (Gal-3) is a marker of organ fibrosis, including cardiac fibrosis [7, 43]. Plasma levels are increased in patients with HF and can have additional prognostic value to NT-proBNP levels [7, 44]. Many clinical association studies have shown that plasma levels of Gal-3 are associated with cardiac function and LV-filling pressures [45, 46]. Moreover, studies in animal models of HF revealed that Gal-3 was involved in cardiac remodeling, and both genetic disruption and pharmacological inhibition of Gal-3 resulted in reduced cardiac remodeling, including myocardial fibrosis [43, 47-52]. However, because HF is a multi-organ syndrome, other organs could also contribute to increased Gal-3 levels in HF. Gal-3 is expressed in multiple tissues and in different types of cells, including macrophages, eosinophils, neutrophils and mast cells [47, 53]. Gal-3 is also involved in renal fibrosis, as shown by multiple animal studies [51, 54], and plasma levels of Gal-3 are increased in several other diseases, including chronic obstructive pulmonary disease (COPD), and several types of cancer [55-57]. Therefore, it is likely that the observed increases in plasma levels of Gal-3 in HF are associated with increased cardiac Gal-3 production, but also with production in other organs and/or tissues. In line with this, Gal-3 is associated with HF comorbidities, including obesity [58]. Unfortunately, the HF clinical association studies and animal studies that have been performed do not provide full clarity on this matter. In some clinical studies, Gal-3 plasma levels were not directly related to specific cardiac parameters of HF, including echocardiographic parameters [59]. Moreover, a study in which endomyocardial biopsies were taken from dilated and inflammatory cardiomyopathy patients revealed that Gal-3 levels in these biopsies did not reflect plasma Gal-3 levels [60]. Finally, in HF patients with elevated Gal-3 plasma levels prior to heart

transplantation, the levels did not decline post-transplantation, which indicated that other non-cardiac sources were predominantly responsible for elevated Gal-3 plasma levels in these patients [61]. Altogether, Gal-3 is not a cardiac-specific marker and it is unclear which organs and tissues contribute to the increased Gal-3 plasma levels, and to what extent, in HF. The use of Gal-3 as a biomarker for stratification of HF patients and as a marker of cardiac remodeling should therefore be interpreted in view of this possible multi-organ contribution.

Fibrosis marker ST2

Some of the issues discussed above also apply to sST2. Several clinical studies have shown that sST2 plasma levels are increased in patients with both acute and chronic HF and are predictive for HF outcome [62, 63]. In both humans and mice, sST2 plasma levels are temporarily increased post-myocardial infarction, indicating that it could also act as a marker for myocardial injury [64]. ST2 has four isoforms, including sST2, ST2L, ST2V, and ST2LV; ST2L is the transmembrane isoform, and sST2 lacks transmembrane properties [41]. ST2L can interact with interleukin-33 (IL-33), and the ST2/IL33 interaction is involved in several diseases, including cardiovascular disease [41, 42]. Triggered by cardiac strain or myocardial injury, cardiomyocytes and cardiac fibroblasts produce IL-33, which, by binding to ST2L, exerts cardioprotective effects by reducing cardiac hypertrophy and myocardial fibrosis [41, 42]. sST2 is also produced by cardiomyocytes and cardiac fibroblasts, but it is associated with adverse cardiac remodeling via its competitive binding to IL-33, thereby limiting the protective effects of the ST2L/IL33 interaction [41,42,65]. Thus, a relationship exists between sST2 and cardiac dysfunction [41, 66]. A recent study showed that sST2 plasma levels normalized within 3 months post-implantation of a left ventricular assist device (LVAD) in endstage HF patients [67] ; this indicates that unloading the heart by LVAD placement lessens fibrosis. Because these are end-stage HF patients, many of whom will have multi-organ involvement, this finding may not be limited to the heart. In the ACC/AHA HF guidelines, sST2 has been included as a biomarker for myocardial fibrosis for further stratification of HF patients [1, 2]. Like Gal-3, increased sST2 plasma levels are also present in other diseases, including gastric cancer, breast cancer, nephropathy and liver disease [68-71]. Thus, although sST2 is able to promote cardiac remodeling locally, plasma levels of sST2 may be influenced by production in other organs in HF patients; hence circulating levels do not necessarily mirror cardiac production and remodeling.

Fibrosis marker HE4

The marker human epididymis protein 4 (HE4) is a recently discovered novel HF biomarker. HE4 is also known as the whey acidic protein four-disulfide core domain 2 (WFDC2 or WAP-4C). Though the exact function of HE4 is yet unknown, a role for HE4 in fibrosis formation

has been suggested because it shows similarities to extracellular proteinase inhibitors [72, 73]. In a mouse model of renal disease, reduced fibrosis was observed in mice treated with HE4-neutralizing antibodies [74]. In patients with both acute and chronic HF, HE4 levels were correlated with HF severity and could predict outcome in a multivariable model [75, 76]. In both studies, HE4 levels in HF were correlated with Gal-3 and, therefore, probably with organ fibrosis. HE4, however, is not cardiac specific; its expression was first identified in the epididymis and later in many other tissues and organs [72, 77, 78]. Moreover, HE4 plasma levels are associated with several types of cancer [77-79], including ovarian cancer [80], and with chronic kidney disease (CKD) severity [81]. The association of HE4 levels with kidney function has also been replicated in cohorts comprised of acute and chronic HF patients [75,76]. It has been suggested that the elevated levels of HE4 in CKD patients may complicate its use in monitoring patients with epithelial ovarian cancer [82], and the same is probably true in the HF setting. These multi-disease effects on HE4 plasma levels will mean that HE4 will not be useful for HF diagnosis, but, as part of a multi-biomarker model, it may have potential in the stratification of HF patients. HE4 has been included in such a model as an instrument to identify populations with a distinct therapy response. Patients with acute HF were investigated for response to treatment with the selective A1 adenosine receptor antagonist, rolofylline; in this study, the authors assessed tools to distinguish responders from non-responders to therapy [83]. A multi-marker model, including HE4 plasma levels, tumor necrosis factor alpha receptor 1 (TNF-R 1a), sST2 and total cholesterol, appeared to be superior to clinical characteristics, including age, sex, and cardiac function, to differentiate non-responders from responders. This study showed that multi-marker tools provide opportunities to improve clinical testing of novel drugs [83]. Moreover, this study is an example of how plasma biomarkers can be used in a multi-marker setting for stratification of HF patients.

Metabolic markers

Metabolic marker IGFBP-7

Insulin-like growth factor-binding protein 7 (IGFBP-7) can bind to insulin-like growth factor-1 (IGF-1) and, by regulating the activity of the growth hormone/insulinlike growth factor-1 (GH/IGF-1) system, it influences growth in various tissues. Its affinity for IGF-1 is relatively low compared to other IGFBPs [84,85]. It has, however, strong affinity for insulin, thereby reducing the binding of insulin to its receptor [86]. Interestingly, IGFBP-7 was investigated in a HF mouse model in which IGFBP-7 expression and plasma levels were increased in relation to cardiac hypertrophy, which showed a link between IGFBP-7 and HF development [87]. IGFBP-7 levels were also elevated in serum of patients with both HFrEF and HFpEF [88-90] and levels in these patients were also associated with prognosis [88, 89]. As a biomarker,

IGFBP-7 may be interesting especially for the HFpEF population [90]. First, IGFBP-7 has been associated with diastolic dysfunction, an important characteristic of HFpEF patients [88-90]. Second, IGFBP-7 levels were associated with insulin resistance and metabolic syndrome risk [91], which were associated with HFpEF development by causing chronic low-grade inflammation [9]. It has been suggested that, in a multi-marker approach, IGFBP-7 levels can be used to link abnormalities in cardiac function and morphology to disturbances in the metabolic status of patients [90]. Further investigations will be needed to establish this association with HFpEF. Urinary IGFBP-7 levels are, together with tissue inhibitor of metalloproteinase 2 (TIMP-2), predictive for acute kidney injury in decompensated HF and post-coronary artery bypass surgery [92-95]. Thus, in addition to being a plasma biomarker, levels in other body fluids such as urine can provide diagnostic and/or prognostic information about patients. Again, IGFBP-7 serum levels have also been associated with several other diseases, amongst them endometriosis, soft tissue sarcoma, and COPD [96-98].

Cardiac injury marker

Cell death marker H-FAB

Heart fatty acid-binding protein (H-FABP), which is produced predominantly in the heart, shows similarities to troponins as a marker. In cardiomyocytes, H-FABP is involved in cardiac metabolism through supplying mitochondria with long-chain fatty acids [99]. H-FABP is released upon ischemic myocardial damage and has been shown to be a myocardial injury marker in mice and in humans, especially in the early hours after myocardial infarction [100-103]. Interestingly, the prognostic capacity of H-FABP appears to be more accurate than hsTn levels; moreover, this also applies to patients with suspected acute coronary syndrome but with negative troponin levels [99,104]. Also, non-acute HF patients show ongoing myocardial damage and therefore, like hsTn, H-FABP is increased in chronic HF patients and will be a potential biomarker of myocardial damage [105,106]. It has been suggested that H-FABP is involved in a vicious cycle of deterioration in HF patients because extracellular H-FABP affects cardiac contraction by reducing intracellular Ca^{2+}, which leads to more damage and therefore more extracellular HFABP [99]. Indeed, increased H-FABP levels were observed in HF patients with ongoing myocardial damage, and these levels were prognostic for HF outcome [105,106]. Importantly, myocyte H-FABP levels are also influenced by exercise, plasma lipid levels, and PPARalpha agonists; hence, its intracellular levels reflect metabolic capacity [107]. In accordance with this, HFABP/mice showed a reduced tolerance to physical activity and were rapidly exhausted by exercise [108]. In cardiac tissue, reduced fatty acid uptake was observed in these *H-FABP*$^{-/-}$ mice [109]. Although speculative, this suggests that the H-FABP/troponin plasma ratio could provide information about cardiomyocyte metabolic function in HF patients;

thus, plasma H-FABP may not be limited to being a marker of only myocardial damage.

Inflammation markers

Inflammation marker IL-6

Inflammation is an important process in HF, and substances related to inflammation, such as interleukin-6 (IL-6), could serve as HF biomarkers [5,110]. In the acute phase after myocardial infarction, IL-6 elevation is beneficial because it induces anti-apoptotic mechanisms in cardiomyocytes, and it is believed to limit infarct size [110]. However, IL-6 can also alter Ca^{2+} handling, and long-term IL-6 signaling is associated with depressed cardiomyocyte function, myocardial hypertrophy, and decreased contractility [110,111]. Limiting the long-term effects of IL-6 on the failing heart by blockade of the IL-6 receptor could therefore result in improved cardiac function, and the IL-6 receptor has been identified as an HF treatment target [112]. In an ischemia/reperfusion mouse model of HF, IL-6 receptor blockade resulted in neither reduced cardiac remodeling nor smaller infarct size; however, treatment was started during the acute phase, which could explain why no effects were observed [113]. Also, in humans, inhibition of IL-6 has been tested, but it was not able to improve coronary flow reserve in patients post-myocardial infarction [114]. Nevertheless, because IL-6 has been shown to be involved in HF development, and because levels of this inflammatory marker are increased in HF and are able to predict HF outcome in various types of HF [5,115-119], it could serve as a HF biomarker. The use of IL-6 in a multi-biomarker model has been suggested [120], but circulating IL-6 levels are also affected by factors like stress, physical exercise, gender and age [119]. Moreover, circulating levels are also increased in non HF patients; for example, elevated IL-6 plasma levels were predictive for post-operative complications in patients post-abdominal surgery [121], and IL-6 levels were associated with outcome in acute stroke patients [122]. Thus, in HF, IL-6 could be a marker of inflammation in a multi-marker model, but this should be complemented by other markers to provide specificity and exclude other causes of elevated IL-6 levels.

Inflammation marker GDF-15

Growth differentiation factor 15 (GDF-15) is another inflammatory protein associated with HF. GDF-15 is a member of the transforming growth factor-beta superfamily [123]. Several studies have shown the involvement of GDF-15 in cardiac remodeling. In mouse cardiomyocytes cultured in vitro, GDF-15 expression and secretion were readily upregulated by ischemia/ reperfusion stress, which was suggestive of autocrine/paracrine functions [124]. Mice lacking GDF-15 were more prone to ischemia/reperfusion damage, which indicated that GDF-15 could have cardioprotective effects (in contrast to other markers like Gal-3 and sST2) [124]. In particular, GDF-15 deficient mice showed increased recruitment of polymorphonuclear

leukocytes to the infarct zone and had a higher chance to develop myocardial rupture [125]. GDF-15 also appears to be involved in myocardial hypertrophy, most likely through SMAD protein activation [126]. In patient cohorts, it was shown that circulating levels of GDF-15 were independent risk predictors for cardiovascular outcome [127-129]. Circulating levels are also associated with other diseases, for example, pulmonary embolism[130], pulmonary arterial hypertension [131], pneumonia, renal disease and sepsis [132]. Plasma levels of GDF-15 cannot be directly associated with myocardial inflammation, but in a multi-marker model GDF-15, could improve risk prediction as a marker of general inflammation [133].

Inflammation marker PCT

Procalcitonin (PCT) is another inflammatory marker that has been associated with HF and is under clinical evaluation [134]. PCT, the prohormone of calcitonin, is secreted by different types of cells from numerous organs in response to proinflammatory stimulation. PCT levels are strongly elevated in bacterial infections and it is an early marker for systemic inflammation, infection, and sepsis; potentially it could be used to monitor patients and guide antibiotic therapy [135]. The half-life of PCT is about 24 h, and the molecule is stable both in vivo and in vitro [136]. PCT was originally postulated to be a proxy for unrecognized infection in acute HF [135]. Based on the BACH (Biomarkers in Acute Heart Failure) trial, PCT was also included in the ESC-HF guidelines for the potential assessment of acute HF patients with suspected coexisting infection, particularly for the differential diagnosis of pneumonia and to guide antibiotic therapy [137]. Mollar et al. [138]. showed that PCT concentrations were also raised in patients admitted with acute HF with no evidence of infection and that it was associated with renal dysfunction and surrogates of venous congestion and inflammation. PCT has been shown to have prognostic value in acute HF patients, but whether this relates to concomitant infection rather than systemic inflammation requires further investigation [134]. Currently, the IMPACT-EU study (clinical trials. gov; NCT02392689), a large, multicenter, randomized controlled trial, is underway to compare PCT-guided patient management with standard management in emergency department patients with acute dyspnea and/or acute HF [134]. This study should confirm whether PCT guided antibiotic therapy will improve patient outcome by early identification of acute HF patients with elevated PCT.

Inflammation marker ADM

Another member of the calcitonin gene-related peptide (CGRP) superfamily and potential HF biomarker is adrenomedullin (ADM) [139]. ADM is a 52-amino acid multifunctional peptide that exhibits vasodilatory potential and increases renal blood flow, natriuresis, and diuresis. Also, anti-inflammatory, anti-apoptotic, and proliferative properties have been linked to ADM, and it therefore appears to exhibit protective functions under diverse pathological conditions

[139]. ADM is produced as a precursor protein called preproadrenomedullin in numerous tissues including adrenal glands, endothelium, vascular smooth muscles, renal parenchyma, and cardiomyocytes. This protein undergoes complex processing, first generating pro-ADM, which subsequently is cleaved into multiple peptides including mid-regional proADM (MR-proADM) and ADM; the latter can exist in both a bioactive amidated form (bio-ADM) and a glycated inactive form [140]. Whether MR-proADM has biological activity is unclear, but because it is more stable than ADM, it is the preferred biomarker. Like PCT, MR-proADM is strongly elevated in sepsis and could be used as a prognostic marker and to guide the diagnosis and treatment of sepsis [140]. MR-proADM lacks specificity for the diagnosis of HF, but the BACH study demonstrated that MR-proADM had superior accuracy for predicting 90-day mortality compared with BNP in acute HF [141]. Recently, a sandwich immunoassay has been developed to measure bio-ADM in plasma. Like MR-proADM, bio-ADM levels in acute HF patients were predictive for 30-day outcomes in these emergency department patients [142]. MR-proADM was also predictive for cardiovascular events in the general population [143]. Adrenomedullin is a substrate of neprilysin and hence its levels may be affected by treatment with neprilysin inhibitors; it has been suggested that the positive effects of neprilysin inhibition by sacubitril may be due in part to the inhibition of adrenomedullin and other bioactive peptides [144]. Despite many studies, there is no evidence yet that MR-proADM or bio-ADM can be used in a biomarker-guided therapeutic strategy.

Endothelial dysfunction

Endothelial dysfunction marker CD146

Cardiovascular diseases, including HF, are also characterized by endothelial damage [145,146]. Therefore, increased levels of a marker of endothelial cell damage could be a marker of disease severity. Moreover, such a biomarker could provide additional information about endothelial status. Different etiologies of endothelial injury are thought to result in the expression of different endothelial markers [146]. A novel marker of endothelial damage is soluble CD146 (sCD146; CD146, cluster of differentiation 146), which is a part of the junction between endothelial cells and which is responsible for maintaining tissue architecture [147]. Mechanical disruption of endothelial junctions probably results in shedding of the long CD146 isoform (CD146-L) present on endothelial cells, which results in sCD146 that can be found in the circulation [146,148]. sCD146 promotes angiogenesis, but also seems to be a marker of endothelial damage, atherosclerosis, and plaque instability [149-152]. In patients with acutely decompensated HF, circulating sCD146 levels were increased and could aid in diagnosing acute HF in patients who were difficult to stratify based on NT-proBNP levels only (e.g. in patients with NT-proBNP levels that were not high enough to include, but also not low enough

to exclude, HF) [153]. In animal models of cardiac pressure overload, LV CD146 gene expression was increased and correlated with lung weight and therefore with lung congestion [153]. Also, in patients with pulmonary edema, the severity of the disease on chest radiography was associated with plasma levels of sCD146 [154]. Interestingly, in a human model of peripheral venous congestion applied to one of the upper extremities of patients with chronic HF, sCD146 plasma levels increased whilst NT-proBNP remained stable [155]. It appears that circulating sCD146 levels can be related to peripheral vascular stretch, and moreover, that it is a marker of systemic congestion. Its plasma levels are also increased in liver cirrhosis, renal failure, atherosclerosis,and COPD [152,156-159]. Therefore, sCD146 seems to be a general marker of congestion and endothelial status in HF, but also in other disease.

Looking beyond circulating proteins: microRNAs and metabolites as HF biomarkers

MicroRNAs

In addition to using circulating proteins as HF biomarkers, recently several other circulating substances have been marked as potential novel HF biomarkers, including circulating microRNAs (miRNAs). The functions of miRNAs in HF, their role in the circulation and their potential as biomarkers are still elusive [160]. MiRNAs, which are post-transcriptional regulators of gene expression, were originally identified as regulators of embryonic development, including cardiac developmentt [160]. Only later, a link between activation of the fetal gene program, miRNAs and HF development was suggested [160-162]. For some solitary miRNAs, a role in pathological cardiac remodelling in animal models was found [160,163-166]. Also, in humans, the relationship between miRNAs and cardiac remodeling has been investigated. For example, myocardial and circulating miR-21 were both associated with the degree of myocardial fibrosis [167]. Several other studies showed associations between circulating miRNAs, including miR-20a, miR-208b, and miR-34a, and processes of cardiac remodeling, making them potentially interesting biomarkers [168,169]. The miRNAs, miR-22-3p, miR-148b-3p, and miR-409-3p, were also associated with HF [170,171]. Interestingly, in human HF, decreased levels of a cluster of circulating miRNAs were associated with acute HF and were inversely correlated with biomarkers associated with worse clinical outcome [172,173]. Also, lower miRNA levels were associated with worsening of renal function [174]. When this set of circulating miRNAs identified in human samples was investigated in several rodent HF models, the observations in humans could not be replicated [175]. However, closer examination revealed that these miRNAs in humans were downregulated, particularly in acute HF, and not, or to a much lesser extent, in chronic HF. Moreover, a clear association with decreased circulating miRNAs and hemodilution, as a result of fluid overload, was observed in decompensated acute HF patients; this could at least partially explain the lowered circulating

miRNA levels [176]. Also, comorbidities such as diabetes were present in the human HF cohort that were absent in the animal models. Therefore, in HF animal models, the cardiac phenotype was investigated without the influence of other HF comorbidities that may strongly affect miRNA levels. These results strongly hint that these miRNAs do not solely reflect cardiac function.

Metabolites

Metabolic dysfunction is prevalent in HF and subsequent changes in metabolite profiles could potentially be used as HF biomarkers [177]. In HF, both myocardial and systemic changes in glucose oxidation, catabolism, β-oxidation, and the urea cycle are responsible for observed alterations in metabolite levels [177]. Several studies have shown that a collection of metabolites can serve as diagnostic tools for HF [178-181]. However, changes in metabolite profiles seem not to be disease specific, because similar differences were observed in serum samples of patients with diseases such as nonHodgkin lymphoma, congestive HF, and communityacquired pneumonia (CAP) [182]. In separate studies, the levels of the metabolite, trimethylamine N-oxide (TMAO), were shown to be associated with the outcome in both acute HF and CAP patients [183,184]. This is not surprising because systemic metabolic dysfunction is a general process that can be observed in other diseases. A recent study by van der Pol et al. identified the gene, OPLAH, which encodes 5-oxoprolinase (5-oxoprolinase, ATP-hydrolyzing), as a cardiac fetal-like gene that was suppressed in HF [185]. OPLAH functions to scavenge toxic 5-oxoproline, and diminished levels of OPLAH in animal HF models resulted in elevated levels of 5-oxoproline and associated oxidative stress in cardiac tissue. This could be reversed by cardiac-specific overexpression of OPLAH. Not only cardiac, but also plasma levels of 5-oxoproline were elevated in animals. Importantly, plasma 5-oxoproline levels were also elevated in acute HF patients, and patients with elevated levels showed a worse outcome. Although OPLAH is not exclusively expressed in the heart, cardiac levels are relatively high and hence 5-oxoproline levels in the plasma may be predominantly from cardiac expression. This makes 5-oxoproline a potentially interesting metabolite and biomarker that may be less affected by interference from non-cardiac sources as compared to other metabolites.

The promise and major hurdle of new biomarkers

As discussed above, plasma biomarkers have the potential to provide information about specific processes (e.g. cardiac strain, interstitial/replacement fibrosis, endothelial dysfunction, and pathological hypertrophic processes) that drive cardiac dysfunction in the individual HF patient; they may provide added prognostic value and could be used to improve and guide therapy. However, one major pitfall in this line of reasoning is that, except for cardiac strain and cardiomyocyte specific cell death, these cellular and molecular processes are general

processes and hence hallmarks of pathophysiological processes in other organs and tissues. Because HF is a multi-system disease affecting many tissues and organs throughout the body and because it is strongly associated with comorbidities, it is likely that these stress-related processes are also induced in noncardiac tissues in these patients. Therefore, circulating levels of these plasma biomarkers in HF may demonstrate not only cardiac production but also production in other stressed tissues. Because these biomarkers monitor not only cardiac stress but also stress in other organs and tissues, it is not surprising that they have a strong prognostic value. Thus, although these novel biomarkers can provide insights in specific pathological processes, the lack of cardiac and/or HF specificity, as depicted in Figure 1 and Table 1, appears to hamper their clinical use. We postulate that this is the main reason why only the cardiac-specific HF biomarkers, especially natriuretic peptides and troponins, have found their way to the clinic, whereas non-cardiac specific markers are still under evaluation. This emphasizes that we must look at a different aspect of these novel biomarkers in order to exploit their potential. This will require more in-depth research as discussed below.

Include preclinical studies in animals in the investigation of novel HF biomarkers

In a scientific statement from the AHA, criteria for the evaluation of novel biomarkers of cardiovascular risk have been proposed [186]. It is important to determine their clinical utility and, moreover, to determine whether the novel biomarker improves clinical outcomes in a cost-effective way. In a recent paper, Ahmad et al. stated that novel biomarkers should be approached in a more systematic manner with a focus on the clinical utility of the markers [187]. These important improvements in clinical biomarker research should be embraced.

We propose an additional pillar of HF biomarker research that has been largely neglected, namely, preclinical investigations (Figure 2). As described in this review, a major issue is that most novel biomarkers are not cardiac and/or HF specific but are also associated with diseases affecting other organs and tissues. Currently, plasma levels of novel biomarkers are investigated in human clinical cohorts, providing at best associations, whilst plasma biomarkers are rarely investigated in HF animal models (preclinical studies), which could provide more causal insights. A simple PubMed search showed about ten times more published HF biomarker studies in patient cohorts as compared to preclinical HF biomarker studies. In contrast, investigations of cardiac remodeling processes such as cardiac hypertrophy and fibrosis are much more equally distributed between preclinical and clinical studies. Decreasing the gap between preclinical and clinical HF biomarker studies could provide more mechanistic insights required for proving causality (Figure 2). Preclinical animal studies could also provide us with accurate information regarding the exact tissue and cell sources that contribute to the HF biomarker plasma levels. Moreover, animal studies are well suited to investigate the effects

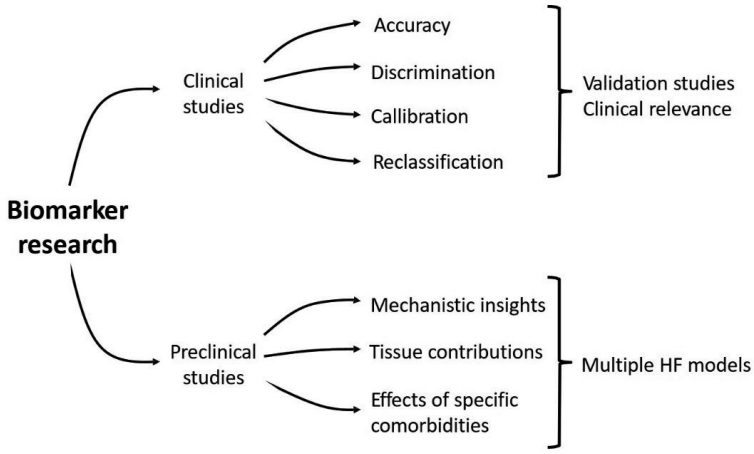

Figure 2. Two pillars of HF biomarker research. HF plasma biomarkers are currently predominantly investigated in clinical cohorts. Despite these investigations, progression in the clinical use of HF biomarkers is limited. Systematic improvements in clinical HF biomarker research have therefore been included to generate the required information [186,187]. We now propose an additional pillar of HF biomarker research, namely, the preclinical pillar, to provide additional information from studies in animals that should enhance our understanding, and together should pave the way to finally exploit these novel biomarkers. As in clinical studies, this will require systematic approaches and reports as indicated.

of comorbidities on the plasma levels of biomarkers and to investigate time-related changes both in plasma and in other tissues (Figure 2). In clinical studies, we will at best be able to perform this in end-stage patients postmortem, but this will not provide information about the dynamics during HF development. Therefore, we suggest expanding translational animal experiments in which novel biomarkers can be studied at multiple levels. In line with clinical studies, these preclinical studies should be performed in a systematic manner and reported following guidelines such as the ARRIVE guidelines for animal studies [188]. It will also be crucial to invest in appropriate reagents to study biomarkers in animal models. So far, this has been a major limitation in animal studies, and even established HF biomarkers such as NT-proBNP and NTproANP are seldom measured in mouse or rat studies because of the lack of good and affordable reagents (e.g. ELISA kits). Obviously, the small plasma volumes also make it challenging to measure these biomarkers in small animals. We believe that the current HF biomarker impasse can be broken by investing in preclinical studies to improve our understanding of these biomarkers, which finally could result in exploiting their full clinical potential.

Discussion

Established HF plasma biomarkers have proven their utility in the evaluation of HF patients, and novel HF biomarkers could further improve stratification of these patients. In the past decades, many novel HF biomarkers have been discovered and investigated in clinical trials. Despite these major efforts, only two novel biomarkers, Gal-3 and sST2, have been included in the ACC/AHA HF guidelines, but also their clinical value is still uncertain. Although these biomarkers can show specific molecular and cellular processes (e.g. fibrosis, hypertrophy), they lack cardiac and/or HF specificity. In Figure 1, a schematic depiction of the cardiac and noncardiac specificity of HF plasma biomarkers is shown.This helps to explain why the investigated novel HF biomarkers have not yet been accepted into clinical use.Therefore, as depicted in Figure 2, we suggest including more in-depth preclinical investigations in animal models to gain insight into the relationship between plasma biomarker levels and the processes of cardiac remodeling, and into the potential contribution of other affected organs and tissues. Eventually, this should result in multi-biomarker models. To increase the predictive value of multi-marker panels requires a comprehensive evaluation of a broad set of biomarkers that represent the many pathophysiological pathways involved in HF, as described here. Serial evaluation of multi-marker panels is needed to maximize their prognostic utility [189]. These models could be used both to determine the right therapy regime and to guide therapy. The idea of a multi-biomarker model is not new, but to date, no real advances have been made in developing these types of models. This is most likely because of our lack of understanding of the contributions of other tissues to biomarker levels, and preclinical studies will therefore be indispensable.

We suggest that analyzing non-cardiac specific HF biomarkers in a cardiac-specific way could be another way forward. Visualizing the local cardiac presence of these proteins and substances, for instance by using specific tracers in cardiac imaging, could provide direct information about the ongoing cardiac remodeling processes. Gal-3 could, for example, serve as a marker of myocardial fibrosis. This approach sounds futuristic, but for Gal-3, these types of tracers already exist [190,191]. Further research is needed to fully investigate this potential path to the utilization of non-cardiac specific biomarkers in a cardiac-specific manner.

In conclusion, most novel HF biomarkers provide evidence of specific molecular and cellular processes, but in a non-cardiac specific fashion. Therefore, it is still unclear whether altered plasma biomarker levels represent solely cardiac production and can be directly associated with the degree of cardiac remodeling. Clinical association studies will not provide sufficient information to solve these issues, because cardiac samples are often not available and full body biomarker profiling will not be realistic or may be impossible. As shown in Figure 2, we

therefore propose that comprehensive biomarker plasma and tissue profiling in preclinical HF models, in addition to biomarker plasma profiling in clinical cohorts, is necessary to fully reveal the potential of these HF biomarkers.

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Conflicts of interests

All authors work for the University Medical Center Groningen (UMCG), Groningen, the Netherlands. The UMCG, which employs Dr. De Boer has received research grants and/or fees from AstraZeneca, Abbott, Bristol-Myers Squibb, Novartis, Roche, Trevena, and ThermoFisher GmbH. Dr. de Boer is a minority shareholder of scPharmaceuticals, Inc. Dr. de Boer received personal fees from MadalMed Inc, Novartis, and Servier. Dr. Silljé received research grants from AstraZeneca.

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Chapter 3

MicroRNA-328, a potential anti-fibrotic target in cardiac interstitial fibrosis

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Abstract

Background: Deregulated myocardial fibrosis is associated with a wide spectrum of cardiac conditions, being considered one of the major causes for heart disease. Our study was designed to investigate the role of microRNA-328 (miR-328) in regulating cardiac fibrosis.

Methods: We induced cardiac fibrosis following MI by occlusion of the left coronary artery in C57BL/6 mice. Real-time PCR was employed to evaluate the level of miR-328. Masson's Trichrome stain was used to evaluate the development of fibrosis. Luciferase activity assay was performed to confirm the miRNA's binding site in the TGFβRIII gene. Western blot analysis was used to examine TGFβRIII, p-smad2/3 and TGF-β1 at protein level.

Results: In this study, we found that miR-328 was significantly upregulated in the border zone of infarcted myocardium of wild type (WT) mice; TGFβRIII was downregulated whereas TGF-β1 was upregulated along with increased cardiac fibrosis. And miR-328 stimulated TGF-β1 signaling and promoted collagen production in cultured fibroblasts. We further found that the pro-fibrotic effect of miR-328 was mediated by targeting TGFβRIII. Additionally, cardiac fibrosis was significantly reduced in infarcted heart when treated with miR-328 antisense.

Conclusions: These data suggest that miR-328 is a potent pro-fibrotic miRNA and an important determinant of cardiac fibrosis in diseased heart.

Introduction

In tissues composed of post-mitotic cells, like heart, new myocytes cannot be regenerated; instead, fibroblasts proliferate to fill the gaps created due to removal of dead cells. In the normal heart, cardiac myocytes are surrounded by a fine network of collagen fibers: two-thirds of the cell population is composed of non-muscle cells, the majority of which are fibroblasts [1]. A growing body of evidence indicates that, along with cardiomyocyte hypertrophy, diffused interstitial fibrosis is a key morphological feature of the structural myocardial remodeling that is a characteristic of all forms of cardiac diseases of different origins (e.g. ischemic, hypertensive, valvular, genetic, and metabolic) [2-6].

Acute myocardial infarction (AMI) due to coronary artery occlusion represents a major cause of morbidity and mortality in humans. Scar formation at the site of the infarct and interstitial fibrosis of adjacent myocardium prevent myocardial repair, contribute to loss of pump function, and predispose individuals to ventricular dysfunction and arrhythmias, which in turn confer an increased risk of adverse cardiovascular events [7, 8]. Fibrosis results in the abnormal myocardial stiffness that contributes to diastolic dysfunction, cardiomyocyte loss, arrhythmias, and the progression of heart failure. Developing novel molecular therapeutic strategies for cardiac fibrosis is still a challenge for basic and clinical scientists.

The transforming growth factor-β1 (TGF-β1), a multifunctional cytokine, mediates the signaling pathway known to be essential regulator of matrix deposition and production of collagen in the development of cardiac fibrosis. TGF-β1 stimulates cardiac fibrogenesis through its receptors. There are three prototypical TGF-β1 receptors: TGFβRI, TGFβRII and TGFβRIII (also known as betaglycan) [9]. TGFβRIII is a transmembrane glycoprotein with large extracellular regions that can bind TGF-β1, and small cytoplasmic regions with no clearly identifiable signaling motif. TGFβRIII is considered an ‘accessory' receptor, since it regulates the interaction of TGF-β1 with TGFβRI/TGFβRII [10]. Recent studies showed that TGFβRIII transduces anti-fibrotic signals through binding TGF-β1 [11, 12]. However, the molecular mechanisms for the regulation of TGFβRIII in the development of cardiac fibrosis are still poorly understood.

MicroRNAs (miRNAs) are a recently discovered class of endogenous non-coding RNAs that silence expression of protein-coding genes by acting on the 3’ untranslated region (3’UTR) of the target genes through a partial base-paring mechanism and have been implicated in a variety of biological and pathological processes including cardiovascular disease [13-15]. A subset of miRNAs is enriched in cardiac fibroblasts compared to cardiomyocytes [16]. A number of studies have demonstrated the involvement of miRNAs in regulating myocardial fibrosis in the

settings of myocardial ischemia or mechanical overload [7, 16-18].

In our previous study, we generated a transgenic mouse line with cardiac-specific overexpression of miR-328 and demonstrated that miR-328 plays a critical role in atrial fibrillation and atrial electrical remodeling process [19]. In this study, we demonstrated that miR-328 is a potent pro-fibrotic miRNA and is potentially involved in cardiac fibrosis by targeting TGFβRIII, which provides new insight into the mechanism for regulation of cardiac fibrosis and indicates that inhibition of miR-328 may be a promising therapeutic strategy for intervention of cardiac fibrosis.

Materials and Methods

Mouse model of myocardial infarction

Adult male (8 week) C57BL/6 mice used in this study were kept under controlled conditions (humidity: 55 ± 5%; temperature: 23 ± 1℃ and a 12 h light/dark artificial cycle). Mice (25-30 g) were randomly divided into sham and myocardial infarction groups. Five animals were included in each group. Myocardial infarction (MI) was established as previous described in detail [20]. Mice were anesthetized with pentobarbital (40 mg/kg) and subjected to open chest surgery. The left anterior descending coronary artery was occluded and then the chest was closed. Sham animals underwent open-chest procedures without coronary artery occlusion. All surgical procedures were performed under sterile conditions. One week after occlusion, the heart was removed for detecting the level of miR-328 and other molecules as to be specified. All experimental procedures were in accordance with and approved by the Institutional Animal Care and Use Committee of Harbin Medical University, P.R. China. The cholesterol-conjugated miR-328 antisense (antagomiR-328, sequence: 5′-ACGGAA GGGCAGAGAGGGCCAG-3′) and mismatch antagomiR-328 (M-antagomiR-328, sequence: 5′-GGCAAGACGAAACGAGACGACA-3′) was purchased from RiboBio Co., Ltd. (Guangzhou, China). The antagomiR-328 and M-antagomiR-328 were injected through mouse tail vein per 24 h at a dose of 40 mg/kg for consecutive three days before MI. One week after MI, the hearts were removed from the mice and 1-2 mm area between the infarct region and normal tissue was selected as the peri-infarct region for the following experiments.

Histochemistry

Masson’s Trichrome stain was used for the detection of collagen fibers in tissues on 4% paraformaldehyde-fixed, paraffin-embedded sections. The collagen fibers were stained in blue and the nuclei in black, and the cytoplasm was stained in red. Fibrous tissue was examined with image analysis software (Image-pro plus 4.0, Meida Cybernetics LP) as previously

described [21, 22].

Western blot analysis

Protein samples were extracted from the heart tissue as previously described[23, 24]. After grinding frozen tissue with RIPA lysis buffer (Beyotime, Jiangsu, China), cells were lysed in standard lysis buffer. After boiling the samples for 5 min, the protein samples were fractionated by SDS-PAGE (10% -15% polyacrylamide gels). Primary antibodies were used to detect TGFβRIII (Cell Signaling, Beverly, MA), p-smad2/3 (Cell Signaling, Beverly, MA), and TGF-β1 (Cell Signaling, Beverly, MA). Western blot bands were quantified using Odyssey v1.2 software by measuring the band intensity (area × OD) for each group and normalizing to GAPDH (anti-GAPDH antibody from Kangcheng Inc., Shanghai, China) as an internal control.

qRT–PCR

Total RNA was isolated from cells and cardiac tissue using Trizol reagent (Invitrogen, Carlsbad, CA) for measurements of mRNAs and miRNA, and mirVana qRT-PCR miRNA Detection Kit (Applied Biosystems, Foster City, CA, USA) was used for quantification of miRNAs according to the manufacturer's protocol. qRT-PCR was carried out as described [19]. GAPDH was used as an internal control for mRNAs quantification and U6 was used as an internal control for miRNA. All primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd (Shanghai, China).

Fibroblast culture and transfection of miRNA

Neonatal mouse myocardial fibroblasts were prepared as previously described [25]. The fibroblasts were incubated with 50 nM SB505124 (Sigma-Aldrich, St. Louis, MO, USA), a selective inhibitor of TGFβRI kinase, for 1 h before transfection of miR-328, AMO-328. miR-328 (5′-CUGGCCCUCUCUGCCCUUCCGU-3′), its antisense oligonucleotides (AMO-328, 5′-ACGGAAGGGCAGAGAGGGCCAG-3′), and a scrambled RNA as negative control for miR-328 (5'-UUCUCCGAACGUGUCACG UAA-3') were synthesized by Integrated DNA Technologies, Inc. These constructs were delivered into cardiac fibroblasts by transfection using Lipofectamine-2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. The miRNA and Lipofectamine-2000 were separately mixed with 500 ml of Opti-MEM I Reduced Serum Medium (Gibco, Grand Island, NY, USA) for 5 min. Then, the two mixtures were combined and incubated at room temperature for 20 min. The Lipofectamine-miRNA mixture was added to the cells and incubated at 37°C for 6 h. Subsequently, 4 ml of fresh medium containing 10% FBS was added to the flask. Then 5 ml fresh medium containing 10% FBS was added to the flasks and the cells were maintained in the culture until following experiments.

Measurement of collagen content

The Sircol Collagen Assay (Biocolor, Belfast, U.K.) is a quantitative dye-binding method for the analysis of total collagen extracted from tissue and cells. Briefly, cells (2×10^5) were cultured for 4 days with various treatments and then were lysed in 0.05 M Tris buffer (pH 7.5). The samples were stirred at 4°C. A transparent solution was obtained, containing salt soluble collagen. After treatments, lysate (100 µl) was stained with 1 µl of Sircol Dye reagent and mixed by rotating for 30 min. The sample was transferred to a microcentrifuge tube and spanned at 10,000 × g for 10 min. The unbound dye solution was removed carefully and 1 ml of the Alkali reagent was added into the tube. When the bound dye had been dissolved by the Alkali (approximately 10 min), the sample (200 µl) was transferred to a 96-well plate for measurement. The dye was solubilized and absorbance was read at 540 nm. Readings were converted into protein units using a linear calibration curve generated from standards (Vitrogen 100, Angiotech Biomaterials, Palo Alto, CA, USA). Total protein was quantified by the bicinchoninic acid (BCA) assay (BCA protein assay kit, Beyotime, Shanghai, China). Quantitative measurement of collagen content was normalized to the total protein. Results are presented as a ratio of collagen content to the total protein.

Luciferase assays

TGFβRIII 3'UTR containing the conserved miR-328 binding sites was amplified by PCR. The PCR fragment was subcloned into the SacI and HindIII sites downstream of the luciferase gene in PGL3 plasmid (Promega). For luciferase assays, HEK293 cells (1×10^5/well) were transfected with 100 ng target DNA (firefly luciferase vector) and 10 ng PRL-TK (TK-driven Renilla luciferase expression vector) which was used as an internal control to normalize transfection efficiencies. At the same time, miRNA was transfected into HEK293 cells using Lipofectamine-2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, the control group was only transfected with PGL3 plasmid and PRL-TK. Luciferase activity was measured using a luminometer (Lumat LB9507) and luciferase assay kit (Promega, Madison, WI) 24 h after transfection.

Statistical analysis

Group data are expressed as mean ± SEM. To analyze the differences between two groups, an unpaired Student's t-test was performed once normality had been proven (the Kolmogorov-Smirnov test); a nonparametric test (the Wilcoxon test for unpaired data) was applied when normality could not be determined due to small size sample. To analyze the differences more than two groups, a one-way analysis of variance (ANOVA) followed by a Bonferroni test was performed once normality had been proven (the Kolmogorov-Smirnov test). $P < 0.05$ was taken to indicate a statistically significant difference.

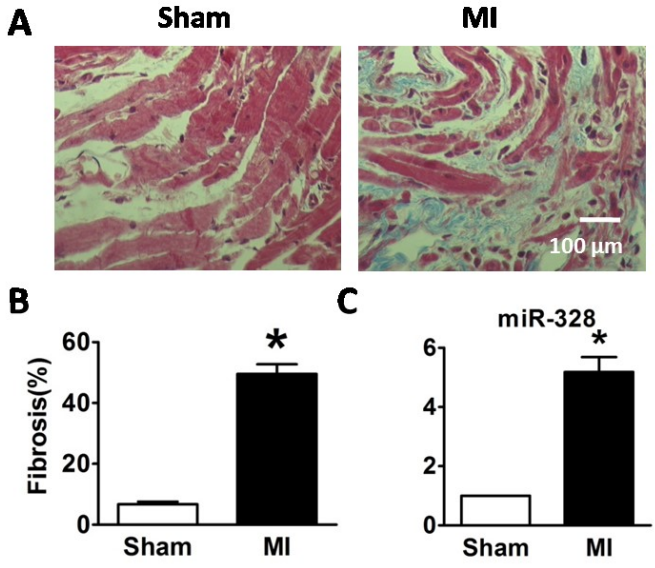

Fig. 1. miR-328 expression in the border zone of the infarcted mouse heart. **(A)** Representative Masson's Trichrome stain cardiac sections of the left ventricle 1 week after MI. **(B)** Averaged percentage of cross-sectional areas comprised of fibrous tissue. **(C)** Upregulation of miR-328 level in infarcted myocardium. n = 5, *P < 0.05 vs. Sham.

Results

MiR-328 is upregulated in the heart of mouse after myocardial infarction (MI)

To explore the potential role of miR-328 in cardiac fibrosis, we firstly determined if miR-328 expression is altered in the peri-infarct region of mice one week after MI. As shown in Fig. 1A & 1B, compared to sham group, Masson's Trichrome stain showed marked collagen production in MI mice. And, quantitative real-time PCR results showed that miR-328 level was ~5-fold higher in MI mice than in sham animals (Fig. 1C).

Effects of miR-328 on collagen production in cultured fibroblasts isolated from neonatal WT mice

TGF-β1 signaling pathway plays important roles in modulating cell function and in the progression of fibrosis. We transfected miR-328 into cultured fibroblasts isolated from neonatal mice to evaluate the effects of miR-328 on TGF-β1 signaling pathway, proteins and collagen production. Western blot analyses revealed remarkable upregulation of TGF-β1 and p-Smad2/3 at the protein level in cultured fibroblasts transfected with miR-328 (Fig. 2A &2B). Moreover, the upregulations were efficiently reversed by AMO-328 and M-miR-328 did not affect the protein levels. Consistently, miR-328 promoted production of collagen in cultured

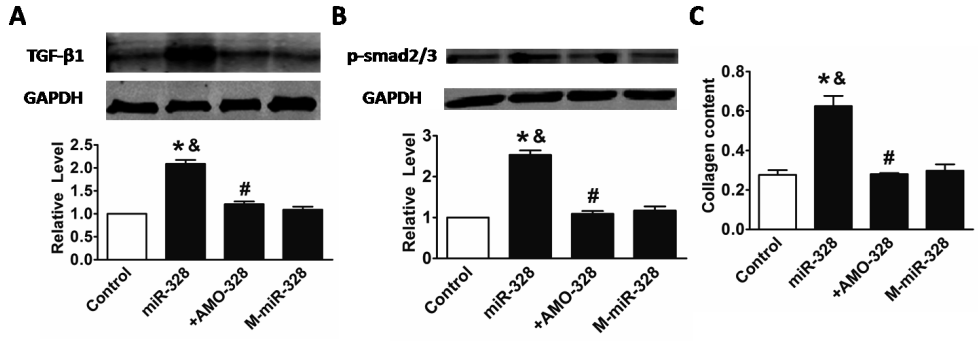

Fig. 2. Effects of miR-328 on expression of proteins in the TGF-β1 signaling pathway and collagen content in neonatal mouse cardiac fibroblasts. (A) Upregulation of TGF-β1 protein by miR-328. n = 4. **(B)** Upregulation of p-smad2/3 by miR-328. n = 5. **(C)** Increase in the collagen contents by miR-328. n = 4. * P < 0.05 *vs*. Control; & P < 0.05 *vs*. M-miR-328; # P < 0.05 *vs.* miR-328.

fibroblasts and AMO-328 nearly abolished the effect (Fig. 2C).

TGFβRIII is a direct target of miR-328 in cardiac fibroblasts

The above results suggest that miR-328 can activate TGF-β signaling and promote fibrogenesis in cultured fibroblasts. We wanted to know if the fibrosis-promoting effect of miR-328 is related to the TGF-β1 signaling mechanism. To test this notion, we first performed computational predictions of target genes for miR-328 using the TargetScan algorithm (http://genes.mit.edu/targetscan/). As anticipated, the 3′UTR of TGFβRIII contains a binding site for miR-328 (Fig. 3A) which is highly conserved among mouse, rat, and human (Fig. 3B). We then experimentally validated the targeting. We constructed a chimeric luciferase reporter gene vector by fusing the 3′UTR of TGFβRIII downstream to the luciferase gene coding sequence. We co-transfected this vector with synthetic miR-328 into HEK293 cells and measured luciferase activities 24 h after transfection. Forced expression of miR-328 resulted in marked reduction of luciferase activities and this effect was abrogated by miR-328 antisense AMO-328 (Fig. 3C). The mismatch miR-328 (M-miR-328) failed to affect luciferase activity, neither did the scrambled miRNA (SC-miRNA) as negative controls (Fig. 3C). The ability of miR-328 to repress the expression of TGFβRIII was further verified by western blot analysis of TGFβRIII protein in fibroblasts isolated from neonatal mouse hearts. The downregulation of TGFβRIII induced by miR-328 was efficiently prevented by AMO- 328. By comparison, the M-miR-328 lost the ability to downregulate TGFβRIII expression (Fig. 3D).

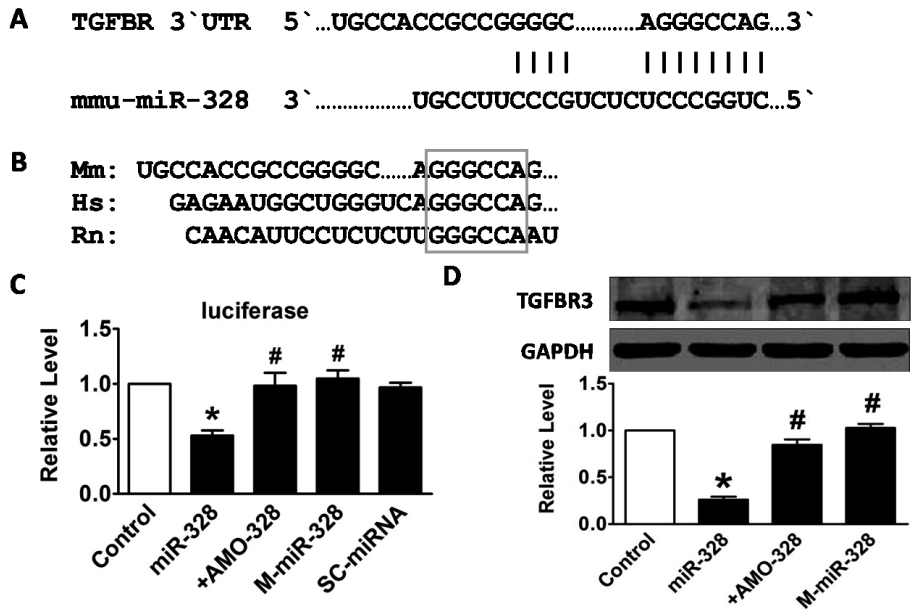

Fig. 3. Experimental verification of TGFβRIII as a target for miR-328. (A) Sequence alignment of miR-328 and the 3′UTR of the TGFβRIII gene predicted by TargetScan algorithm, showing the seed-site complementarity. **(B)** The putative miR-328-binding sites within the TGFβRIII 3′UTR are conserved among mouse, rat, and human. **(C)** Dual luciferase activity assay in HEK293 cells co-transfected with the chimeric vector and miR-328 or other constructs. n = 4. **(D)** Western blot results showing the repressive effects of miR-328 on TGFβRIII in cultured neonatal mouse cardiac fibroblasts. n = 5, * P < 0.05 vs. Control; # P < 0.05 vs. miR-328.

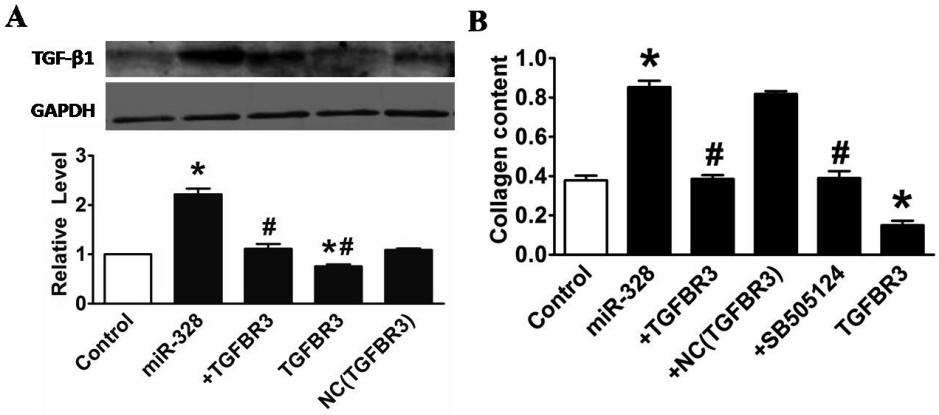

Fig. 4. TGFβRIII attenuates the pro-fibrotic effect of miR-328 in cardiac fibroblasts. (A) TGFβRIII abolished the upregulation of TGF-β1 induced by miR-328. +TGFβRIII: co-transfection of miR-328 and TGFβRIII, n = 4. **(B)** Effects of TGFβRIII on collagen contents when co-transfected with miR-328 or transfected alone. SB505124: a selective inhibitor of TGFβRI kinase. n = 4, * P < 0.05 *vs*. Controls; # P < 0.05 *vs.* miR-328.

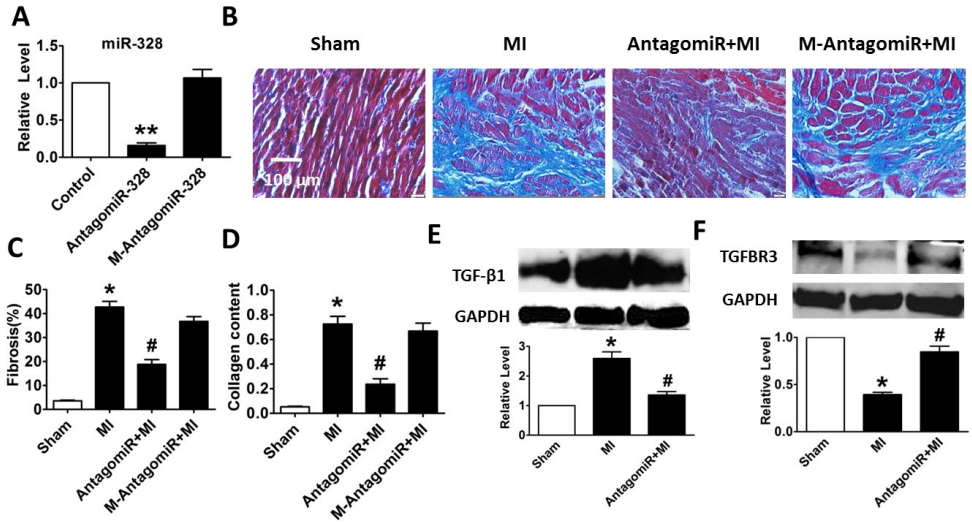

Fig. 5. miR-328 knockdown diminishes cardiac fibrosis induced by MI. **(A)** Expression of miR-328 level in mice administrated with antagomiR-328 or M-antagomiR-328. **(B)** Representative microscopic images of the left ventricular sections from Sham, MI, MI + antagomiR-328, and MI + M-antagomiR-328 mice. Fibrosis was stained in blue by Masson's Trichrome. **(C)** AntagomiR-328 abrogated cardiac fibrosis after MI and M-antagomiR-328 had no significant effect on the fibrosis. **(D)** AntagomiR-328 but not M-antagomiR-328 antagonized collagen production after MI. **(E)** AntagomiR-328 abolished the upregulation of TGF-β1 in MI. **(F)** AntagomiR-328 rescued the downregulation of TGFβRIII in MI. n = 5, * $P < 0.05$ vs. Control or Sham; # $P < 0.05$ vs. MI.

Pro-fibrotic effect of miR-328 is mediated through downregulating TGFβRIII

We subsequently confirmed TGFβRIII as a negative regulator of collagen production. Delivery of the plasmid carrying the TGFβRIII gene into the fibroblasts not only inhibited miR-328 induced expression of TGF-β1, also resulted in decreased TGF-β1 compared with control group. However, negative control (NC) failed to do so (Fig. 4A). As a consequence, when co-transfected, TGFβRIII remarkably attenuated the collagen production induced by miR-328 and when transfected alone, it diminished the collagen content compared with control. Meanwhile, SB505124, a selective inhibitor of TGFβRI kinase, abolished the effects of miR-328 (Fig. 4B). These results indicate that overexpression of miR-328 stimulates TGF-β1 signaling and promotes collagen production through targeting TGFβRIII.

Knockdown of miR-328 attenuates cardiac fibrosis in mice following MI

If miR-328 is indeed crucial to cardiac fibrogenesis, then knockdown of this miRNA should confer a resistance to fibrosis stimulation, being beneficial to improve myocardial interstitial fibrosis. To get onto this point, we assessed whether knockdown of miR-328 alleviates cardiac fibrosis induced by MI. As depicted in Fig. 5A, injection of antagomiR-328 significantly

inhibited the expression of miR-328 in the heart of mice. Furthermore, our data showed that administration of antagomiR-328 substantially diminished cardiac fibrosis (Fig. 5B and 5C) and collagen contents (Fig. 5D). As expected, mismatch antagomiR-328 (M-antagomiR-328) had no such effects. In addition, protein level of TGF-β1 was reduced by antagomiR-328 (Fig. 5E). Conversely, TGFβRIII expression was elevated by antagomiR-328 (Fig. 5F).

Discussion

In this study we evaluated the role of miR-328 in controlling cardiac fibrosis, the underlying signaling mechanisms, and the significance of this cellular function in the pathological fibrogenesis of infarcted myocardium. And for the first time, our results showed that overexpression of miR-328 increased the collagen content in the primary cardiac fibroblasts. On the contrary, normalization of miR-328 level by its antisense reversed this pro-fibrotic effect, which could be a novel approach to protect against cardiac fibrosis. MiR-328 repressed the expression of TGFβRIII protein through directly targeting its encoding gene. Knockdown of miR-328 alleviated cardiac fibrosis and rescued the TGFβRIII expression in infarcted myocardium of mice. These findings indicate that (1) miR-328 is a strong pro-fibrotic miRNA in the heart; (2) Repression of the anti-fibrotic signaling molecule TGFβRIII likely underlies the pro-fibrotic action of miR-328; (3) miR-328 is a critical determinant of fibrosis and therefore miR-328 may be considered a new therapeutic target for cardiac disease.

Recent identification of miRNAs as regulators of myocardial fibrosis has forged new frontiers in understanding cardiac conditions associated with fibrogenesis. In this conceptual framework, characterization of individual miRNAs that are specifically associated with myocardial fibrosis might allow us to develop diagnostic tools and innovative therapies for fibrogenic cardiac diseases. Van Rooij et al., [26] reported that miR-29 exerts an anti-fibrotic effect in myocardium. Artificial overexpression of miR-29b in fibroblasts results in reduced collagen expression. Conversely, downregulation of miR-29b by its antisense both *in vitro* and *in vivo* induces expression of collagens. Another study from Thum et al. showed that miR-21 is selectively upregulated in cardiac fibroblasts in the later stages of heart failure [16]. *In vivo* silencing of miR-21 in mice inhibits interstitial fibrosis and attenuates cardiac dysfunction.

In our previous study, we demonstrated that miR-328 controls atrial fibrillation (AF) via targeting CACNA1C and CACNB1 genes encoding L-type Ca^{2+} channel α1c- and β1- subunits in animal models [19]. Forced expression of miR-328 in canine atrium and in mice recapitulates the phenotypes of AF, diminishes L-type Ca^{2+} current, and shortens atrial action potential duration. However, knockdown of miR-328 level with its antisense oligonucleotide reverses the effects both *in vivo* and *in vitro*. Interestedly, histological examination reveals progressive

fibrosis in the heart of miR-328 transgenic mice, which indicates that miR-328 plays an important role in the process of cardiac fibrosis. We also found that miR-133 and miR-590 contribute to nicotine-induced AF by targeting TGF-β1 and TGF-βRII. Upregulation of miR-133 and miR-590 decreases TGF-β1 and TGF-βRII levels and collagen content. These effects are abolished by antagomiRs against miR-133 or miR-590. In the present study, we presented strong evidence for miR-328 as a pro-fibrotic miRNA critically contributing to the increased cardiac fibrosis in ischemic myocardium through directly repressing TGFβRIII to indirectly upregulate TGF-β1.

TGFβRIII displays different biological functions to mediate TGF-β pathway as an activator [27] or inhibitor [12, 25] dependent on cell and tissue types. Eickelberg et al. reported that TGFβRIII inhibits TGF-β pathway activity in LLC-PK1 renal proximal tubule cells and enhances TGF-β pathway activity in L6 myoblasts [27]. Recent studies provide more evidence that TGFβRIII exhibits antagonistic activities of TGF-β1 signaling by preventing TGFβRI-TGFβRII complex formation. Our previous study showed that TGFβRIII negatively regulates TGF-β pathway by neutralizing TGF-β1 and preventing the formation of TGFβRI/ TGFβRII which in turn attenuates collagen production in cardiac fibroblasts [25]. Moreover, a synthetic peptide from TGFβRIII can inhibit TGF-β1-induced collagen I synthesis by blocking the binding of TGF-β1 to its type I and type II receptors in fibroblast cells [27]. Hermida et al., further demonstrated that this soluble peptide exerts significant inhibition on TGF-β1 dependent signaling activity, collagen type I synthesis, and myocardial fibrosis, in addition to blocking the binding of TGF-β1 to its type I and II receptors [28]. A study by Vilchis-Landeros et al., demonstrated that this soluble peptide binds TGF-β with high affinity and isoform selectivity to block TGF-β2 and TGF-β1 and therefore inhibits the actions of TGF-β [29].

Our results show that miR-328 expression is upregulated in the peri-infarct region. Correspondingly, the level of TGFβIII is downregulated whereas that of TGF-β1 is upregulated after MI. More importantly, application of miR-328 antisense increases TGFβRIII level and reduces collagen contents and protein levels of TGF-β1. These findings suggest that miR-328 plays an important role in cardiac fibrogenesis in ischemic heart as a pro-fibrotic miRNA and reveal a novel signaling mechanism of miRNA in controlling the generation of cardiac fibrosis.

There are a couple of limitations in the present study. The experiments were performed with 5 animals in the study. However, adequate sample size can produce more precise results. Myocardial infarct size is closely related to the cardiac fibrosis. Measurement of infarct size and heart function will provide more evidence to evaluate role of miR-328 in regulation of cardiac fibrosis induced by MI.

In summary, our results indicate that miR-328 as a pro-fibrotic factor plays a critical role in regulation of cardiac fibrosis following myocardial infarction by targeting TGFβRIII. These findings provide new insight into the mechanism of cardiac fibrosis, and miR-328 may be a new target for therapy of heart disease associated with cardiac fibrosis.

Acknowledgements

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Disclosure Statement

The authors state no conflict of interest.

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Chapter 4

A novel oral available myeloperoxidase (MPO) inhibitor delays cardiac remodeling in a pressure overload mouse model

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Manuscript in preparation

Abstract

Background: In patients myeloperoxidase (MPO) plasma levels are associated with heart failure severity. Mice deficient for MPO are less prone to atrial fibrillation and show diminished cardiac dilatation after infarction. In this study we treated mice with a novel MPO inhibitor, AZM198, and investigated its effects on cardiac remodeling.

Methods: Mice were subjected to transverse aortic constriction (TAC) or sham operation and AZM198 was provided via chow. After 4 or 8 weeks magnetic resonance images (MRI) and hemodynamic data were generated and cardiac tissue was collected for further analysis. Food intake was similar between all groups and AZM198 could be detected in the blood plasma of the AZM198 treated groups.

Results: AZM198 treatment significantly reduced MPO levels in both sham and TAC groups. TAC did, however, not elevate plasma MPO levels. Nevertheless, MRI showed significantly less dilatation in the 4 weeks TAC group treated with AZM198. In addition cardiac hypertrophy was temporarily attenuated in TAC animals, as determined by heart weight and cardiomyocyte cross sectional area and this was paralleled by attenuated fetal gene expression (NPPA) in these mice. At 8 weeks, differences between AZM198 and control TAC group were not significant anymore. ECM deposition was similarly increased in all TAC groups. Nevertheless, expression of some ECM remodeling genes were temporarily attenuated in the AZM198 TAC group.

Conclusions: In conclusion, the absence of MPO elevation after TAC indicates that it is not a main driver of cardiac remodeling. Nevertheless, AZM198 was able to delay cardiac dilatation, indicating that MPO influences the rate of cardiac remodeling.

Introduction

Heart failure (HF) is a clinical symptom in which the pump function of the heart is not sufficient to fulfill the body demands. This will result in a severe reduction in quality of life and may culminate in death. Despite advances in (HF) treatment, morbidity and mortality are still extremely high and 50% of all patients die within 5 years after diagnosis [1]. Cardiac remodeling, as a result of myocardial infarction, hypertension, valve insufficiency or other sustained cardiac stress factors, is the underlying cause of heart failure development [2]. Cardiomyocyte hypertrophy (growth) and excessive extracellular matrix deposition, fibrosis, are the most prominent processes driving cardiac remodeling. Sustained ventricular wall stress, (neuro)hormonal activation and inflammation are strong inducers of these pathological cardiac remodeling processes [3]. Low grade systemic inflammation is often observed in heart failure patients and is associated with poor outcome [4].

Myeloperoxidase is an important enzyme in the innate immune system and mainly present in neutrophils. This enzyme is released into the circulation upon neutrophil activation and is able to generate highly toxic hypochlorous acid (HOCl) from hydrogen peroxide. HOCl contributes to the inactivation of pathogens and deficiency of MPO activity leads to increased susceptible to various fungal and bacterial infections, both in human and in mice [5, 6]. There is ample evidence that besides its function in host defense, MPO also controls other processes relevant to health and disease, including cardiac remodeling and HF [7]. MPO polymorphisms have been shown to be associated with Alzheimer disease and cardiovascular diseases (CVDs) [8, 9]. MPO levels are strongly associated with HF, even after adjustment for age and the heart failure biomarker brain natriuretic peptide (BNP) [10]. Moreover, MPO levels have been shown to be associated with HF severity [11]. Although the exact actions of MPO on the heart are not well understood, it is generally believed that MPO mediated oxidation of circulatory and cellular proteins modulates inflammatory and fibrotic processes and cellular signaling [12]. Studies in MPO knockout mice have shown that MPO prevents cardiac dilatation post myocardial infarction [13, 14]. In an angiotensin II (AngII) infusion model it was shown that MPO contributes to atrial fibrosis [15]. Together these data indicate that MPO could contribute to progressive cardiac remodeling and that targeting of MPO by small molecules could be a promising strategy to suppress progressive cardiac remodeling.

In this study, we used a novel oral available MPO inhibitor, together with a mouse transverse aortic constriction (TAC) pressure overload mouse model to induce cardiac remodeling. The effect of MPO inhibition on cardiac remodeling in this clinical relevant mouse model of pressure overload was subsequently investigated by MRI and intra-cardiac pressure

measurements. It is shown that MPO inhibition can temporarily attenuate cardiac remodeling and delay dilatation in this TAC model. Although the exact mechanisms remain vague, this study provides a rationale to further explore this novel treatment option in heart failure models.

Materials and Methods

Mouse experimental protocol

Animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethical Committee of the University of Groningen. (permit number: DEC6920A). Male mice were housed on a 12/12 hours day/night cycle in a controlled environment and ad libitum access to water and chow. At an age of 8-10 weeks mice were randomized based on body weight to generate 4 equal groups (Figure 1A). Mice underwent either a transverse aortic constriction (TAC) to generate cardiac pressure overload or as a control were sham operated, as described before [16]. In summary, mice were anesthetized with 2% isoflurane/oxygen, intubated and mechanically ventilated (Minivent, type 845, Harvard apparatus, Massachusetts, USA) and placed on a heated pad to maintain adequate body temperature. After skin disinfection, a 0.5-1.0cm incision was made to the chest. The thoracic cavity was opened between the second and third rib. Thereafter, a reproducible stenosis was created by tightening a 7-0 silk suture around the aortic arch between the brachiocephalic and left carotid arteries and a blunt 27G needle. After ligation this needle was removed and the thoracic cavity and skin were closed using sutures. To reduce post-operative pain, all mice received a subcutaneously dose of carprofen (5.0mg/kg) directly after operation. Sham procedures were identical except for the aortic arch ligation. After surgery, mice were placed in clean cages and chow with or without AZM198 was provided. Mice were regularly monitored and sacrificed at 4 or 8 weeks as shown in Figure 1A.

Cardiac MRI measurements

Approximately 4 and 8 weeks after surgery, MRI measurements were performed using a vertical 9.4-T, 89mm bore size magnet equipped with 1500 mT/m gradients combined with a 400 MR system (Brucker Biospin, Ettlingen, Germany). Mice were anesthetized during the whole procedure (induction: O_2, isoflurane 5%, maintenance: O_2, isoflurane 2%). With an ECG trigger unit (RAPID biomedical GmBH, Germany), respiration and cardiac electrophysiology, including heart rate, were monitored. By adjusting the dosage of anaesthetics, heart rate and respiration rate were maintained between 400-600 and 20-60 per minute, respectively. Depending on the size of the heart, 8-9 slices/images were recorded to cover the whole heart. MRI acquisition and reconstruction was performed using ParaVision and IntraGate software (Bruker BioSpin GmBH, Germany). LV end-diastolic volume (LVEDV) and LV end-systolic

volume (LVESV) were determined using semi-automatic contour detection software (QMass, version MR 6.1.5, Medis Medical Imaging Systems, the Netherlands). This data was used to calculate stroke volume (SV) by subtracting LVESV from LVEDV. Subsequently, ejection fraction (EF) was calculated by dividing SV by LVEDV.

PV-loop measurements

Prior to sacrifice, heamodynamics were recorded by aortic and LV cathetherization. During this procedure, mice were anesthetized with 2% isoflurane/oxygen and catheterization was performed with a Millar pressure transducer catheter (Mikro-Tip pressure catheter 1.4F, Transonic Scisense, Transonic Europe, The Netherlands). The catheter tip was inserted via the left carotid artery and pressures in the aorta and LV were monitored. Parameters of cardiac function were recorded, including maximal LV pressure (LV P_{max}), minimal LV pressure (LV P_{min}), dP/dT_{max} (an indicator for maximal LV contraction capacity), dP/dT_{min} (an indicator for maximal LV relaxation capacity), Tau (a measure for the isovolumetric relaxation of the LV), maximal aortic pressure (aorta P_{max}) and heart frequency (HF). Thereafter, the catheter was removed and animals were sacrificed and tissues and organs were collected for molecular and histological analysis.

Enzyme-linked immunosorbent assay (ELISA)

Plasma MPO levels were determined using MPO ELISA kits (Hycult biotech, the Netherlands). All used reagents and buffers were supplied in the kit and were prepared for analysis as described in the manual. Plasma samples were thawed, mixed and diluted 20 times with dilution buffer. Next, 100µL of standard, diluted samples and controls were transferred to MPO-antibody coated ELISA plates and plates were processed according to the manufacturer instructions. Tetramethylbenzidine (TMB) was used as a substrate in the final peroxidase reaction and absorbance of samples, standards and controls was measured at 450nm using a plate reader (Synergy H1 microplate reader, Biotek, Vermont, USA). Plasma MPO levels were calculated using GEN5 software (GEN5 version 2.04, Biotek, Vermont, USA).

Complete blood count

Complete blood count was determined using a hematology analyzer (pocH-100i automated hematology analyzer, Sysmex, Illinois, USA). EDTA tubes with full blood were analyzed within 48 hours after collection. White blood cell concentration (WBC), red blood cell concentration (RBC), mean corpuscular volume (MCV), hemoglobin (Hb) and platelet concentration (PLT) were determined.

Plasma levels of AZM198

Blood plasma AZM198 levels were determined in EDTA plasma in a blinded fashion by Astra Zeneca. These measurements were performed using a validated HPLC-MS protocol.

Histological assessment of fibrosis and cardiomyocyte size

Mid-transverse sections of the LV were fixed in 4% paraformaldehyde for paraffin embedding. Sections of 4 μm were stained with either Masson's trichrome for collagen detection, or FITC-labeled wheat germ agglutinin (WGA) (Vector laboratories, Burlingame, CA, USA) to quantify myocyte cross-sectional area (CSA). Whole Masson's trichrome stained sections were automatically imaged using a Nanozoomer 2.0 HT (Hamamatsu, Japan). Fibrosis fraction as a percentage of the entire section was quantified from a 20 fold magnification (ScanScope, Aperial Technologies, Vista, CA, USA). Cardiomyocyte size was determined on WGA stained sections mounted in DAPI mounting medium, in order to visualize the nucleus. Five randomly selected fields from the WGA-stained LV sections were imaged using a Leica DMI6000B inverted fluorescent microscope (Leica Microsystems B.V., Amsterdam, The Netherlands) and approximately 50 cells per mouse heart were used to measure CSA using Image J software (NIH, Bethesda, MD, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Ribonucleic acid (RNA) was extracted from powdered tissues using Trizol reagent (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA). cDNA was synthesized using QuantiTect Reverse Transcriptional kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. Relative gene expression was determined by qRT-PCR on the Bio-Rad CFX384 real time system (Bio-Rad, Veenendaal, the Netherlands) using ABsolute QPCR SYBR Green mix (Thermo Scientific, Landsmeer, the Netherlands). Gene expressions were corrected for reference gene values (*36B4*), and expressed relative to the control group. Primer sequences used are depicted in supplemental table 1.

Western blot

Protein was isolated with RIPA buffer (50 mM Tris pH 8.0, 1% nonidet P40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl) supplemented with 40 ul/ml phosphatase inhibitor cocktail 1 (Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands), 10 ul/ml protease inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN, USA) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Roche Diagnostics Corp., Indianapolis, IN, USA). Protein concentrations were determined with a DC protein assay kit (Bio-Rad, Veenendaal, the Netherlands). Equal amounts of proteins were separated by SDS-PAGE and proteins were transferred onto PVDF membranes. The following antibodies were used: Phospho-Akt (Ser473)

(#4060S), Akt (#4691S), p44/42 MAPK (Erk1/2) (#4695), p38 MAPK (#9212), phosphor-p38 MAPK (Thr180/Thr182) (#9211), S6 (#2217), Phospho-S6 (#2211) (Cell Signaling); P-Erk (#sc-7383) (Santa Cruz); glyceraldehyde-3-phosphate dehydrogenase (10R-G109A, Fitzgerald, USA). Signals were visualized with ECL and analyzed with densitometry (ImageQuant LAS4000, GE Healthcare Europe, Diegem, Belgium).

Statistical analysis

All values are presented as means ± standard errors of the mean (SEM). Student's paired two-tailed *t*-test was used for two-group comparisons, and one-way analysis of variance (ANOVA) for multigroup comparisons, followed by Tukey's post-hoc correction. For non-normally distributed data or data without homogeneity of variance non-parametric tests were performed. In this case Mann-Whitney tests were used for two group comparisons and Kruskal-Wallis followed by Mann-Whitney tests for multiple group comparisons. $P < 0.05$ was considered to be significant. SPSS software (IBM SPSS statistics, version 22, IBM, USA) was used for statistical analyses.

Table 1. Hemodynamic parameters of the 4 weeks groups

	Sham	**TAC**	
	Ctrl/AZM198	Ctrl	AZM198
Hemodynamics	(N=20)	(N=9-10)	N=8
Heart rate (bpm)	446,3 ± 13	484 ± 15	480 ± 14
SAP (mmHg)	99,8 ± 1,2	147,3 ± 7,5*	149,3 ± 7,5*
LV P_{max} (mmHg)	102,9 ± 1,2	141,3 ± 5,1*	151,5 ± 5,2*
LVESP (mmHg)	97,4 ± 1,2	137,4 ± 5,4*	143,0 ± 5,0*
LVEDP (mmHg)	4,2 ± 0,7	13,9 ± 4,0*	9,7 ± 2,6*
Corrected dP/dT_{max} (1/s)	74,1 ± 2,6	48,8 ± 3,7*	52,6 ± 2,2*
Corrected dP/dT_{min} (-1/s)	-80,7 ± 3,4	-50,0 ± 2,8*	-58,1 ± 3,8*
Tau (ms)	6,6 ± 0,3	8,2 ± 0,8	6,9 ± 0,6

Data are presented as mean ± standard error of the mean. HR = heart rate (bpm); SAP = systolic arterial pressure (mmHg); LV P_{max} = maximal left ventricular pressure (mmHg); LVESP = left ventricular end-systolic pressure (mmHg); LVEDP = left ventricular end-diastolic pressure (mm Hg); Corrected dP/dT_{max} = maximal left ventricular contraction corrected by maximal ventricular pressure (1/s); Corrected dP/dT_{min} = maximal left ventricular relaxation corrected by maximal ventricular pressure (-1/s); TAC: transverse aortic constriction; AZM198 = Myeloperoxidase inhibitor. N=8-12. * P<0.05 as compared to the respective non-treated AZM198 group.

Table 2. Hemodynamic parameters of the 8 weeks groups

	Sham	**TAC**	
	Ctrl/ AZM198	Ctrl	AZM198
Hemodynamics	N=19	N=8	N=10
Heart rate (bpm)	507 ± 17	502 ± 9	511 ± 11
SAP (mmHg)	99,1 ± 1,3	133,0 ± 5,6*	138,3 ± 6,0*
LV P_{max} (mmHg)	104,0 ± 1.3	131,3 ± 6,2*	142,4 ± 6,1*
LVESP (mmHg)	88,6 ± 3,3	119,1 ± 9,7*	136,0 ± 6,0*
LVEDP (mmHg)	5,2 ± 0,9	22,0 ± 2,0*	13,7 ± 2,7* #
Corrected dP/dT_{max} (1/s)	89,6 ± 4,3	44,9 ± 2,0*	50,3 ± 1,6*
Corrected dP/dT_{min} (-1/s)	-91,7 ± 4,2	-39,9 ± 1,6*	-46,8 ± 3,2*
Tau (ms)	6,2 ± 0,3	11,8 ± 0,8*	9,7 ± 1,1*

Data are presented as mean ± standard error of the mean. HR = heart rate (bpm); SAP = systolic arterial pressure (mmHg); LV P_{max} = maximal left ventricular pressure (mmHg); LVESP = left ventricular end-systolic pressure (mmHg); LVEDP: left ventricular end-diastolic pressure (mm Hg); Corrected dP/dT_{max} = maximal left ventricular contraction corrected by maximal ventricular pressure (1/s); Corrected dP/dT_{min} = maximal left ventricular relaxation corrected by maximal ventricular pressure (-1/s); TAC: transverse aortic constriction. N=8-12. * P<0.05 as compared to the respective non-treated AZM198 group.

Results

AZM198 and MPO levels in a mouse TAC model

Prior to TAC and AZM-198 treatment all mice had comparable measures of body weight. Directly after TAC or sham operation mice were transferred to a normal chow diet or an identical diet including the MPO inhibitor, AZM198. Food intake was similar between the control- and AZM198-chow diet groups and also body weights were similar (Supplemental Table 2-4). After 4 and 8 weeks mice were sacrificed and blood plasma levels of AZM198 and MPO were determined. As shown in Figure 1B, D, AZM198 could be detected in the plasma of AZM198 treated mice and reached a level of approximately 1 μM. No AZM198 could be detected in the chow control groups. AZM198 also reduced MPO plasma protein levels itself and although this does not directly reflect MPO activity it further supports proper targeting of MPO in our mouse models (Fig. 1C, E). No assays are available for MPO specific activity measurements in blood plasma. However, since MPO activity can be specifically determined in peritoneal fluid [17], a separate experiment was used to show AZM198 mediated MPO inhibition after zymosan induced peritonitis. As shown in Supplemental figure 1, a 1 uM plasma AZM198 concentration, as observed in this study (Fig. 1B, D), diminished peritoneal MPO activity by approximately 95%. Our data, also revealed that TAC mediated pressure overload did not result in elevated MPO levels (Fig. 1C, E). Together these results show that MPO plasma levels are not influenced by TAC mediated pressure overload, but that AZM198 can significantly attenuate baseline MPO levels in blood plasma. No significant differences were observed in the sham AZM198 and sham control groups in the parameters discussed hereafter. Thus, except for MPO levels, AZM198 did not affect any other measured parameters in the sham groups and for simplification sham groups (control and AZM198) were combined for each time point (for comprehensiveness, all separate sham group values are included in Supplemental Table 3 & 4).

AZM198 attenuates cardiac dilatation

In all TAC groups left ventricular (LV) weight corrected by tibia length were significantly increased as compared to the sham groups (Fig. 2A-B). Also, atria weight increased significantly (data not shown), but right ventricular weight and lung weight showed an increase only at 8 weeks, indicative for progressive remodeling of the heart and of lung congestion Fig. 2C-F). Spleen weight was not affected (Fig. 2G-H). At 4 weeks the increase in LV and atrial weights were significantly lower in the AZM198-TAC4 group as compared to the Ctrl-TAC group (Fig. 2A). At 8 weeks this difference was not anymore significant (Fig. 2B). Cardiac imaging by MRI, confirmed an increase in cardiac dimensions after TAC in all groups (Fig.3A).

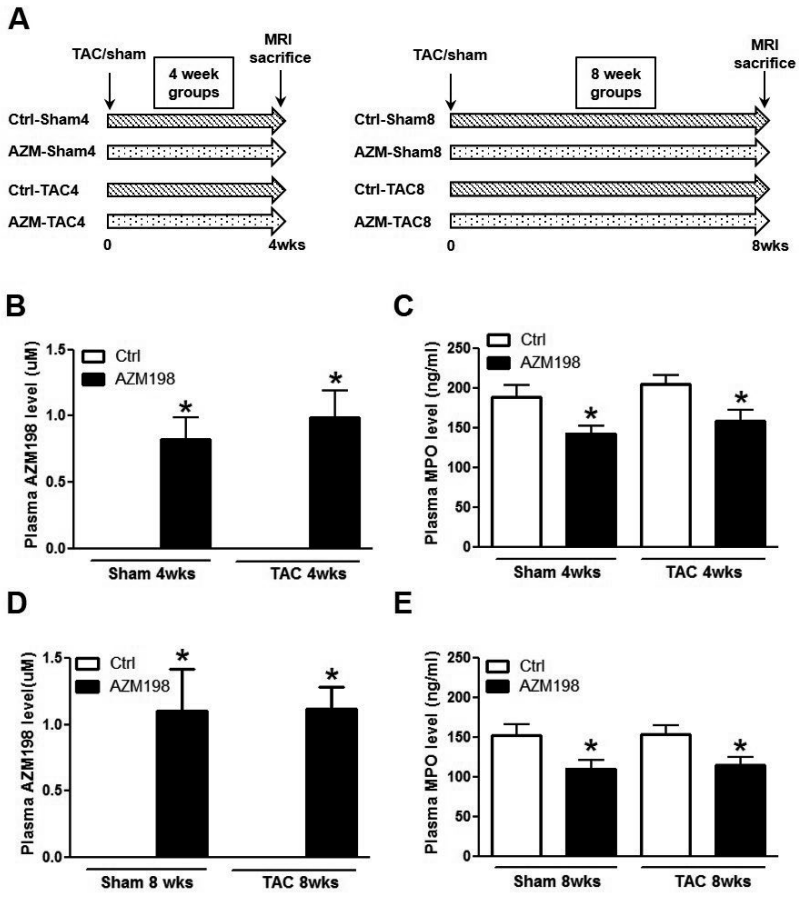

Figure 1. AZM198 and MPO levels in control and TAC mice. (A) Schematic depiction of all mice groups used in this study. **(B)** AZM198 levels in control (Ctrl) and AZM198 (AZM) treated animals after 4 weeks of sham or TAC operation. **(C)** MPO plasma levels after 4 weeks of sham or TAC operation. **(D)** The same as (B), but after 8 weeks. **(E)** The same as (C), but after 8 weeks. n=8-11 * $p<0.05$ as compared to the respective non-treated AZM198 group.

In particular, the left ventricular end-diastolic (LVEDV) and end-systolic volumes (LVESV) were significantly increased and consequently the ejection fraction (EF) was decreased (Fig 3B-G). At 8 weeks LV diastolic and systolic volumes were even higher supporting progressive remodeling. Importantly, AZM198 partially prevented this cardiac dilatation and the reduction in EF was significantly less in the AZM-TAC4 group, as compared to the Ctrl-TAC4 group (Fig. 3B-D). At 8 weeks these effects of AZM198 were no longer significant (Fig. 3E-G). Thus, AZM198 treatment temporarily attenuated or delayed cardiac dilatation in mice subjected to cardiac pressure overload.

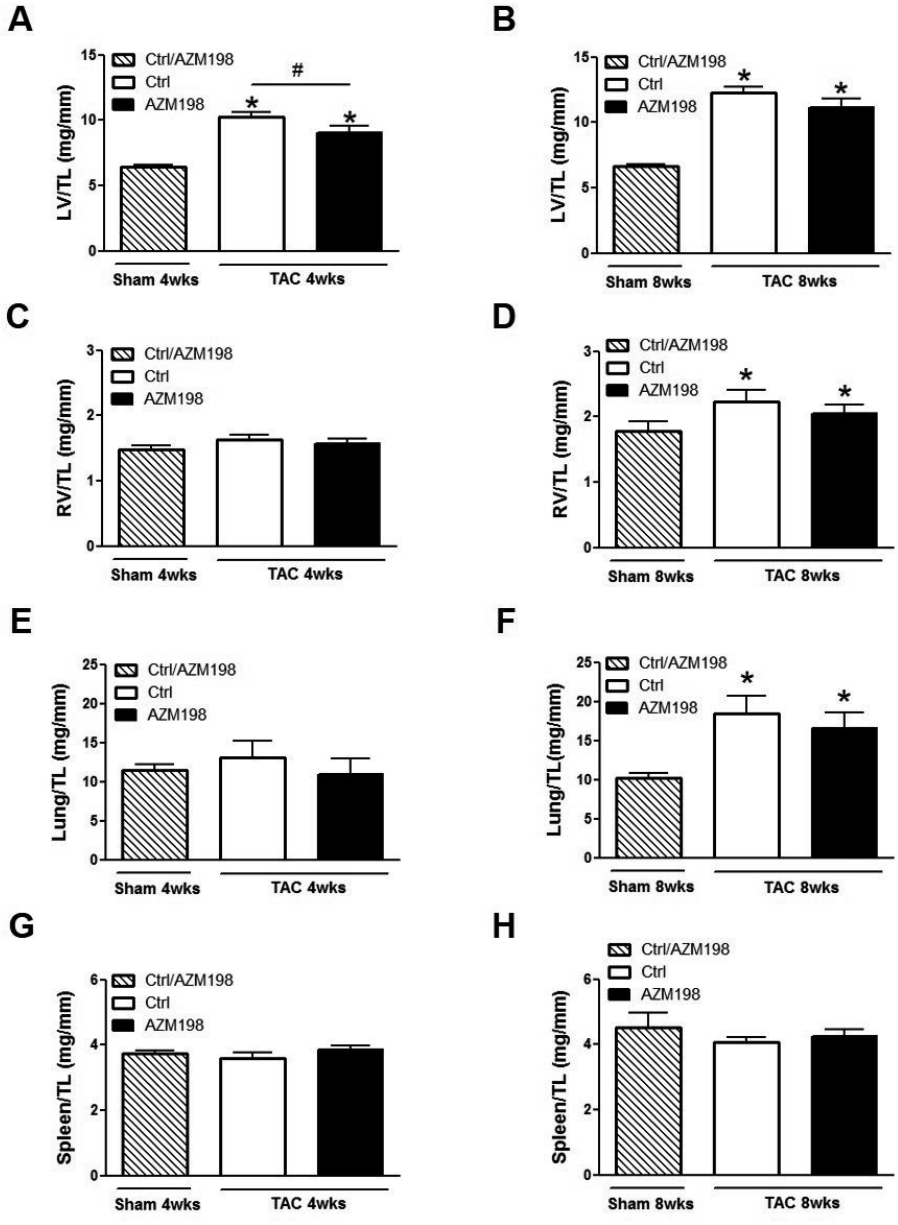

Figure 2. Organ weights. (A-D) Quantification of the left ventricular (LV), right ventricular (RV), lung and spleen weights, respectively, of the 4 week groups. All weights were corrected by tibia length. E-H Quantification of the left ventricular (LV), right ventricular (RV), lung and spleen weights, respectively, of the 8 week groups. All weights were corrected by tibia length. n= 7-20, * $P<0.05$ as compared to Ctrl/AMZ198 group, # $P<0.05$ as compared to the TAC non-treated AZM198 group.

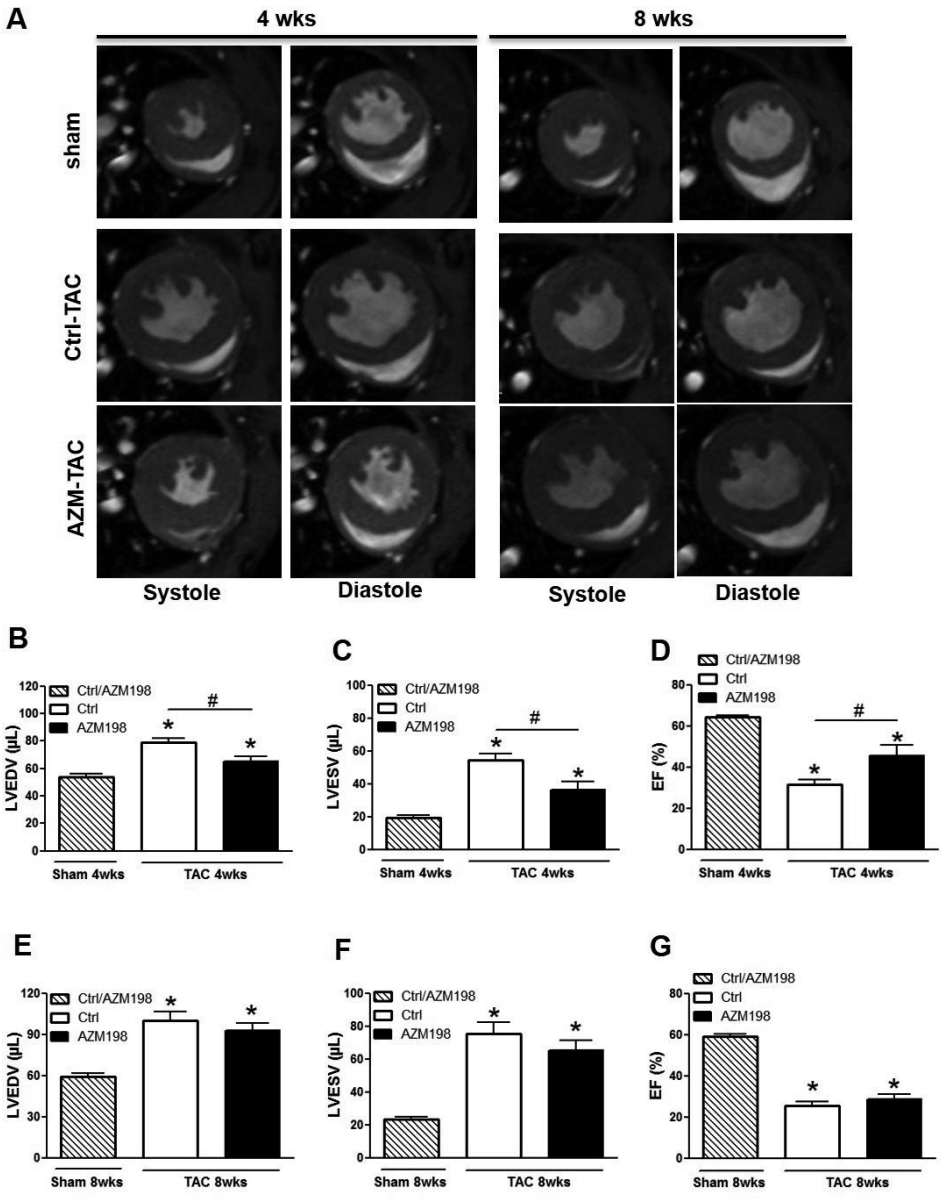

Figure 3. Cardiac MRI data. (A) Cardiac MRI images. Images show representative mid-ventricular slices in short axis view of the indicated animal groups in both diastole and systole. **(B)** Quantification of the left ventricular end diastolic volume (LVEDV in µl) of the 4 week animal groups. **(C)** Quantification of the left ventricular end systolic volume (LVESV in µl) of the 4 week animal groups. **(D)** Quantification of the ejection fractions (EF in %) of the 4 week animal groups. **(E)** Same as B, but for 8 week animal groups. **(F)** The same as (C) but for 8 week animal groups. **(G)** The same as (D), but for 8 week animal groups. n=8-20, * P<0.05 as compared to Ctrl/AMZ198 group, # P<0.05 as compared to the TAC non-treated AZM198 group.

AZM198 does not affect hemodynamic data

At 4 and 8 weeks after sham or TAC operation arterial and intra-cardiac pressures were assessed using in situ catheterization (Table 1, 2). Peak arterial pressure and LV end systolic (LVESP) and end diastolic (LVEDP) were significantly increased in all TAC groups. In the 8 weeks TAC groups aortic pressure and LVESP where somewhat lower as compared to the 4 weeks group (compare Table 1 with 2) suggestive for further remodeling and corroborating the MRI data. The corrected dP/dT_{max}, a measure for contractile function, and corrected dP/dT_{min} and Tau, measures of relaxation, were significantly decreased in all TAC groups. Based on LVEDP, and dP/dT_{max}, dP/dT_{min} and Tau values there appeared to be a tendency of improved function in the AZM198 treated groups, both at 4 and 8 weeks, but only the LVEDP at 8 weeks was significantly better in the AZM-TAC group.

Cardiac histology and gene expression analysis confirms temporal effect on hypertrophy

Histological sections of the left ventricle were stained with WGA-FITC in order to determine cardiomyocyte size. Microscopic analysis showed that cardiomyocyte cell size was significantly increased in all TAC groups, but this increase was significantly attenuated in the AZM-TAC4 group, as compared to the Ctrl-TAC4 group (Fig. 4A-B). At 8 weeks this AZM198 effect on hypertrophy was not significantly different anymore from the Ctrl-TAC8 group (Fig 4C-D). To corroborate these data, hypertrophic gene expression was investigated. As shown (Fig. 4E-F), expression of the natriuretic genes NPPA (ANP) and NPPB (BNP) were significantly elevated in all TAC groups. Also myosin heavy chain isoform expression was altered (β-MHC/α-MHC switch) and activation of the pathological NFAT pathway, as determined by RCAN1 expression, was apparent in all TAC groups (Fig. 4E-F). Importantly, in line with the other data, expression of some of these hypertrophic marker genes, NPPA and the β-MHC/α-MHC switch was attenuated in the AZM198-TAC4 group as compared to the Ctrl-TAC group. Again, these affects were no longer apparent at 8 weeks TAC. Since AZM-198 only showed effects at 4 weeks, we also investigated some hypertrophic signaling pathways at this time point. As shown in supplemental figure 2, AZM198 had minor effects on the hypertrophic p38, Akt and S6 signaling pathways. Pathological Erk phosphorylation was, however, significantly increased in the Ctrl-TAC group and this was attenuated by AZM198.

AZM198 does not affect fibrosis

Histological sections were also stained with Masson's trichrome to investigate cardiac fibrosis. In all TAC groups an increase in fibrosis was observed, as compared to the sham groups (Fig. 5A-D). No differences were observed between the TAC groups with or without AZM198 and fibrosis. This was corroborated by gene expression profiling. Collagens (ColI and ColIII) were

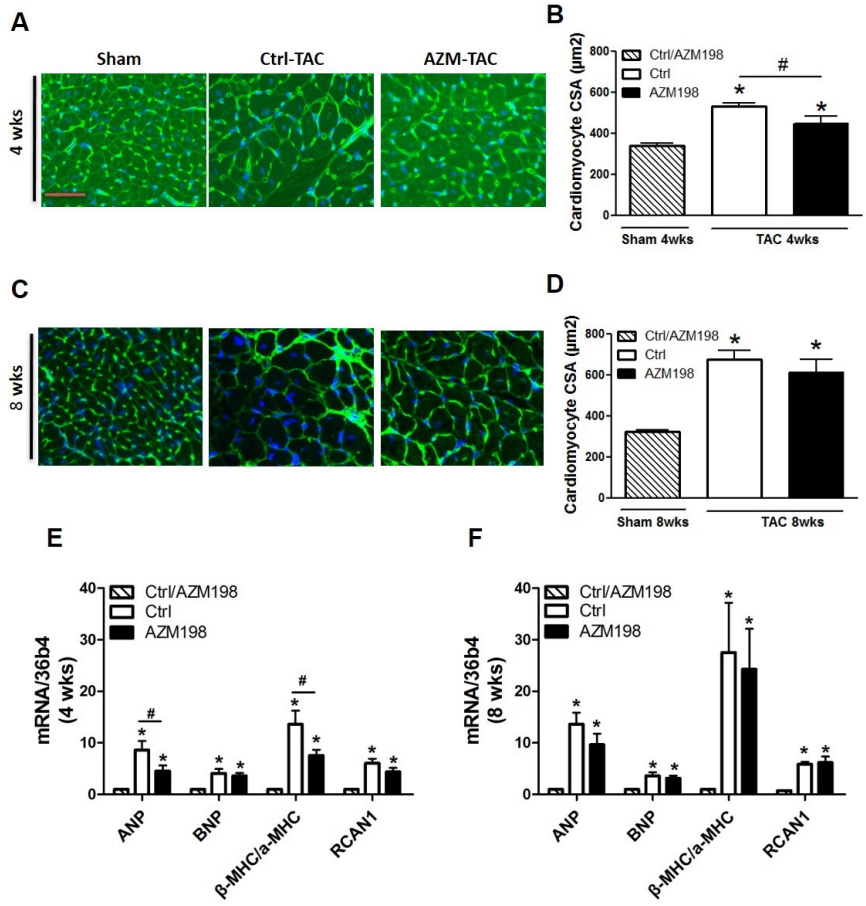

Figure 4. Cardiac hypertrophy. (A, C) Representative images of FITC-WGA-stained LV sections for quantification of cardiomyocyte hypertrophy of the 4 and 8 week animal groups (bar = 50 µm). **(B, D)** Quantification of cardiomyocyte cell sizes (>40 cells quantified per moue heart). **(E, F)** Quantification of gene expression of the indicated hypertrophic genes. All values were normalized to 36b4 gene expression and expressed as fold change. For β-MHC and α-MHC the ratio is shown. n= 6-20, * P<0.05 as compared to Ctrl/AMZ198 group, # P<0.05 as compared to the TAC non-treated AZM198 group.

induced to a similar extend in the TAC groups with and without AZM198 (Fig. 5E-F). Interestingly, however, in the AZM198-TAC4 group, genes involved in extracellular matrix remodeling, including TIMP1 and CTGF, were differentially expressed between the AZM-TAC4 and Ctrl-TAC groups. Thus, although the total level of collagen expression and deposition is not altered, we cannot rule out changes in ECM quality in the AZM-TAC4 group.

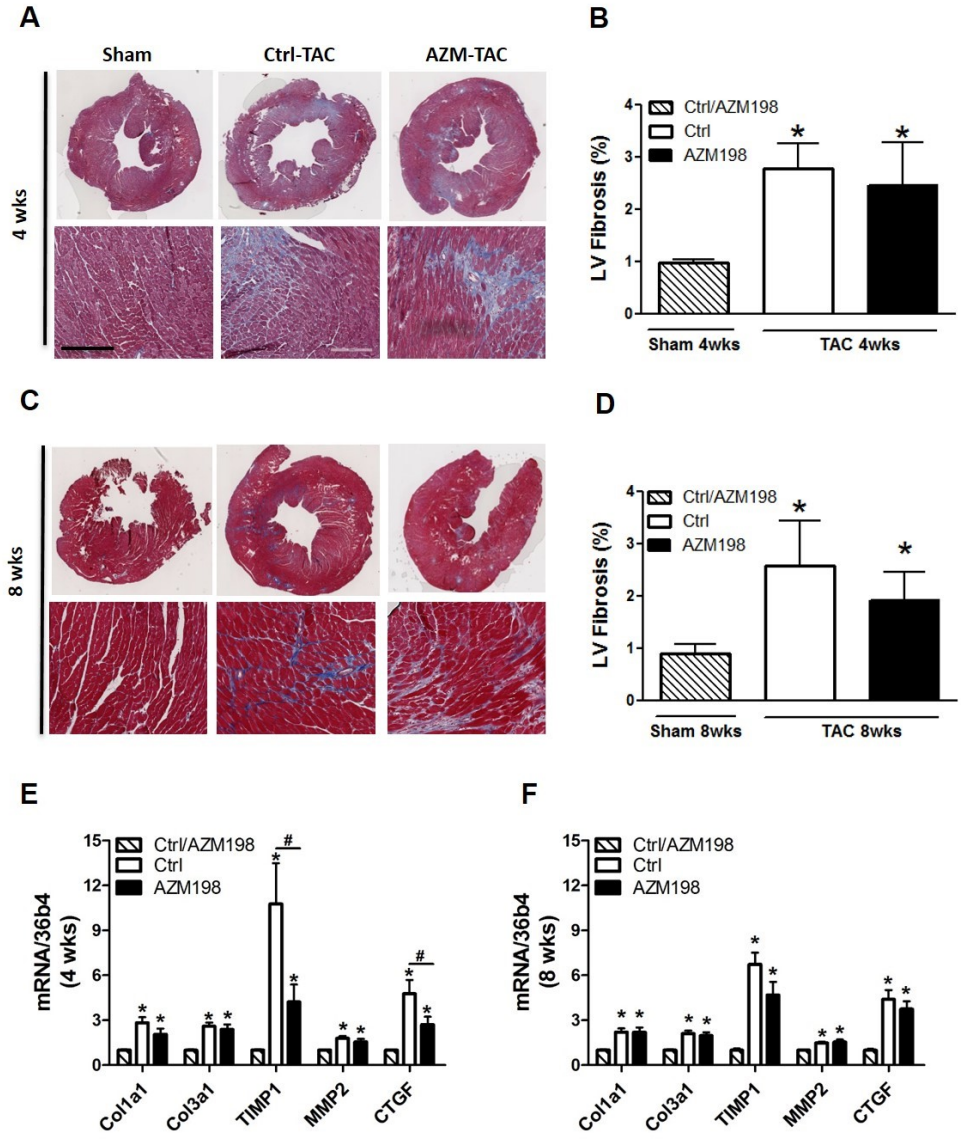

Figure 5. Cardiac fibrosis. (A, C) Representative Masson Trichrome-stained LV sections for the detection of fibrosis in the 4 and 8 week animal groups (bar = 200 µm). **(B, D)** Quantification of fibrotic area as percentage of the whole LV of the 4 week animal groups. **(E, F)** Quantification of fibrotic gene expression of the indicated genes. All values were normalized to 36b4 gene expression and expressed as fold change. n= 3-10, * P<0.05 as compared to Sham Ctrl/AMZ198 group, # P<0.05 as compared to the TAC non-treated AZM198 group.

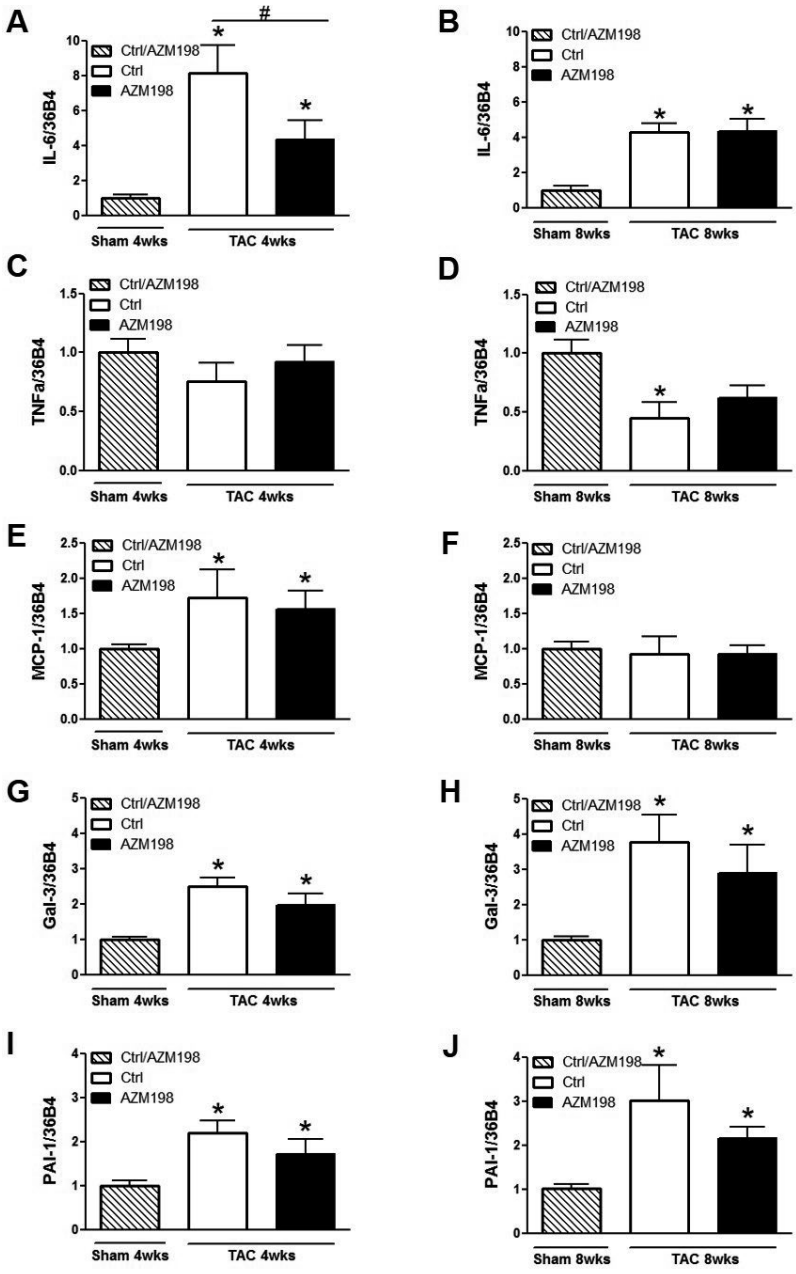

Figure 6. Inflammatory gene expression (A-F) Relative gene expression of the cytokines IL-6, TNFα and MCP-1at 4 and 8 weeks after sham or TAC operation. All values were normalized to 36b4 gene expression and expressed as fold change. **(G-J)** Relative gene expression of Gal-3 and PAI-1 at 4 and 8 weeks after sham or TAC operation. All values were normalized to 36b4 gene expression and expressed as fold change. n= 6-20, * P<0.05 as compared to Sham Ctrl/AMZ198 group, # P<0.05 as compared to the TAC non-treated AZM198 group.

Cardiac inflammation

Since MPO is a component of the inflammatory system, we also investigated gene expression of several cytokines and other inflammation related markers in the heart. In particular gene expression of TNFα, IL-6, MCP1, Gal-3 and PAI-1 were determined. As shown in figure 6A-B the cytokine IL-6 was clearly elevated after TAC and suppressed in the 4 weeks TAC group by AZM198. TNFα, on the other hand was not elevated by TAC. The chemokine, monocyte chemoattractant protein-1 (MCP-1), was temporarily increased after TAC, but this was not altered by AZM198 (Fig. 6E-F). The heart failure biomarker, Galectin-3 (Gal-3) was also elevated after TAC, but the expression of this inflammatory and pro-fibrotic marker was not altered by AZM198. The same was true for plasminogen activator inhibitor-1 (PAI-1), which is associated with thrombotic and fibrotic processes. Thus, except for IL-6, AZM198 had little to no effects on other cardiac inflammation related markers.

Discussion

Here we investigated the effect of a novel oral available MPO inhibitor, AZM198, on cardiac function in response to TAC mediated cardiac pressure overload. Our results show that AZM198 can be detected in blood plasma at sufficiently high levels to generate approximately 90% inhibition of MPO activity. Moreover, this is accompanied by about 25% reduction in MPO protein levels. Despite this inhibition the effects on cardiac remodeling were minor and only a delayed dilatation was observed at 4 weeks post pressure overload, which was supported by a difference in histological and molecular parameters. This effect was, however, not present anymore at 8 weeks post pressure overload.

Whereas in human heart failure patients a clear correlation has been observed between plasma MPO levels and heart failure severity, we did not observe any increase in plasma MPO levels in our TAC mouse model. A possible explanation is that the number of neutrophils in mice is much less than that in humans (10–15% in mice vs. 60–70% in humans) and also the level of MPO in murine neutrophils has been estimated to be around of 10-20% of that in human neutrophils [18, 19]. Hence small alterations in humans, might not become visible in mice. Alternatively, our TAC model might not be severe enough and not sufficiently mimic human HF, including other co-morbidities. Neurohormonal activation plays an important role in human heart failure and in mice infusion of AngII did raise plasma MPO levels [15]. Thus, sufficient neurohormonal activation might be an important factor in elevating MPO levels and is apparently insufficient in this TAC model. The absence of MPO changes makes it unlikely that MPO is a driving force in cardiac remodeling, at least not in a mouse TAC model. Despite this, we did observe temporal effects upon AZM198 treatment in cardiac hypertrophy and

dilatation, suggesting that baseline MPO activity may contribute or accelerate cardiac remodeling that is driven by other factors. In that case only subtle and time delayed effects on cardiac remodeling would be expected, as observed in this study. A stronger effect of this MPO inhibitor might be observed in heart failure with inflammatory involvement, like ischemic heart disease. Also heart failure with preserved ejection fraction (HFpEF) is believed to be driven by inflammation and hence MPO could also have a more pronounced role in this syndrome. Good animal models that mimic human HFpEF are, however, still lacking.

Studies using MPO knockout mice have provided clear evidence that MPO is a contributing factor in cardiac remodeling. In post myocardial infarcted $MPO^{-/-}$ mice, thinning of the ventricular walls was reduced and ventricular dilatation was attenuated [13, 14]. No effect was observed on infarct area, indicating that this was due to diminished post-MI remodeling. In our TAC study we also observed delayed cardiac dilatation. This was not accompanied by alteration in deposition of fibrotic proteins, although some genes encoding fibrotic modulating factors were altered. So, we cannot exclude that other cell types and processes could be involved. This is also in line with a study in which a role for MPO mediated inhibition of plasminogen activator inhibitor I (PAI-1) was suggested that would result in ECM remodeling and prevent cardiac rupture in $MPO^{-/-}$ mice after myocardial infraction [14]. Thus, subtle changes observed in our study are probably mediated by multiple mechanisms acting on cardiomyocytes, fibroblasts and other cells.

There are several limitations for this study. Considering the small effects observed, significant differences at later time points (8 weeks) might have been missed due to lack of sufficient power. As mentioned, mice have lower neutrophil blood counts as compared to humans and mouse neutrophils also contain less MPO. We can therefore not exclude that small effect observed in mice may be more prominent in humans. One deficit of this study is we have not been able to show direct effects of MPO on protein or lipid oxidation. Since many other oxidative mechanisms are present in vivo it has been difficult to access MPO specific oxidation. Although 3-Chlorotyrosine has been suggested to be relatively specific surrogate markers for MPO activity, others have questioned the in vivo reliability [20, 21]. Moreover, this modification has only been measured in highly inflammatory conditions and not in low grade inflammatory conditions, like our TAC model. The effects that we measured on cardiac hypertrophy, gene expression, and ERK activity in relation to AZM198 treatment do, however, provide indirect evidence for MPO specific effects.

In conclusion, we have shown that MPO plasma levels do not change after TAC induced cardiac pressure overload in mice. Hence there is no direct association with cardiac ejection

fraction and MPO plasma levels. Importantly, we did observe a temporal attenuation of cardiac dilatation by the MPO inhibitor AZM198. This indicates that MPO contributes to cardiac dilatation in this model, but is not the main driver. AZM198 could therefore have a more pronounced effects in cardiac diseases in which inflammation plays a more dominant role, like in HFpEF.

Acknowledgements

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Conflict of Interest

This work was supported by a grant from AstraZeneca. E.L.L. and E.M. are employees of AstraZeneca, R&D, Mölndal, Sweden

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Supplemental Tables

Supplemental table 1. Oligonucleotide sequences used for qRT-PCR

Gene	5' - 3' forward	5' - 3' reverse
ANP	ATGGGCTCCTTCTCCATCAC	TCTACCGGCATCTTCTCCTC
BNP	AAGTCCTAGCCAGTCTCCAGA	GAGCTGTCTCTGGGCCATTTC
RCAN1	GCTTGACTGAGAGAGCGAGTC	CCACACAAGCAATCAGGGAGC
α-MHC	GTTAACCAGAGTTTGAGTGACA	CCTTCTCTGACTTCGGAGGTACT
β-MHC	AAGTCCTAGCCAGTCTCCAGA	GAGCTGTCTCTGGGCCATTTC
TIMP1	CTGCTCAGCAAAGAGCTTTC	CTCCAGTTTGCAAGGGATAG
MMP2	CCCTGATGTCCAGCAAGTAG	GGAGTCTGCGATGAGCTTAG
CTGF	GCATCTCCACCCGAGTTAC	ACTGGTGCAGCCAGAAAG
Col1a1	CTTCACCTACAGCACCCTTGTG	CTTGGTGGTTTTGTATTCGATGAC
Col3a1	GCGATTCAAGGCTGAAG	GGGTGCGATATCTATGATGG
IL-6	TCCCAACAGACCTGTCTATAC	CAGAATTGCCATTGCACAACTC
TNF-α	AAACCACCAAGTGGAGGAGC	ACAAGGTACAACCCATCGGC
Gal-3	CCCGCTTCAATGAGAACAAC	ACCGCAACCTTGAAGTGGTC
MCP-1	CAATGAGTAGGCTGGAGAG	CTGGACCCATTCCTTCTTG
PAI-1	CACGCCTGGTGCTGGTGAAT	CGGTGCTGCCATCAGACTTG
36B4	AAGCGCGTCCTGGCATTGTC	GCAGCCGCAAATGCAGATGG

Supplemental table 2. Food intake of mice receiving chow containing AZM198 or normal chow

	Sham		TAC	
Food intake (%BW/day)	Ctrl	AZM198	Ctrl	AZM198
	(N=3)	(N=3)	(N=3)	(N=3)
Day 1 – 3	21,6 ± 2,1	20,8 ± 0,3	29,5 ± 6,0	24,9 ± 6,2
Day 4 – 6	15,0 ± 1,1	15,6 ± 0,6	20,2 ± 3,5	24,0 ± 9,2
Day 7 – 9	15,8 ± 0,8	14,2 ± 2,1	20,1 ± 4,9	16,8 ± 5,0
Day 10 – 12	16,3 ± 1,7	15,8 ± 1,5	14,0 ± 0,2	20,1 ± 2,7
Average	17,2 ± 1,1	16,6 ± 1,0	20,9 ± 2,7	19,0 ± 1,5

Data are presented as means ± SEM. TAC=Transverse aortic constriction. AZM198=Myeloperoxidase inhibitor; BW=Body weight.

Supplemental table 3. Organ weight, magnetic resonance imaging, hemodynamic, cardiomyocyte CSA, LV fibrosis (%) and gene expression profiles of mice after 4 weeks of sham surgery

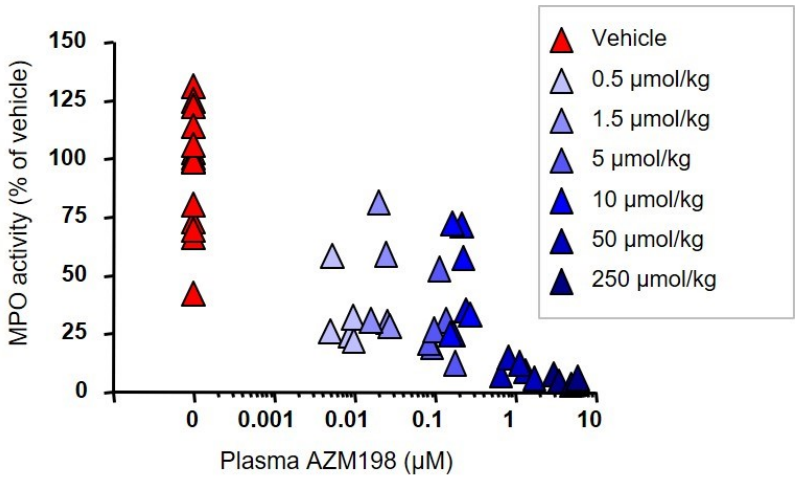
	Sham	
	Ctrl	AZM198
Organ weight	(N=10)	(N=10)
Body weight (g)	26,1 ± 0,4	26,4 ± 0,5
Tibia length (mm)	17,4 ± 0,1	17,3 ± 0,1
LV/tibia length (mg/mm)	6,1 ± 0,1	6,6 ± 0,3
RV/tibia length (mg/mm)	1,5 ± 0,1	1,5 ± 0,1
Atria/tibia length (mg/mm)	0,3 ± 0,0	0,4 ± 0,0
Lung/tibia length (mg/mm)	12,2 ± 1,1	10,7 ± 0,8
Spleen/tibia length (mg/mm)	3,8 ± 0,1	3,7 ± 0,1
MRI	(N=9)	(N=10)
LVEDV (µL)	56,4 ± 2,2	51,8 ± 3,7
LVESV (µL)	20,6 ± 1,5	18,5 ± 1,7
SV (µL)	35,9 ± 1,1	33,3 ± 2,3
EF%	63,9 ± 1,5	64,5 ± 1,3
Hemodynamics	N=10	N=10
Heart rate (bpm)	435 ± 20	457 ± 15
SAP (mmHg)	99,2 ± 1,7	100,5 ± 1,6
LV P_{max} (mmHg)	101,8 ± 1,9	104,0 ± 1,6
LVEDP (mmHg)	3,3 ± 0,7	5,0 ± 1,2
LVESP (mmHg)	96,6 ± 1,9	98,2 ± 1,5
Corrected dP/dT_{max} (1/s)	74,6 ± 4,0	73,6 ± 3,6
Corrected dP/dT_{min}(-1/s)	-80,8 ± 3,8	-80,6 ± 5,9
Tau (ms)	6,4 ± 0,5	6,7 ± 0,4
Histology	(N=9)	(N=10)
Cardiomyocyte CSA (μm^2)	329,5 ± 19,1	345,8 ± 21,9
LV fibrosis (%)	0.98 ± 0.08	0.96 ± 0.10
Hematology	(N=9)	(N=9)
WBC ($x10^9$/L)	6,2 ± 0,5	4,7 ± 0,6
RBC ($x10^9$/L)	9,7 ± 0,2	9,4 ± 0,2
MCV (fL)	48,1 ± 0,3	48,1 ± 0,8
Hemoglobin (mmol/L)	8,4 ± 0,1	8,1 ± 0,2
Platelets ($x10^9$/L)	1410,7 ± 132,9	1189,4 ± 194,2
Gene expression	(N=10)	(N=10)
Hypertrophy		
ANP	1,00 ± 0,12	1,14 ± 0,15
BNP	1,00 ± 0,15	1,14 ± 0,08
RCAN1	1,00 ± 0,18	1,40 ± 0,17
α-MHC	1,00 ± 0,08	1,07 ± 0,06
β-MHC	1,00 ± 0,14	1,28 ± 0,21
Fibrosis		
TIMP1	1,00 ± 0,05	1,10 ± 0,12
MMP2	1,00 ± 0,07	1,10 ± 0,06
CTGF	1,00 ± 0,05	1,08 ± 0,07
Col1a1	1,00 ± 0,09	1,02 ± 0,08
Col3a1	1,00 ± 0,92	0,96 ± 0,08
Inflammation		
IL-6	1,00 ± 0,35	0,58 ± 0,09
TNFα	1,00 ± 0,09	1,10 ± 0,10
Gal-3	1,00 ± 0,10	0,92 ± 0,09
MCP-1	1,00 ± 0,10	0,87 ± 0,06
PAI-1	1,00 ± 0,17	0,95 ± 0,15

Data are presented as means ± SEM. TAC=Transverse aortic constriction. AZM198=Myeloperoxidase inhibitor; LV=Left ventricle; RV=Right ventricle; SAP=Systolic arterial pressure; LV P_{max}=Maximal left ventricular pressure; dP/dT_{max} is an indicator for maximal LV contraction capacity and is corrected for LV P_{max}; dP/dT_{min} is an indicator for maximal LV relaxation capacity and is corrected for LV P_{max}; LVEDP/ESP=Left ventricular end/systolic pressure; Tau is a measure for isovolumetric relaxation of the LV; LVEDV/ESV=Left ventricular end/systolic volume; SV=Stroke volume; EF=Ejection fraction; CSA=Cross sectional area; WBC=White blood cell; RBC=White blood cell; MCV=Mean corpuscular volume; Gene expression was normalized to 36B4 gene expression and is presented as fold change. No significant differences were observed.

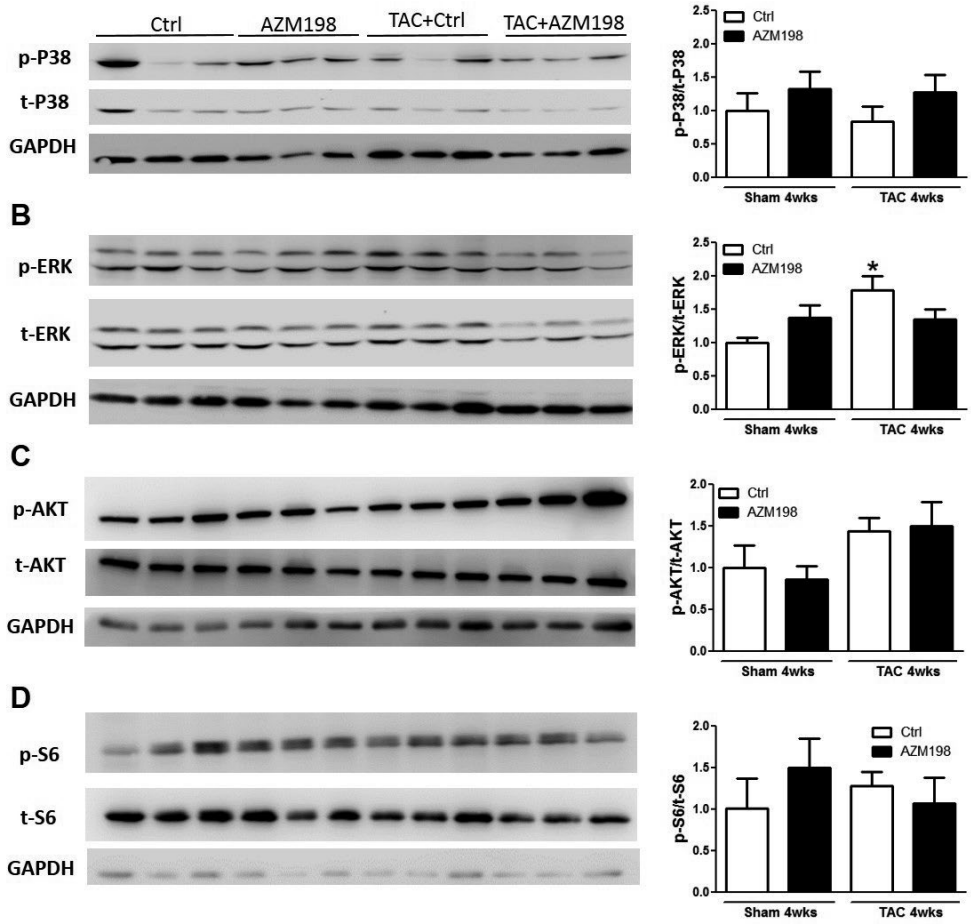
Supplemental table 4. Organ weight, magnetic resonance imaging, hemodynamic, cardiomyocyte CSA, LV fibrosis (%) and gene expression profiles of mice after 8 weeks of sham surgery

	Sham	
	Ctrl	AZM198
Organ weight	(N=9-10)	(N=10)
Body weight (g)	29,3 ± 0,4	29,5 ± 0,3
Tibia length (mm)	17,6 ± 0,1	17,6 ± 0,2
LV/tibia length (mg/mm)	6,7 ± 0,2	6,6 ± 0,2
RV/tibia length (mg/mm)	1,6 ± 0,1	2,0 ± 0,3
Atria/tibia length (mg/mm)	0,4 ± 0,0	0,5 ± 0,1
Lung/tibia length (mg/mm)	9,5 ± 0,9	10,8 ± 1,1
Spleen/tibia length (mg/mm)	5,0 ± 0,8	4,0 ± 0,1
MRI	(N=10)	(N=10)
LVEDV (µL)	61,4 ± 3,0	57,5 ± 3,1
LVESV (µL)	24,5 ± 1,7	23,5 ± 1,5
SV (µL)	36,9 ± 1,6	33,9 ± 2,0
EF%	60,4 ± 1,4	58,9 ± 1,7
Hemodynamics	(N=10)	(N=9)
Heart rate (bpm)	521 ± 25	490 ± 24
SAP (mmHg)	98,7 ± 1,8	99,5 ± 1,9
LV P_{max} (mmHg)	103,3 ± 1,8	104,9 ± 2,1
LVESP (mmHg)	85,0 ± 5,1	92,6 ± 3,9
LVEDP (mmHg)	5,3 ± 1,6	5,0 ± 0,9
Corrected dP/dT_{max} (1/s)	90,3 ± 5,8	88,7 ± 6,7
Corrected dP/dT_{min} (-1/s)	-90,0 ± 6,0	-93,6 ± 6,0
Tau (ms)	6,2 ± 0,5	6,1 ± 0,4
Histological	(N=3-10)	(N=3-10)
Cardiomyocyte CSA (µm2)	325,4 ± 10,1	319,8 ± 16,7
LV fibrosis (%)	1,16 ± 0,3	0,68 ± 0,2
Hematology	(N=10)	(N=8)
WBC ($x10^9$/L)	6,1 ± 0,8	4,4 ± 0,5
RBC ($x10^9$/L)	9,0 ± 0,2	9,3 ± 0,2
MCV (fL)	48,6 ± 0,8	47,6 ± 0,7
Hemoglobin (mmol/L)	7,8 ± 0,2	7,7 ± 0,1
Platelets ($x10^9$/L)	994,8 ± 183,6	1138,6 ± 157,1
Gene expression	(N=10)	(N=8-10)
Hypertrophy		
ANP	1,00 ± 0,10	1,22 ± 0,20
BNP	1,00 ± 0,12	1,02 ± 0,16
RCAN1	1,00 ± 0,12	0,83 ± 0,16
α-MHC	1,00 ± 0,06	1,18 ± 0,18
β-MHC	1,00 ± 0,15	0,77 ± 0,12
Fibrosis		
TIMP1	1,00 ± 0,09	1,22 ± 0,22
MMP2	1,00 ± 0,06	1,08 ± 0,08
CTGF	1,00 ± 0,09	1,05 ± 0,15
Col1a1	1,00 ± 0,06	1,07 ± 0,14
Col3a1	1,00 ± 0,09	1,24 ± 0,13
Inflammation		
IL-6	1,00 ± 0,38	0,66 ± 0,13
TNFα	1,00 ± 0,15	0,98 ± 0,18
Gal-3	1,00 ± 0,12	1,25 ± 0,12
MCP-1	1,00 ± 0,10	1,19 ± 0,17
PAI-1	1,00 ± 0,14	1,07 ± 0,14

Data are presented as means ± SEM. TAC=Transverse aortic constriction. AZM198= Myeloperoxidase inhibitor; LV=Left ventricle; RV=Right ventricle; SAP=Systolic arterial pressure; LV P_{max}=Maximal left ventricular pressure; dP/dT_{max} is an indicator for maximal LV contraction capacity and is corrected for LV P_{max}; dP/dT_{min} is an indicator for maximal LV relaxation capacity and is corrected for LV P_{max}; LVEDP/ESP=Left ventricular end/systolic pressure; Tau is a measure for isovolumetric relaxation of the LV; LVEDV/ESV=Left ventricular end/systolic volume; SV=Stroke volume; EF=Ejection fraction; CSA=Cross sectional area; WBC=White blood cell; RBC=White blood cell; MCV=Mean corpuscular volume; Gene expression was normalized to 36B4 gene expression and is presented as fold change. No significant differences were observed.



Supplemental figure 1. Effect of plasma AZM198 concentration on MPO activity in zymosan-induced peritonitis. MPO activity was determined in peritoneal fluid after thioglycolate and zymosan induced peritonitis. AZM198 was administered 2 hours after zymosan treatment and peritoneal fluid was collected 2 hours later. AZM198 levels in blood plasma and MPO activity in peritoneal fluid were subsequently determined.



Supplemental figure 2. Protein levels after treatment with AZM198 in TAC mouse heart. Western blot was performed in the LV of mice subjected to TAC with or without AZM198 treatment. **(A)** phosphorylated p38 (Thr180/182) to total p38. **(B)** phosphorylated ERK (Tyr204/187) to total Perk. **(C)** phosphorylated AKT (Ser473) to total AKT. **(D)** phosphorylated S6 (ser235/236) to total S6, normalized to GAPDH. Quantitative values are expressed as fold change; n=3-10/group. Data are expressed as means ± SEM, * P<0.05 as compared to the TAC non-treated AZM198 group

Chapter 5

Plasma levels of heart failure biomarkers are primarily a reflection of extracardiac production

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ABSTRACT

Plasma heart failure (HF) biomarkers, like natriuretic peptides, are important in diagnosis, prognosis and HF treatment. Several novel HF biomarkers have been identified, including Gal-3, GDF-15 and TIMP-1, but their clinical potential remains vague. Here we investigated plasma biomarker levels in relation to tissue expression and structural and functional cardiac changes.

Methods: Cardiac remodeling, cardiac function, and plasma and tissue biomarker levels were investigated in mice after myocardial infarction induced by temporal and permanent LAD ligation (tLAD and pLAD). In addition, a pressure overload model induced by transverse aortic constriction (TAC) and an obese/hypertensive HFpEF-like mouse model were investigated.

Results: Plasma levels of ANP and its cardiac expression were strictly associated with cardiac remodeling and function. Gal-3, GDF-15 and TIMP-1 cardiac expressions were also related to cardiac remodeling and function, but not their plasma levels. Only directly after myocardial infarction could elevated plasma levels of Gal-3 and TIMP-1 be detected. Eight weeks after infarction, plasma levels were not elevated despite enhanced cardiac expression and low EF (18.3±3.3%, pLAD). Plasma levels of TIMP-1 and GDF-15 were elevated after TAC, but this also correlated with increased lung expression and congestion. In obese-hypertensive mice, elevated plasma levels of Gal-3, GDF-15 and TIMP1 were associated with increased adipose tissue expression and not with cardiac function.

Conclusions: The Gal-3, GDF-15 and TIMP-1 plasma pool levels are hardly influenced by dynamic changes in cardiac expression. These biomarkers are not specific for indices of cardiac remodeling, but predominantly reflect stress in other affected tissues and hence provide health information beyond the heart.

Introduction

Standard clinical examination of heart failure (HF) patients does not provide means for detailed patient stratification and therefore other tools are needed. Plasma biomarkers provide great promise to further dissect the underlying disease processes, may aid in further stratification and allow tailored therapy for HF patients [1].

Natriuretic peptides are the gold standard HF plasma biomarkers, have improved the management and evaluation of HF patients and have been included in the American Heart Association (AHA) and European Society of Cardiology (ESC) HF guidelines [2, 3]. These peptides are mainly produced and released by the heart in response to sustained wall stretch [4]. Cardiac-specific troponins were originally used as acute damage markers in diagnosis of myocardial infarction, but with the appearance of high-sensitivity troponin (hsTn) assays are now also being used as HF markers and are mentioned in the HF guidelines. Most other novel HF biomarkers appear to be non-cardiac specific [5]. Hence, the diagnostic value of these biomarkers is limited, but these biomarkers could provide essential information about underlying biological processes, like fibrosis, and aid in additive risk stratification of HF patients [6]. For this purpose, Gal-3 (Galectin-3) and sST2 (soluble suppression of tumorogenicity-2) have been included in the AHA HF guidelines [3]. Gal-3 is associated with organ fibrosis, including cardiac fibrosis, and is elevated in HF patients [4, 7, 8, 9]. sST2 is a soluble decoy receptor for IL-33 and is associated with adverse cardiac remodelling, including hypertrophy and fibrosis, by inhibiting the protective effects of IL-33 [10]. Although not yet mentioned in the HF guidelines, many other biomarkers have been identified that could act as prognostic biomarkers in HF [11]. The fibrotic marker TIMP-1 (tissue inhibitor of metalloproteinase-1), for instance, is elevated in HF [12-14] and increased levels were detected in coronary sinus blood, suggesting cardiac origin [15]. GDF-15 (growth differentiation factor-15), another emerging biomarker, is elevated in HF patients and protects the heart from cardiac damage [16-19]. Most interestingly, these biomarkers are also elevated in patients with HF with preserved ejection fraction (HFpEF), are related to disease severity and can have predictive power [20-25]. The correlation between the plasma levels of the above mentioned novel biomarkers and the presence and severity of cardiac dysfunction, and the relation to their production in other tissues, is, however, largely unknown. Understanding the origin of plasma biomarkers and whether other organs or tissues contribute to their elevated levels is therefore needed.

In contrast to clinical HF studies, preclinical animal studies allow us to investigate biomarkers at multiple levels (RNA and protein) and within multiple organs and tissues. Nevertheless,

biomarker studies in small animals are hampered by the small plasma/serum volumes and the lack of appropriate reagents. We therefore focused on three novel heart failure biomarkers, Gal-3, GDF-15, and TIMP-1 and the established HF biomarker ANP, for which suitable reagents were available. We studied them in three mouse models of cardiac remodeling, including two models of HF with reduced ejection fraction (HFrEF), consisting of a myocardial infarction model and a transverse aortic constriction (MI and TAC), and one model with HFpEF characteristics generated by high fat diet (HFD) and angiotensin II (AngII) infusion (obesity/hypertension).

Methods

Animals

All animal experimental protocols were approved by the Animal Care and Use Committee of University of Groningen, The Netherlands (permit numbers: DEC6827A, DEC6920A and IVD16487-03-01) and were conducted according to the existing guidelines for the care and use of laboratory animals. Approximately 10-week-old male C57BL/6J mice were obtained from Envigo, the Netherlands. Mice were housed on a 12 h light/ 12 h dark cycle with ad libitum access to chow and water. In these exploratory studies, mice were randomized based on bodyweight. Exclusion criteria included abnormal bodyweight (>2 SD) and abnormal behavior. Prior to surgery, mice received a subcutaneous injection of Carprofen (5.0 mg/kg) to alleviate wound pain. For anesthesia, 2% isoflurane/oxygen was used. Cardiac functional measurements (MRI and pressure loops) were performed in a blinded fashion by an independent operator. Molecular and histological analyses were blinded. No mice were excluded from analysis, except for those mice that died or reached a humane endpoint before the end of the experiment.

Myocardial infarction

Myocardial infarctions (MI) were induced as previously described [26]. In brief, mice were anesthetized, intubated and placed supine on a heated pad for mechanical ventilation. To create large MIs, the left anterior descending coronary artery (LAD) was permanently ligated using a suture. To create small MIs, a temporal ligation was performed: a suture was tied onto a polyethylene tube placed on the LAD coronary artery and was removed again after 60 min. Control mice were sham operated. The experimental set-up is outlined in **Figure S1A**.

TAC

Transverse aortic constriction (TAC) was performed as previously described [26, 27]. In brief, mice were anesthetized, intubated and placed supine on a heated pad for mechanical ventilation. A suture was tied around a blunt 27-gauge needle placed on the aortic arch between the branchiocephalic and left carotid arteries, establishing a reproducible aortic stenosis. Control

mice were sham operated. No differences were observed between 4 and 8 weeks sham-operated mice and these groups were therefore combined in later analyses. The experimental set-up is outlined in **Figure S1B**.

Obesity/hypertension

Mice were fed high-fat diet (HFD) (60 kcal% fat, D12492, Research diets, USA) or a control diet indicated with LFD (low fat diet) (10 kcal% fat, D12450J, Research diets, USA) for a period of 16 weeks. After 12 weeks, the diet was supplemented with a 4 week infusion of either angiotensin II (AngII) (1 mg/kg/day; Bachem, Switzerland) or saline using osmotic minipumps (Alzet 1004, Durect corporation, USA). Pumps were combined with polyetheretherketone tubing (Alzet PEEK tubing 0002612, Durect corporation, USA) to allow magnetic resonance imaging (MRI). Placement of pumps was performed as described before [7]. In brief, mice were anesthetized and placed in the prone position on a heating pad. A subcutaneous pocket was created in the right flank for pump insertion. The experimental set-up is outlined in **Figure S1C**.

Body mass composition

In the obesity/hypertension model, body mass composition was determined after 15 weeks of diet intervention using a minispec LF90II body composition analyzer (Bruker Optics, USA).

Magnetic resonance imaging (MRI)

Cardiac MRI was performed on anaesthetized mice using a 9.4 T, 89 mm bore size magnet equipped with 1500 mT/m gradients and connected to an advanced 400 MR system (BrukerBiospin, Germany) as previously described [26, 28]. Images were reconstructed and left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV) and left ventricular (LV) mass were determined using QMass® software (version MR 6.1.5, Medis Medical Imaging Systems, the Netherlands) and stroke volume (SV) and ejection fraction (EF) were calculated.

Hemodynamic measurements and sacrifice

Prior to sacrifice, hemodynamic measurements were performed using a Millar pressure transducer catheter (Mikro-Tip pressure catheter 1.4F, Transonic Scisense, Transonic Europe, The Netherlands). Mice were anaesthetized and the catheter was inserted via the right carotid artery. Heart rate, arterial pressures, left ventricular end-systolic pressure (LVESP), left ventricular end-diastolic pressure (LVEDP) and maximal and minimum rates of pressure change during contraction and relaxation, respectively dP/dt_{max} and dP/dt_{min}, were recorded. The dP/dt_{max} and dP/dt_{min} values were adjusted for LV maximal pressure (Pmax). After catheter removal, blood was collected, transferred into ethylenediaminetetraacetic acid (EDTA) tubes

and immediately centrifuged at 1500×*g* for 10 min, followed by plasma collection. Organs were flushed with 10 mL saline to remove remaining red blood cells. Thereafter, LV and other tissues were collected. Blood plasma and tissues were frozen in liquid nitrogen and stored at -80 °C. An LV mid-slice of each heart was fixed in formalin and processed for histology and immunohistochemistry.

Immunohistochemistry

Formalin-fixed paraffin-embedded mid-transverse LV sections were cut in 4 μm thick slices and stained with Masson's trichrome to detect fibrosis. Whole stained sections were automatically imaged using a Nanozoomer 2.0 HT (Hamamatsu, Japan). Fibrosis fraction as a percentage of the entire section was quantified from a 20× magnification (ScanScope, Aperial Technologies, USA).

For ANP staining, paraffin sections were deparaffinized and, after blocking endogenous peroxidases with H_2O_2, these sections were incubated for 1 h at room temperature with rabbit anti-ANP (ab91250, ABCAM, UK) in PBS with 1% BSA. For Gal-3 staining, antigen retrieval was performed with 10 mM citrate buffer (pH 6.0) on deparaffinized sections and, after blocking endogenous peroxidases with H_2O_2, these sections were incubated for 1 h at room temperature with rat anti-Mac2 (CL8942AP, Cedarlane, Canada) in PBS with 1% BSA. For ANP, goat anti-rabbit IgG/HRP was used as secondary antibody and rabbit anti-goat IgG/HRP as tertiary antibody. For Gal-3, rabbit-anti rat IgG/HRP was used as secondary antibody. Subsequently 3, 3–diaminobenzidine (DAB) staining was performed and thereafter haematoxilin counterstaining, followed by mounting using DPX mounting medium (Sigma-Aldrich, USA). For microscopy, an Olympus BX50 microscope (Olympus, Japan) was used with 4×, 10× and 20× objectives and images were collected with an Olympus DP70 camera (Olympus, Japan).

RT-qPCR

Total RNA was isolated from organs using TRIzol reagent (Invitrogen Corporation, the Netherlands) and from visceral adipose tissue (VAT) using RNeasy lipid tissue mini kits (Qiagen, the Netherlands). cDNA was synthesized using QuantiTect Reverse Transcription kits (Qiagen, The Netherlands). RNA concentration of samples was determined by spectrophotometry (NanoDrop 2000, ThermoScientific, the Netherlands). Gene expression levels were determined using Absolute QPCR SYBR Green ROX mix (Abgene, Epsom, UK) using 7.5 ng cDNA. Real-time quantitative PCR (RT-qPCR) was performed on a C1000 Thermal Cycler CFX284 Real-Time Detection system (Bio-Rad Laboratories, The Netherlands). Gene expressions were corrected by ribosomal protein, large, P0 (36B4)

reference gene expression. This gene showed minimal variation in expression between tissues, in contrast to many other reference genes (B2M, TBP, Ppia, GAPDH and PRL13A) that showed at least a 1.5-fold difference between LV and one of the tested organs (data not shown). Gene expressions in different organs were corrected by the values in LV of control mice. Oligonucleotide pairs are listed in **Table S1**.

ELISA and Western blot analysis of biomarkers

The following commercial enzyme-linked immunosorbent assays (ELISA) were used to determine protein levels in plasma: Gal-3 (DY1197, R&D, USA); GDF-15 (MGD150, R&D, USA); TIMP-1 (MTM100, R&D, USA); NT-proANP (BI-20892, BIOMEDICA, Austria). Plasma biomarker quantities are reported per volume of plasma.

The above-mentioned ELISA kits were also used for measurement of Gal-3, GDF-15 and TIMP-1 protein levels in cardiac, lung and adipose tissue. For LV and lung, tissue homogenization was performed in phosphate buffered saline (PBS) containing 0.5% Triton-x100 (Sigma-Aldrich, USA) and protease inhibitor (Roche 11873580001, complete, EDTA-free, Sigma-Aldrich, USA). After centrifugation at 12000 ×*g* for 10 min at 4 °C, the supernatants were used. For measurement of Gal-3, GDF-15 and TIMP-1 protein levels in VAT, total protein was extracted from tissues using Minute Total Protein Extraction kits for Adipose Tissues/Cultured adipocytes (Invent Biotechnologies, USA). Total lysate protein content was determined using Pierce BCA protein assay kits (ThermoScientific, USA). Tissue biomarker levels determined by ELISA were corrected for total protein lysate.

The NT-proANP ELISA kit was not suitable for detection of ANP in tissue lysates and therefore detection of ANP was performed by Western blotting. Proteins were isolated from frozen organs homogenized in ice-cold lysis buffer (50 mM Tris pH 8.0, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM PMSF, 15 mM sodium vanadate) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, USA), and from VAT using the Minute Total Protein Extraction kit for Adipose Tissues/Cultured adipocytes (Invent Biotechnologies, USA). Proteins were loaded on SDS-PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad, USA). The following antibodies were used for immunoblotting: anti-ANP (ab91250, Abcam, UK); anti-GAPDH (10R-G109A, Fitzgerald, USA); anti-MAC-2 (Gal-3) (Tebu-Bio, The Netherlands); anti-rat immunoglobulins/HRP (ab6734, Abcam, USA); anti-rabbit immunoglobulins/HRP (P0448, Dako, Denmark); and rabbit anti-mouse immunoglobulins/HRP (P0260, Dako, Denmark). Signals were detected by enhanced chemiluminescence (ECL) (PerkinElmer, USA), and intensities of bands were quantified with ImageQuant LAS 4000 (GE Healthcare Eurpe GmbH, Belgium). Protein levels were corrected

by GAPDH reference protein levels. Fold changes relative to internal control were calculated and shown.

Statistics

All values are presented as mean ± standard error of the mean (SEM). Student's paired two-tailed *t*-test was used for two-group comparisons and one-way analysis of variance (ANOVA) followed by Tukey's post-hoc correction for multigroup comparisons. For non-normally distributed data or data without homogeneity of variance, non-parametric tests were performed. In this case, Mann-Whitney tests were used for two group comparisons and Kruskal-Wallis followed by Mann-Whitney tests for multiple group comparisons. Normality of data and homogeneity of variance were tested using the Shapiro-Wilk test for normality and Levene's test for homogeneity of variances, respectively. Correlation was determined using Spearman's correlation test. $P < 0.05$ was considered to be significant. SPSS software (IBM SPSS statistics, version 22, IBM, USA) was used for statistical analyses.

Table 1. Hemodynamic effects and effects on cardiac dimensions and function in tLAD/pLAD, TAC and HFD/AngII mice.

		P-catheter					MRI		
	HR	**MAP**	**LVESP**	**LVEDP**	**dP/dt$_{max}$**	**dP/dt$_{min}$**	**LVEDV**	**LVESV**	**EF**
Sham	519 ± 18	86.7± 0.9	100.3 ± 2.6	7.8 ± 1.4	81.7 ± 4.6	-78.3 ± 5.4	62.5 ± 2.6	26.4 ± 1.9	58.1 ± 1.6
tLAD	536 ± 9	86.6± 2.1	96.7 ± 3.1	9.4 ± 1.4	72.7 ± 2.4	-63.73 ± 4.0*	80.3 ± 3.8*	49.3 ± 3.6*	39.0 ± 2.1*
pLAD	489 ± 14	72.3± 3.5*#	84.2 ± 3.8*#	11.6 ± 0.4*	55.9 ± 1.4*#	-46.0 ± 1.8*#	175.1 ± 21.7*#	148.2 ± 22.9*#	18.3 ± 3.3*#
Sham	478 ± 18	81.6± 1.3	90.8 ± 3.0	4.3 ± 0.9	82,5 ± 3.9	-85,4 ± 3,6	59.0 ± 1.9	22.6 ± 1.2	62.0 ± 1.1
TAC4	484 ± 15	91.5± 5.7*	137.4 ± 5.4*	13.9 ± 4.0*	48,8 ± 3.7*	-50,0 ± 2,8*	79.1 ± 3.2*	54.5 ± 3.6*	31.5 ± 2.2*
TAC8	502 ± 9	85.7± 2.7	119.1 ± 9.7*	22.0 ± 2.0*	44,99 ± 1.6*	-39,9 ± 1.6*	100.2 ± 6.5*#	77.4 ± 7.0*#	23.6 ± 2.2*#
LFD	485 ± 21	93.6± 3.7	97.3 ± 2.3	10.5 ± 2.0	74.8 ± 4.7	-66.4 ± 6.4	49.9 ± 2.1	22.7 ± 1.8	55.1 ± 1.8
HFD	472 ± 10	99.9± 2.4	105.1 ± 3.9	17.2 ± 2.7	72.5 ± 4.1	-60.5 ± 3.6	46.3 ± 1.4	17.8 ± 0.7	61.7 ± 0.9*
HFD+AngII	460 ± 30	107.8± 2.9*	114.1 ± 9.2*	17.7 ± 1.8	59.0 ± 4.8*	-47.4 ± 4.1*	51.5 ± 2.5	24.7 ± 3.1	53.7 ± 3.7

Data are presented as mean ± standard error of the mean. dP/dt$_{max}$: maximal left ventricular contraction corrected by maximal ventricular pressure (1/s); dP/dt$_{min}$= maximal left ventricular relaxation corrected by maximal ventricular pressure (1/s); EF: ejection fraction (%); HFD: high fat diet; HR: heart rate (bpm); MAP: mean arterial pressure (mmHg); P-catheter: pressure catheter; MRI: magnetic resonance imaging; LFD: low fat diet; LVESP: left ventricular end-systolic pressure (mmHg); LVEDP: left ventricular end-diastolic pressure (mm Hg); LVEDV: left ventricular end-diastolic volume (µl); LVESV: left ventricular end-systolic volume (µl); pLAD: permanent ligation of the left anterior descending coronary artery; TAC: transverse aortic constriction; tLAD: temporal ligation of the left anterior descending coronary artery. N=8-12. *P<0.05 versus respective control group. #P<0.05 for pLAD versus tLAD, TAC 4 wks versus TAC 8 wks or HFD+AngII versus HFD.

Results

Cardiac remodeling and biomarker expression after temporal LAD ligation (tLAD)

Myocardial infarctions (MIs) were generated by temporal and permanent ligations of the left anterior descending coronary artery (tLAD and pLAD, respectively). The tLAD groups were investigated at multiple time points, whereas the pLAD group was investigated at 8 weeks only (Figure S1A). The effects of tLAD in time are presented in Figure 1, and the comparison between tLAD and pLAD at 8 weeks is shown in Figure 2 and Table 1.

Serial MRI measurements of the tLAD groups revealed a rapid drop in ejection fraction (EF) within one week post-MI and histological analysis of LV sections by Masson's trichrome staining confirmed the presence of infarcted fibrotic areas (Figure 1A-B). Cardiac gene expression of NPPA, encoding ANP, increased directly after MI and remained at a similar level thereafter (Figure 1C, left panel). Tissue ANP levels, determined by Western blotting, were also significantly elevated after tLAD (Figure 2C, middle panel, and Figure S2) and immunohistochemical analysis revealed that ANP was predominantly elevated in cardiomyocytes in the non-infarcted area (Figure S3). This also resulted in elevated NT-proANP plasma levels (Figure 1C, right panel), which increased even faster than tissue levels. This may be explained by ischemia-induced ANP secretion after infarction [29].

In contrast to NPPA, gene expression of LGALS3 (encoding Galectin-3), GDF-15 and TIMP-1 strongly increased within 3 days after tLAD, but subsequently decreased again, showing only a modest elevation at 8 weeks (Figure 1D-F, left panels). These gene expression profiles were similar to the expression profiles of extracellular matrix genes, including alpha-1 type 1 collagen (Col1a1) and alpha-3 type 1 collagen (Col3a1), and inflammatory genes, like interleukin-6 (IL-6) (Figure S4A). Gene expression changes were specific to the heart and no changes were observed in other tissues investigated (lung, kidney, liver) (Figure S4B-D). Gal-3, GDF-15 and TIMP-1 protein levels in tissue lysates and blood plasma were determined by ELISA. LV protein levels of Gal-3, GDF-15 and TIMP-1 largely paralleled gene expression patterns, with highly elevated protein levels within 3 days after MI (Gal-3 ~10-fold; GDF-15 ~3-fold; TIMP-1 ~20-fold increased) and subsequent decrease in the weeks thereafter (Figure 1D-F, middle panels). Immunohistochemical analysis confirmed this strong temporal increase in Gal-3 levels in the heart and importantly Gal-3 expression was confined to the infarcted area and localized to both non-cardiomyocytes and cardiomyocytes (Figure S5). The increase in expression also resulted in a temporal elevation of blood plasma levels of both Gal-3 and TIMP-1 at 3 days post-infarction (Figure 1D, F, right panels). None of them remained elevated in the weeks thereafter, despite reduced cardiac EF. Thus, only highly elevated LV expression

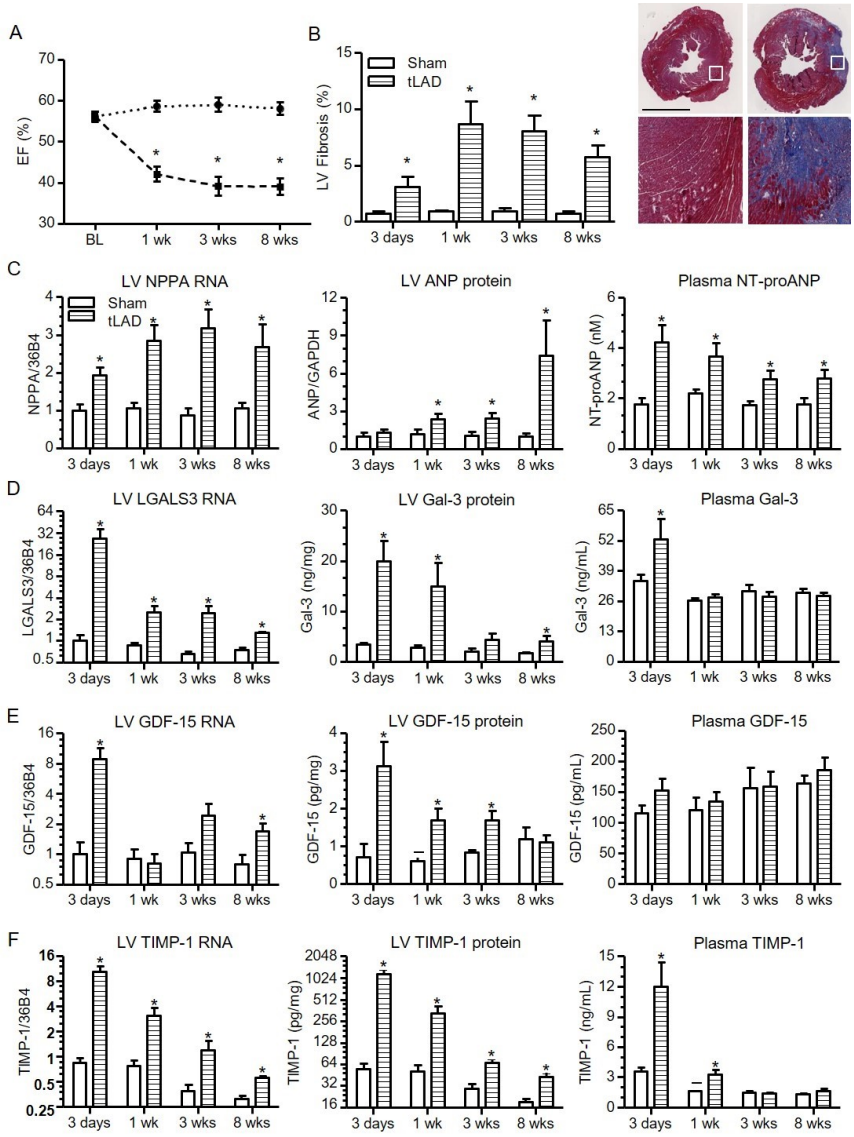

Figure 1. Cardiac function, remodeling and biomarker expression after tLAD. (A**)** Percent ejection fraction (EF) after tLAD, as determined by MRI (N=8-10). **(B)** Quantification of percent LV fibrosis as determined by Masson's trichrome staining (left panel) (N=8-10). Representative images of stained mid-left ventricular slices of mice of the 8 weeks group are shown on the left with higher magnifications of the indicated areas at the bottom. **(C)** NPPA gene expression (left panel), ANP protein levels in LV (middle panel) and NT-proANP biomarker plasma levels (right panel). **(D-F)** The same as (C), but for, respectively (D) Gal-3, (E) GDF-15 and (F) TIMP-1. Gene expression changes were corrected for 36B4 and are shown as fold changes relative to the 3 days sham group (N=6-10). ANP protein levels in the LV were determined by Western blot and corrected for GAPDH levels. All other proteins were determined by ELISA. Plasma protein levels N=6-9. LV protein levels N=4-9. Bars represent means. Error bars represent SEM. *P<0.05 versus respective control group. Black bar in (B) represents 2 mm.

directly after tLAD resulted in a temporal increase in Gal-3 and TIMP-1 plasma levels.

Cardiac remodeling and biomarker expression after permanent LAD ligation

The absence of elevated biomarker plasma levels (except NT-proANP) 8 weeks post-tLAD could be due to small MIs and hence limited decrease in cardiac function (e.g., EF from 58.1±1.6% in control to 39.0±2.1% in tLAD). Permanent LAD ligations (pLAD) that were performed in parallel generated much stronger cardiac dilatation at 8 weeks and a significant further decrease in EF to 18.3±3.3% (Figure 2A and Table 1). Moreover, cardiac fibrosis, indicative of infarct size, was much more pronounced at 8 weeks pLAD as compared to tLAD and the same was true for cardiac hypertrophy and diminished contraction and relaxation (Figure 2B and Table 1).

Since cardiac protein levels reflected gene expression levels at 8 weeks, only gene expression levels are shown for simplicity. For comprehensiveness, cardiac protein levels of all experiments are included in Figure S6. NPPA gene expression was much more elevated in the 8 weeks pLAD group as compared to the tLAD group and the same was true for cardiac protein levels and plasma levels (Figure 2C and Figure S6A). Cardiac gene expression and protein levels of Gal-3, GDF-15 and TIMP-1 were all higher in pLAD as compared to tLAD, but again this did not result in elevated plasma levels of these biomarkers (Figure 2D-F and Figure S6A). Thus, despite strongly reduced EF (18.3±3.3%) and concomitantly elevated cardiac expression, blood plasma levels did not change, except for NT-proANP.

Cardiac remodeling and biomarker expression after transverse aortic constriction (TAC)

To corroborate the above-mentioned findings, we decided to include a cardiac hypertrophy model by inducing LV pressure overload via transverse aortic constriction (TAC) (Figure S1B). In this model, EF progressively worsened from 4 to 8 weeks and, as expected, induced strong and progressive cardiomyocyte hypertrophy (Figure 3A-B). Cardiac fibrosis was modestly elevated in this cardiac hypertrophy model (Figure S7A) and hemodynamic analysis revealed a strong increase in LV end-systolic and end-diastolic pressures (LVESP, LVEDP), and contractility (dP/dT_{max}) and relaxation (dP/dT_{min}) worsened after 4 and 8 weeks post-TAC (Table 1).

Cardiac gene expression and protein levels of all four biomarkers were significantly elevated 4 and 8 weeks after TAC (Figure 3C-F and Figure S4B). In plasma, NT-proANP levels were increased in both TAC groups. Plasma Gal-3 was again not elevated, despite increased cardiac expression and protein levels (Figure 3D). However, plasma TIMP-1 was clearly elevated and GDF-15 was elevated in the 8 weeks TAC group only (Figure 3E-F). This suggests that the the underlying etiology of cardiac remodeling rather than cardiac function (e.g., %EF) per se

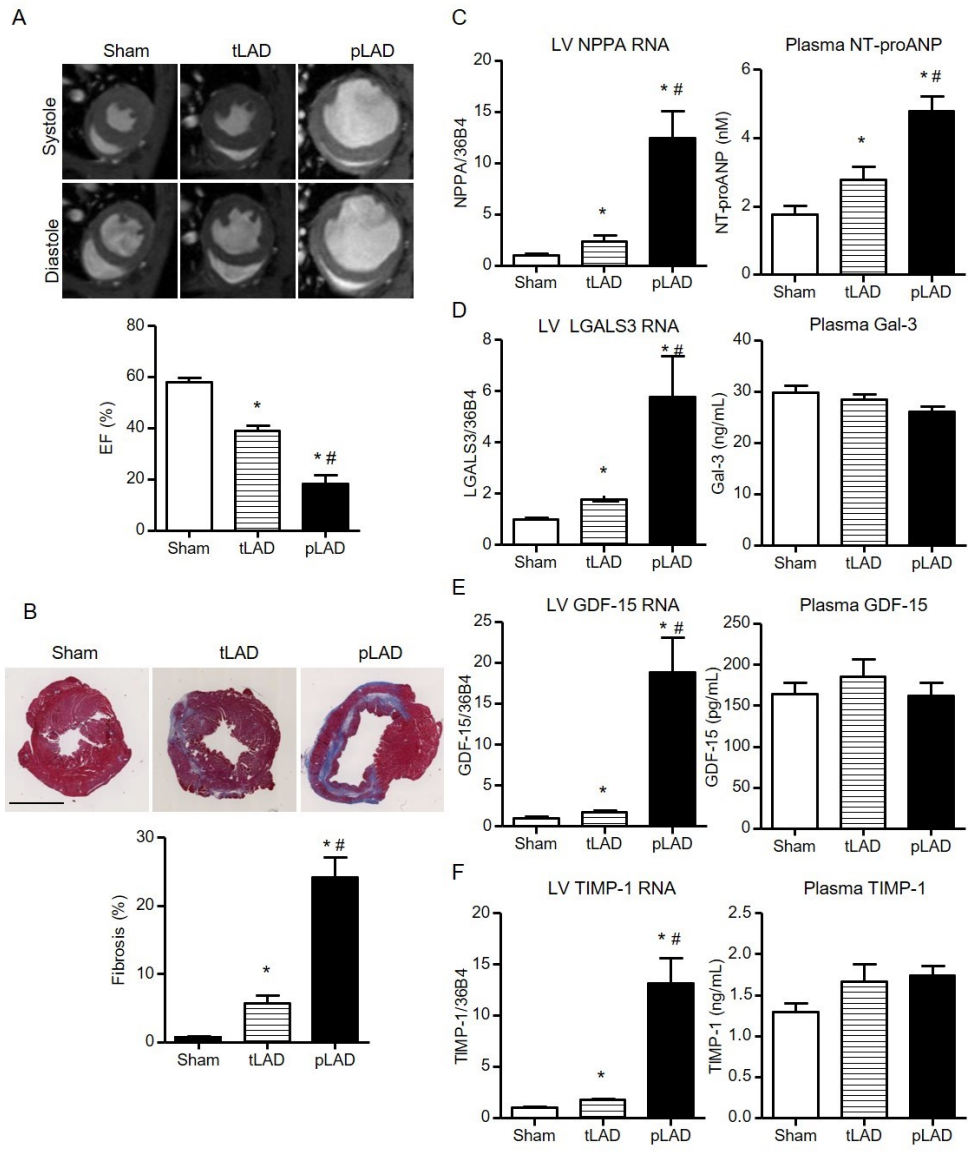

Figure 2. Cardiac function, remodeling and biomarker expression 8 weeks after tLAD and pLAD. (A) Representative MRI images of short axis in systole and diastole (upper panel). EF (%) 8 weeks after tLAD and pLAD, as determined by MRI (N=8-9). **(B)** Representative images of Masson's trichrome-stained transverse mid slices of LVs after 8 weeks follow up are shown (upper panel). **(C)** Quantification of percent LV fibrosis (lower panel, N=8-9), NPPA gene expression (left panel), and NT-proANP biomarker plasma levels (right panel). **(D-F)** The same as (C), but for, respectively (D) LGALS3/Gal-3, (E) GDF-15 and (F) TIMP-1 (N=6-10). Gene expression corrected for 36B4 and shown as fold changes relative to the 8 weeks sham group (N=6-9). Plasma levels N=8-9. Bars represent means. Error bars represent SEM. *P<0.05 versus control group. #P<0.05 versus tLAD. Black bar in (B) represents 2 mm.

determines the plasma levels of these biomarkers. The higher cardiac Gal-3, GDF-15 and TIMP-1 protein levels in the 8 weeks post-TAC group as compared to the post-MI groups are in accordance with this suggestion (Figure S6, comparison of panels A and B).

Comparison of biomarker expression in other tissues post-MI and post-TAC

Since cardiac failure can cause stress in distal organs, gene expression in other tissues was also investigated. As shown in Figure 4 (TAC study) and Figure S8 (tLAD and pLAD data), NPPA expression was confined to the heart, but the other biomarkers showed strong expression in other investigated organs (kidney, lung, liver). GDF-15 gene expression was about 100-fold higher in the liver and Gal-3 gene expression was almost 30-fold higher in lung as compared to LV tissue. This indicates that other organs may profoundly contribute to the plasma levels of these markers.

Despite reduced EF, no expression changes were identified in other organs post-MI (tLAD and pLAD) (Figure S8). After TAC, however, elevated expressions of Gal-3, GDF-15 and TIMP-1 were observed in lung tissue and Gal-3 and GDF-15 also showed some increase in the liver (Figure 4A-D). Since GDF-15 and TIMP-1 showed the highest fold increase in lung, lung protein levels, they were also investigated. Although, Gal-3 protein levels did not increase in the lungs, GDF-15 and TIMP-1 protein levels were significantly elevated (Figure 4E). The lung protein levels of GDF-15 and TIMP-1 also correlated with plasma protein levels and also with lung weight, suggesting that lung congestion in TAC animals could stimulate lung expression (Figure 4F-G). These results indicate that increased plasma levels after TAC may involve dynamic contribution of other affected organs. No congestion and increased lung expression were observed after MI, which could readily explain the lack of elevated biomarker plasma levels post-MI.

Biomarker levels in a high fat/ AngII mouse model

To further investigate the role of underlying etiology, we decided to include a mouse model that incorporates two important HFpEF risk factors, obesity and hypertension (Figure S1C). Compared to the control LFD mice, the HFD and HFD/AngII groups showed a significant increase in fat mass (Figure S9). In none of the tested conditions was cardiac dilatation or reduction in cardiac EF observed (Figure 5A). Low-dose AngII infusion induced cardiac fibrosis (Figure 5B), and cardiac mass increased significantly in the HFD+AngII group (Table S2). Importantly, in the combined HFD+AngII treatment group, cardiac dysfunction was observed, including diminished relaxation, indicative of a HFpEF-like phenotype (Table 1).

Investigation of LV NPPA gene expression and NT-proANP plasma levels revealed that these were elevated in the HFD+AngII group, in line with observed cardiac remodeling in this group

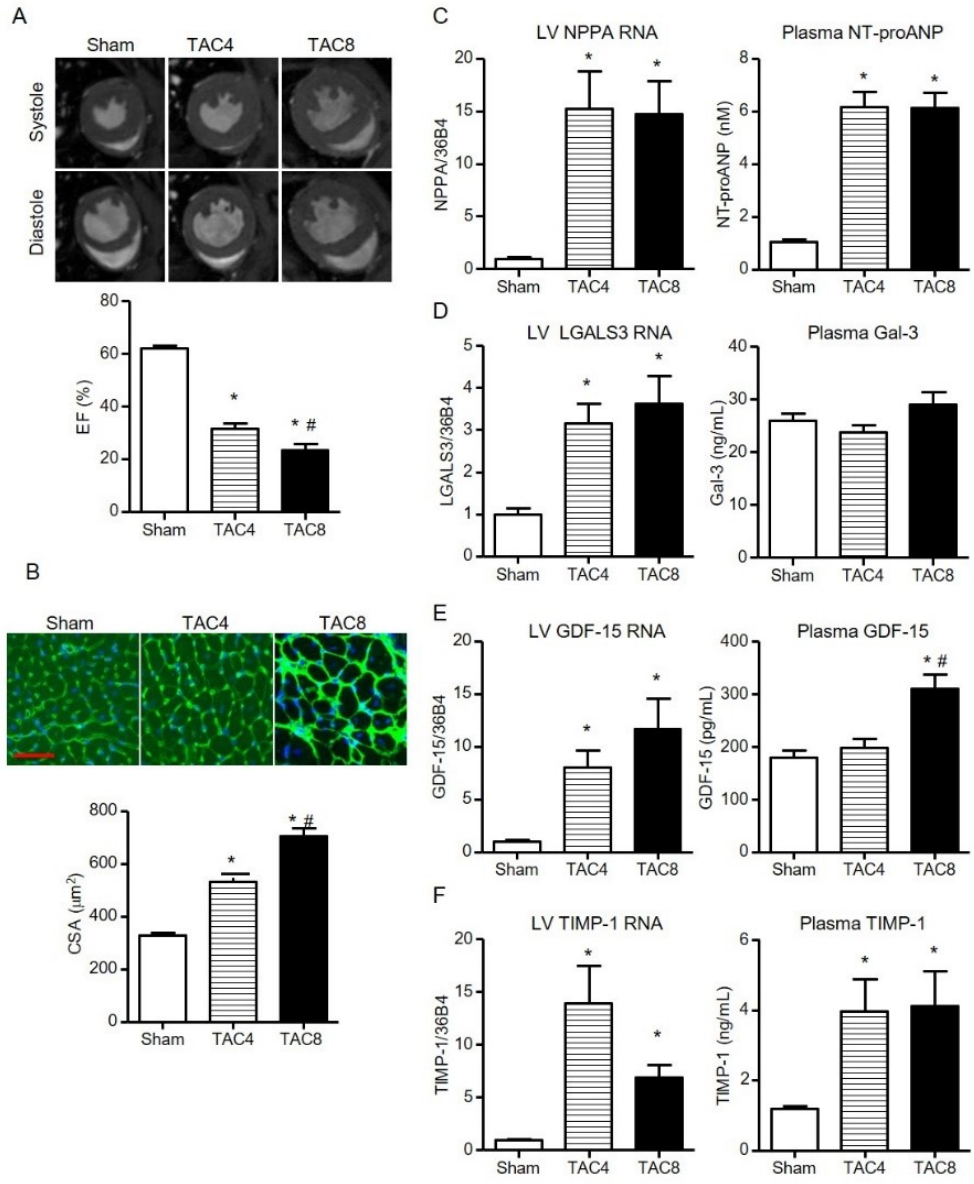

Figure 3. Cardiac function, remodeling and biomarker expression 4 and 8 weeks after TAC. (A) Representative MRI images of short axis in systole and diastole (upper panel). EF (%) 8 weeks after tLAD and pLAD, as determined by MRI. **(B)** Representative images of mid-ventricular slices stained with FITC-WGA to determine cell size (upper panels). Quantification of cell size based on FITC-WGA staining (lower panel). **(C)** NPPA gene expression (left panel), and NT-proANP biomarker plasma levels (right panel). **(D-F)** The same as (C), but for, respectively (D) LGALS3/Gal-3, (E) GDF-15 and (F) TIMP-1. All analysis N=15-20 for sham, N=8-10 per TAC group. Bars represent means. Error bars represent SEM. *P<0.05 versus control group. #P<0.05 versus TAC 4 wks. Red bar in (B) represents 50 µm

(Figure 5C). Gal-3, GDF-15 and TIMP-1 were all elevated in plasma of the HFD groups, irrespective of AngII treatment, and hence plasma levels were not associated with cardiac remodeling (Figure 5F-H). In the LV, Gal-3 and TIMP-1 gene expressions did not change in the HFD group and GDF-15 gene expression showed only a moderate elevation, indicating that other tissues must be responsible for the elevated plasma levels (Figure 5 and Figure S6C). In lung and liver, no gene expression changes were observed for Gal-3, GDF-15 and TIMP-1 and in kidney, minor changes were observed (Figure S10). Since adipose tissue was strongly increased in the HFD groups, gene and protein expressions were also investigated in visceral adipose tissue (VAT). Interestingly, Gal-3, GDF-15 and TIMP-1 gene expression and proteins were readily detected in VAT and, importantly, strongly induced in the HFD groups (Figure 6). Gal-3 levels in HFD groups were almost 300-fold higher in VAT as compared to LV. Importantly, VAT protein levels of Gal-3 and GDF-15 strongly correlated with plasma biomarker levels (Figure S11).

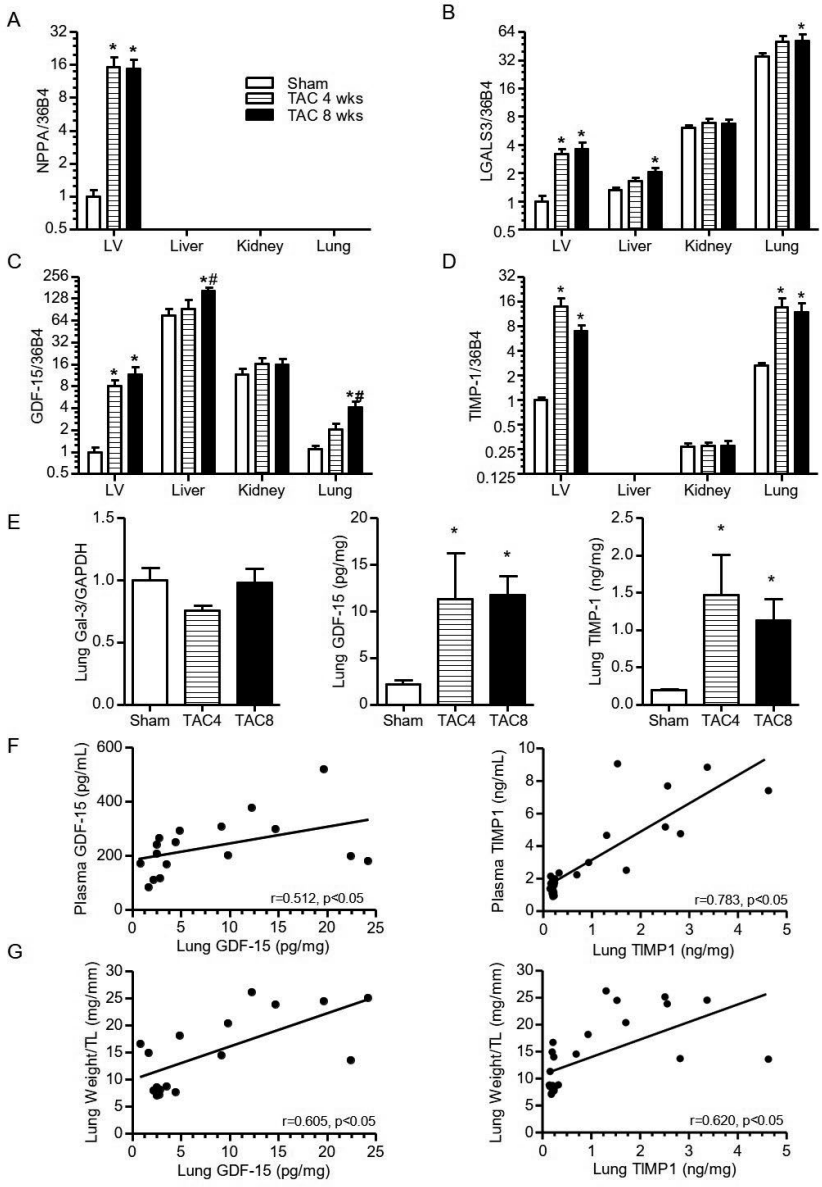

Figure 4. Biomarker gene expression in different organs post-TAC and lung-associated protein levels. **(A-D)** Gene expression levels in LV, liver, kidney and lung at 4 and 8 weeks post-TAC. (A) NPPA, (B) LGALS3, (C) GDF-15, (D) TIMP-1. N=15-20 for sham group of TAC experiment. For other groups, N=7-10. Bars represent means. Error bars represent SEM. *P<0.05 versus control group. #P<0.05 versus TAC 4wks. **(E)** Quantification of lung protein levels of Gal-3 (left), GDF-15 (middle) and TIMP-1 (right). **(F)** Association between GDF-15 and TIMP-1 plasma levels and their respective lung protein levels. **(G)** Association between GDF-15 and TIMP-1 lung protein levels and lung weight. Points in graph represent individual measurements of combined groups (N=17-25). Spearman's correlation test was performed.

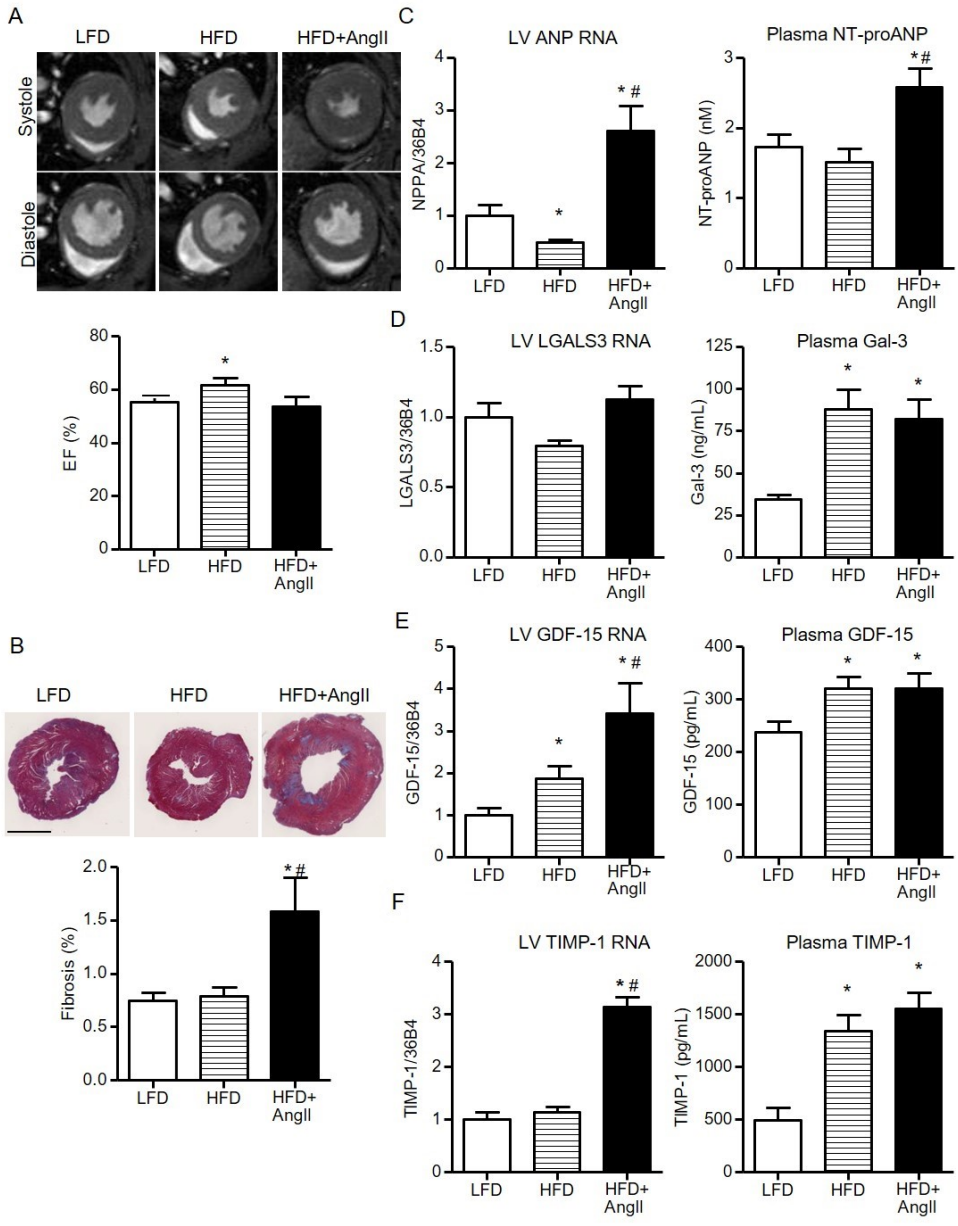

Figure 5. Cardiac function, remodeling and biomarker expression in hypertensive/obese mice. (A) Representative cardiac magnetic resonance imaging (MRI) images of the short axis of the LFD, HFD and HFD+AngII groups at 16wks. EF (%) as determined by MRI (lower panel). **(B)** Representative images of Masson's trichrome-stained mid-ventricular sections of the LFD, HFD and HFD+AngII groups. Quantification of percent LV fibrosis (lower panel). **(C)** NPPA gene expression (left panel), and NT-proANP biomarker plasma levels (right panel). **(D-F)** The same as (C), but for, respectively (D) Gal-3, (E) GDF-15 and (F) TIMP-1. N=7-13. Bars represent means. Error bars represent SEM. *P<0.05 versus LFD. #P<0.05 versus HFD. Black bar in (B) represents 2 mm.

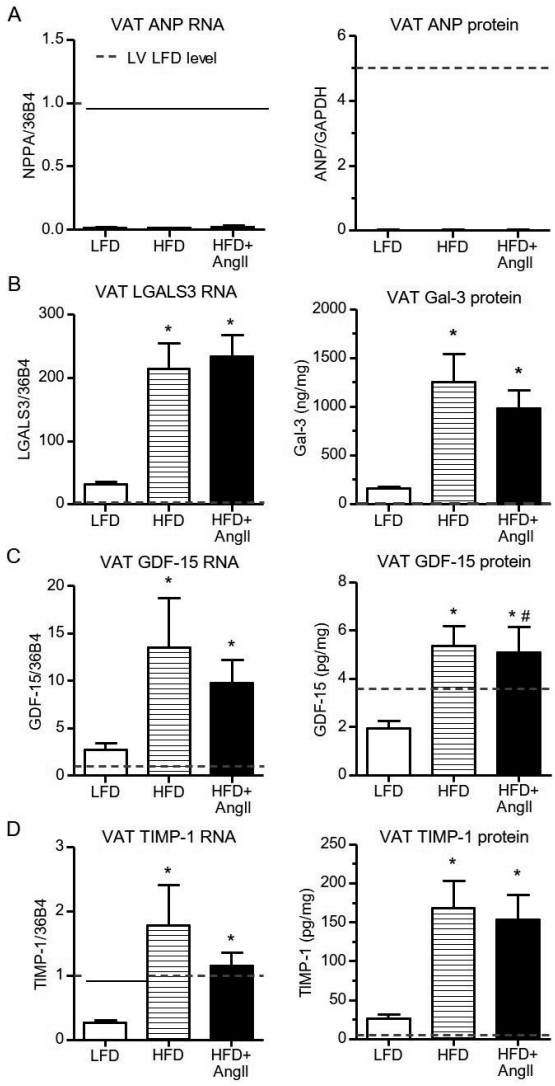

Figure 6. HF biomarkers in visceral adipose tissue of hypertensive/obese mice. Gene expression changes are presented as fold change relative to LV levels in the LFD group (dashed lines). **(A)** NPPA gene expression (left) and ANP protein levels (right) in VAT. **(B-D)** The same for, respectively, (B) Gal-3, (C) GDF-15 and (D) TIMP-1. The dashed line shows the levels observed in the LV of the LFD group. N=4-10. Bars represent means. Error bars represent SEM. *P<0.05 versus LFD. #P<0.05 versus HFD.

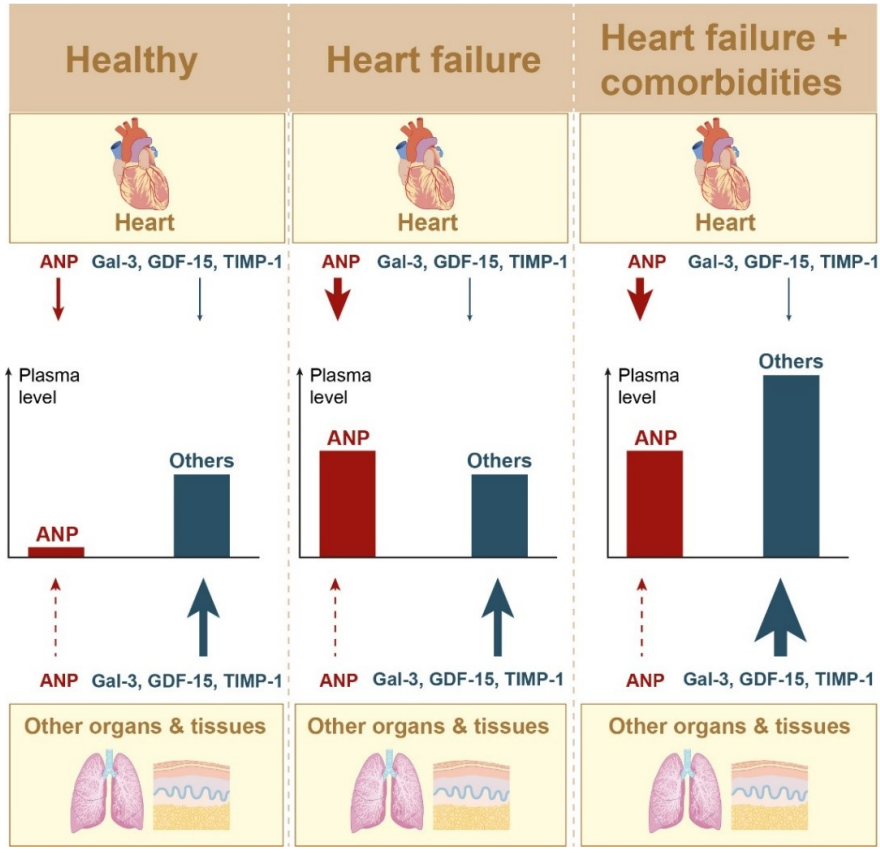

Figure 7. Model of organ/tissue involvement in plasma biomarker levels. Model with simplified schematic depiction of organ/tissue contribution to plasma biomarkers levels. Included organs/tissues: heart, lungs, kidney, liver and visceral adipose tissue (VAT). Larger arrow represents stronger relative contribution.

Discussion

Here we investigated HF biomarkers ANP, Gal-3, GDF-15 and TIMP-1 at three different levels: i) organ gene expression, ii) organ protein quantities and iii) plasma protein quantities, all in relation to cardiac function and structure. This was performed in post-MI and TAC HFrEF mouse models and in an obese/hypertensive mouse model with HFpEF characteristics. Our comprehensive dataset revealed that cardiac gene expression and/or protein levels of these markers significantly increased upon cardiac remodeling. However, in blood plasma, only NT-proANP levels were specifically and significantly elevated in response to cardiac remodeling in all mouse models. Circulating levels of Gal-3, GDF-15 and TIMP-1 were strongly influenced by extra-cardiac tissues and their elevation also reflects enhanced stress and concomitant productions in these extra-cardiac tissues (Figure 7).

Cardiac gene expression of all four biomarkers paralleled in most cases the cardiac protein levels. Changes in cardiac ANP gene expression and protein levels showed some divergence under some conditions and this may be related to additional levels of control, including protein secretion and post-translational processing of ANP. Whereas ANP cardiac gene expression and plasma levels showed a very strong correlation in all cases, this was not true for the other biomarkers. At 8 weeks post-MI, cardiac expressions of Gal-3, GDF-15 and TIMP-1 were clearly elevated, but not their plasma levels. Although elevated plasma levels of GDF-15 and TIMP-1 were observed 8 weeks post-TAC, this may not simply reflect increased GDF-15 and TIMP-1 cardiac expression, since in these animals lung expression was also clearly elevated. TIMP-1 protein levels in lung tissue were even 4.5 times higher as compared to LV levels. Taking organ size into consideration, this suggests that under these conditions lungs may contribute stronger to the elevated plasma levels. The expression also correlated with lung weight, suggesting that TAC-mediated congestion may be responsible for increased GDF-15 and TIMP-1 plasma levels. It is therefore tempting to suggest that hemodynamic stress in other organs and tissues can contribute to elevated plasma levels of these biomarkers and hence these markers are indirectly associated with cardiac function.

In contrast to NPPA (ANP), the other biomarkers showed a fast temporal increased expression in the heart after cardiac infarction that resembled the pattern of inflammatory (IL-6) and pro-fibrotic genes (Col1a1, Col3a1), which is in accordance with their suggested functions in these processes. Whereas Gal-3 expression was confined to the infarcted area, ANP expression was mostly elevated in the remote area and absent from the infarcted (fibrotic) regions. Although we were unable to stain mouse TIMP-1 and GDF-15, the latter has previously been shown to be highly expressed in cardiomyocytes in the infarcted area in human hearts [30]. Therefore, in contrast to ANP, these novel biomarkers appear to play a role in the infarcted area, which

again stresses the differences between these markers. Furthermore, as shown for Gal-3, different cell types may be involved at different stages after infarction, which further adds to the complexity of this biomarker.

The highly elevated expressions directly after infarction resulted in temporally elevated plasma levels of Gal-3 and TIMP-1. Although Gal-3 and TIMP-1 plasma levels have been reported to be elevated in patients with acute MI, the exact temporal changes have never been systematically investigated. Performing such clinical investigations could be rewarding, but results may be more complex to interpret. In patients, cardiovascular risk factors and comorbidities are involved in both causing and maintaining cardiovascular disease, whereas our MI mouse model was an isolated ischemic model, lacking these confounding factors that might influence biomarker levels, as discussed below.

Our observation that the plasma levels of the tested novel biomarkers did not change despite severely reduced EF (8 weeks post-MI), may sound remarkable, but we would like to point out that these are otherwise healthy mice without other co-morbidities. This seems to reflect the results of the HF-ACTION clinical study in which patients of NYHA class III and IV were included; but, in order to allow them to follow a structured exercise program, these patients were generally healthier than patients included in other HF studies. Not surprisingly, in this study, much lower Gal-3 plasma levels were measured as compared to other HF studies [31]. The observations that these biomarkers are mostly elevated in patients with additional co-morbidities and poor outcome further strengthens our observations [14, 15, 17, 32]. In particular, we showed that Gal-3, GDF-15 and TIMP-1 were strongly influenced by obesity. The elevated plasma levels in obese animals were not associated with cardiac function. Adipose tissue was the major producer of Gal-3 and its levels were even more than 300 times higher in adipose tissue as compared to LV tissue. Additional induction of a cardiac HFpEF-like phenotype in these animals using AngII infusion did not result in further enhancement of their plasma levels, despite alterations in cardiac expressions. Positive associations of these biomarkers have been reported with several other diseases and with obesity, and this provides clear evidence that non-cardiac sources are important determinants of their circulating levels in humans as well [5, 33, 34, 35, 36]. We therefore would like to postulate that elevated levels of these biomarkers in HF patients most likely reflect the presence of other comorbidities and hint to stress and involvement of other organs.

In many clinical studies, circulating levels of Gal-3, GDF-15 and TIMP-1 have been correlated to HF severity based on clinical symptoms (NYHA class) or tested for their predictive power for mortality and HF rehospitalization in HF patients [12, 14, 20, 21, 32, 34, 37]. Even though

several clinical studies have shown that these biomarkers are associated with cardiac function (e.g., EF or LV strain) [38], cardiac remodeling (e.g., LV dimensions) [39, 40] and LV-filling pressures[37, 41, 42], none of these studies provided evidence for the cardiac nature of the increased systemic levels. Not surprisingly, several studies also provided evidence that these biomarkers could not be directly related to specific cardiac indices in HF patients, including echocardiographic parameters [43]. Moreover, in an elegant study, it was shown that elevated Gal-3 levels did not decline after heart transplantation, a clear indication that non-cardiac sources were responsible for high Gal-3 levels in these HF patients [44]. In cardiomyopathy patients, it was recently reported that plasma Gal-3 levels did not correlate with Gal-3 levels in endomyocardial biopsies [45]. Therefore, we postulate that correlating circulating biomarker levels to parameters of cardiac dysfunction does not take into account the above-mentioned issues, and such results might therefore generate false results. Thus, although these biomarkers are intensely studied in HF, our results call into question the feasibility of relating plasma/serum biomarkers levels to cardiac-specific indices of HF severity including function and dimensions. Our data clearly indicate that these measures have a strong relation to cardiac biomarker expression and production, but not necessarily to systemic biomarker levels.

Since ANP expression is highly cardiac specific, its plasma levels are not obscured by contribution of non-cardiac tissues. This is not true for the other HF biomarkers studied here and this is most likely also true for other non-cardiac specific HF biomarkers. Unfortunately, we could not test this for another HF biomarker that has been included in the AFFC/AHA clinical guidelines, soluble ST2 (sST2), due to lack of proper mouse reagents. From literature, it is known that sST2 gene expression is not cardiac specific and it is therefore likely that non-cardiac contributions will also affect plasma levels of sST2 [10, 46, 47]. Our results clearly show that it will be important to rigorously test any novel plasma biomarker in animal models, allowing the simultaneous investigation of the dynamic contribution of multiple tissues to the plasma levels.

Conclusion

In conclusion, we have shown that only plasma NT-proANP levels are directly related to cardiac function, whereas plasma levels of the novel HF biomarkers Gal-3, GDF15 and TIMP-1 are heavily influenced by dynamic contribution of non-cardiac tissues. Therefore, rather than being specific for indices of cardiac remodeling, these biomarkers reflect health status beyond cardiac function. They also reflect stress in other organs, either as a consequence of the failing heart and/or as a consequence of other underlying comorbidities, like metabolic syndromes.

Abbreviations

AngII: angiotensin II; ANP: atrial natriuretic peptide; EF: ejection fraction; Gal-3: galectin-3; GDF-15: growth differentiation factor-15; HF: heart failure; HFD: high fat diet; HFrEF: heart failure with reduced ejection fraction; HFpEF: heart failure with preserved ejection fraction; LAD: left anterior descending coronary artery; LFD: low fat diet; LV: left ventricle; LVEDP: left ventricular end-diastolic pressure; LVESP: left ventricular end-systolic pressure; MI: myocardial infarction; MRI: magnetic resonance imaging; pLAD: permanent LAD ligation; TAC: transverse aortic constriction; tLAD: temporal LAD ligation; TIMP-1: tissue inhibitor of metalloproteinase-1; VAT: visceral adipose tissue

Acknowledgements

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Competing Interests

The UMCG, which employs a number of the authors, received research grants and consultancy fees from AstraZeneca, Roche, Bristol Myers Squibb, Pfizer, Trevena, Thermofisher GmbH, and Sphingotec GmbH, for work done by UMCG employers. Dr. Voors received funding from Roche Diagnostics and Sphingotec. Dr. de Boer received speaker fees from Novartis. Dr. H. Silljé received research grants from AstraZeneca.

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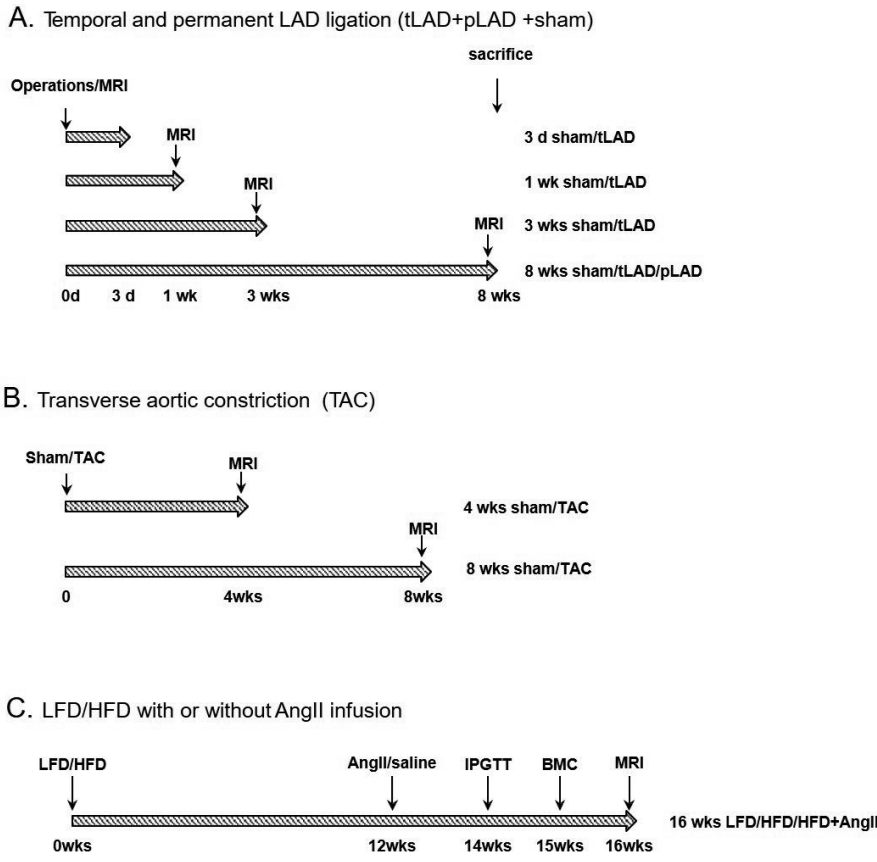
Supplementary Table 1. Oligonucleotide pairs used for qPCR

Gene	5' - 3' forward	5' - 3' reverse
NPPA	ATGGGCTCCTTCTCCATCAC	TCTACCGGCATCTTCTCCTC
NPPB	AAGTCCTAGCCAGTCTCCAGA	GAGCTGTCTCTGGGCCATTTC
TIMP1	CTGCTCAGCAAAGAGCTTTC	CTCCAGTTTGCAAGGGATAG
Col1a1	CTTCACCTACAGCACCCTTGTG	CTTGGTGGTTTTGTATTCGATGAC
Col3a1	GCGATTCAAGGCTGAAG	GGGTGCGATATCTATGATGG
IL6	TCCCAACAGACCTGTCTATAC	CAGAATTGCCATTGCACAACTC
LGALS3	CCCGCTTCAATGAGAACAAC	ACCGCAACCTTGAAGTGGTC
GDF-15	TGACCCAGCTGTCCGGATAC	GTGCACGCGGTAGGCTTC
VCAM-1	ACCCAAACAGAGGCAGAGTG	CACTTGAGCAGGTCAGGTTC
36b4	AAGCGCGTCCTGGCATTGTC	GCAGCCGCAAATGCAGATGG

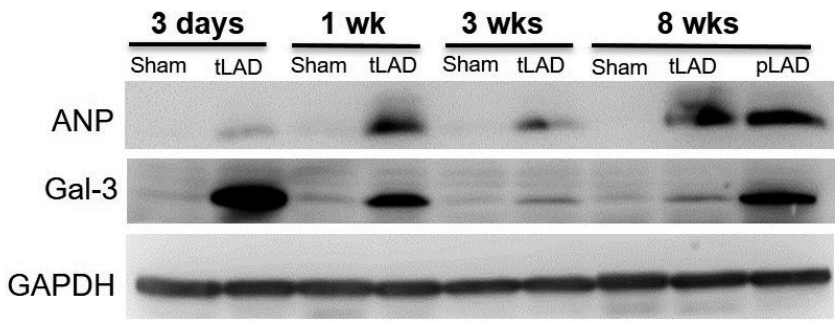
Supplementary Table 2. Bodyweight, and corrected LV weights

Group	BW	LV/TL (mg/mm)
Sham (8 weeks)	31.2 ± 0.6	6.5 ± 0.2
tLAD (8 weeks)	28.9 ± 0.6	6.5 ± 0.2
pLAD (8 weeks)	31.4 ± 0.5$^{\#}$	8.9 ± 0.3*$^{\#}$
Sham	27,7 ± 0,5	6,4 ± 0,1
TAC4	25,1 ± 0,4*	10,3 ± 0,4*
TAC8	27,1 ± 0,8	12,3 ± 0,4*$^{\#}$
Sham	31.7 ± 0.8	7.0 ± 0.2
HFD	42.8 ± 1.9*	7.3 ± 0.3
HFD+AngII	38.5 ± 1.1*$^{\#}$	9.4 ± 0.4*$^{\#}$

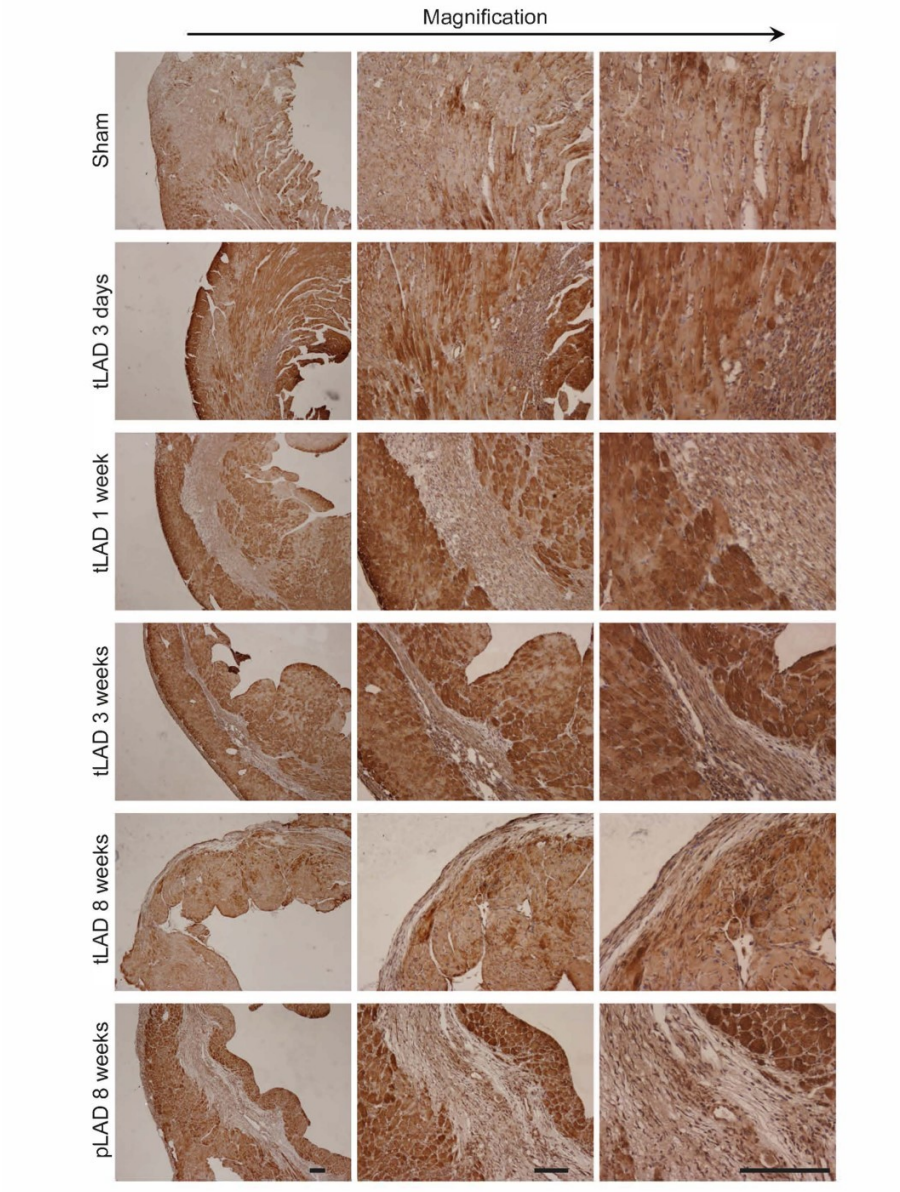
BW=bodyweight; LV/TL=Left ventricular weight divided by tibia length. N=7-12* $p<0.05$ as compared to sham control; # $p<0.05$ as compared to tLAD, TAC4 or HFD, respectively.



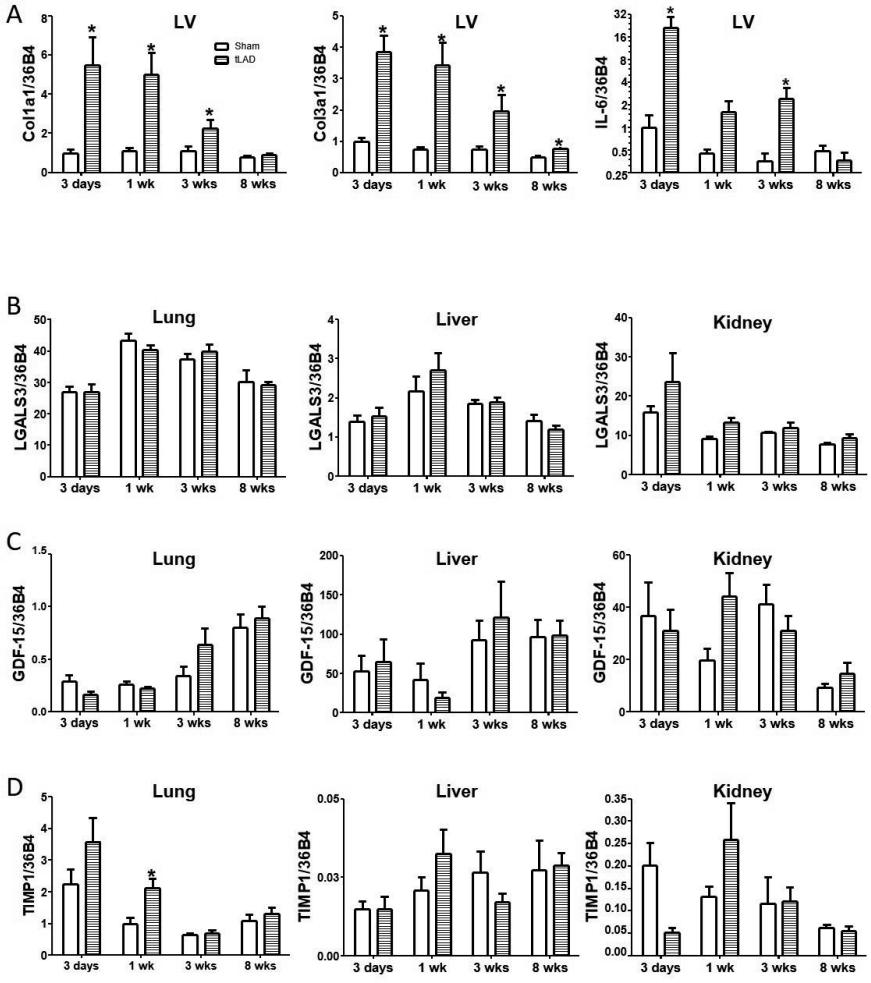
Supplemental figure 1. Schematic depictions of the mouse experimental setups. (A) Schematic depiction of the post-myocardial infarction (MI) experiments including temporal LAD ligation (tLAD) , permanent ligation (pLAD) and sham groups. tLAD was investigated at multiple time points, whereas pLAD animals were only investigated at 8 weeks. Magnetic resonance imaging (MRI) measurements were performed in each group (except 3d), as indicated. **(B)** Schematic depiction of the transverse aortic constriction (TAC) experiment. With 4 and 8 weeks and sham groups. MRIs are indicated. **(C)** Schematic depiction of the obese-hypertensive mouse experiment. Mice received either a high fat diet (HFD) with or without Angiotensin II (AngII) infusion. Also a control LFD group was included. Body mass composition (BMC) and MRI measurements are indicated. AngII or saline infusion was performed during the last 4 weeks of the experiment.



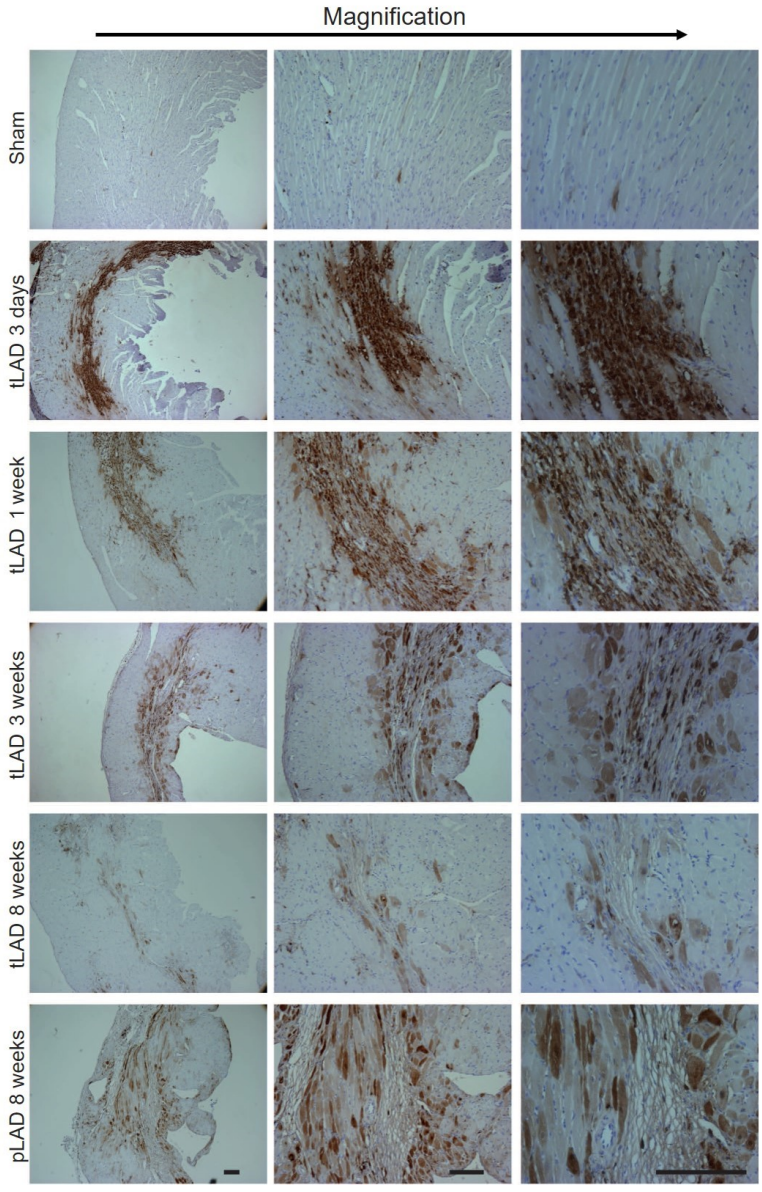
Supplemental figure 2. Cardiac protein ANP levels as determined by Western blotting. Representative Western blot of ANP detection in left ventricular tissue post-infarction (tLAD and 8 weeks pLAD). For comparison Gal-3 is also included and showed major elevation at 3 days (in agreement with ELISA results). As a loading control GAPDH is shown.



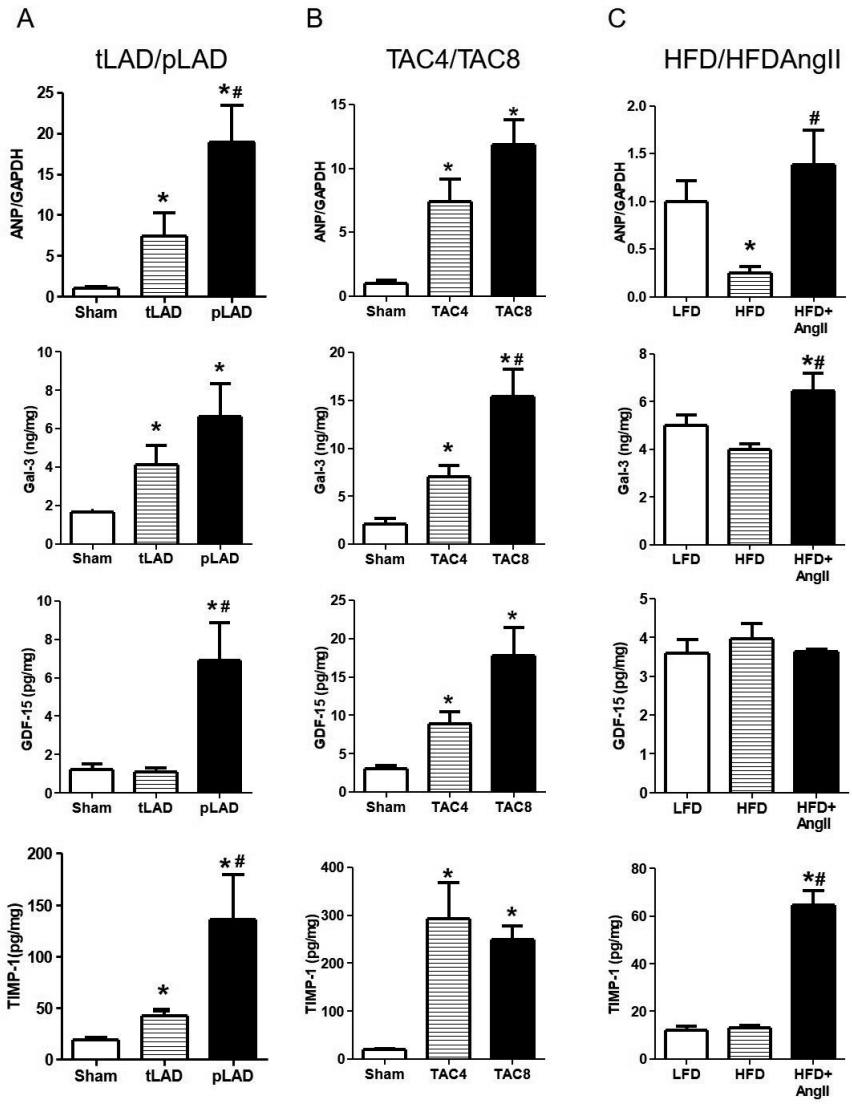
Supplemental figure 3. Immunohistological ANP staining of cardiac left ventricular slices. Left ventricular cardiac slices of hearts from sham, tLAD and pLAD mice were stained with anti-ANP (brown) and counter stained with haematoxylin (blue). Representative images are shown at different magnifications. Scale bar indicates 150 µm.



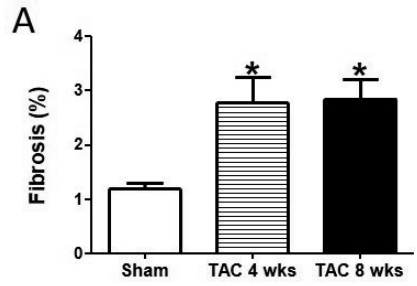
Supplemental figure 4. Gene expression in LV and other tissues after tLAD. (A) Cardiac gene expression at 3 days, 1 week, 3 weeks and 8 weeks post tLAD. Of Alpha-1 type 1 collagen (Col1a1) (left), Alpha-3 type 1 collagen (Col3a1) (middle) and Interleukin-6 (IL-6) (right panel). (B-C) gene expression in lung (left panels), liver (middle panels) and kidney (right panels). **(B)** Gal-3. **(C)** GDF-15 and **(D)** TIMP-1. Gene expression is corrected for 36B4 expression levels and is presented as fold change. N=5-9 per group. Bars represent means. Error bars represent SEM. *P<0.05 versus control group.



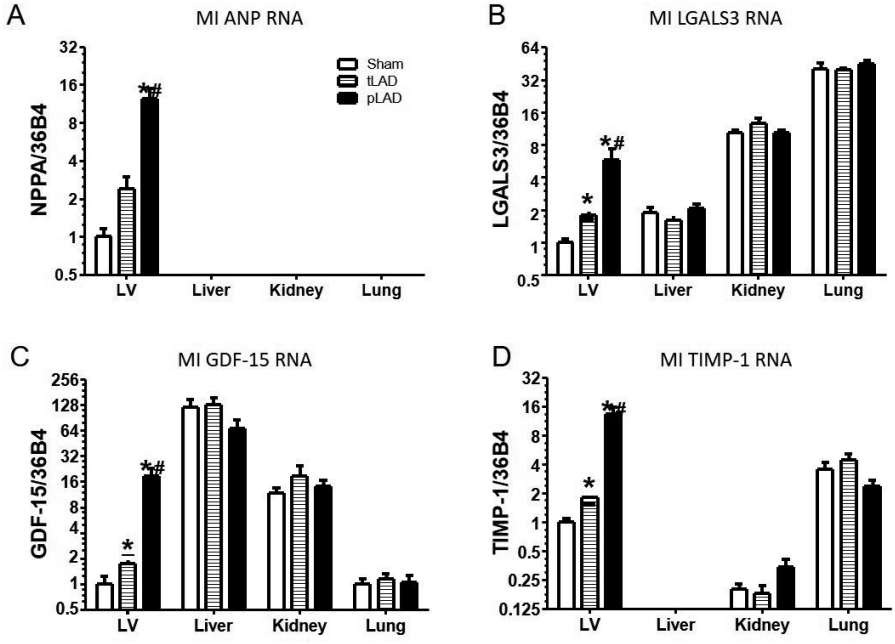
Supplemental figure 5. Immunohistological Gal-3 staining of cardiac left ventricular slices. Left ventricular cardiac slices of hearts from sham, tLAD and pLAD mice were stained with anti-Gal-3 (brown) and counter stained with haematoxylin (blue). Representative images are shown at different magnifications. Scale bar indicates 150 µm.



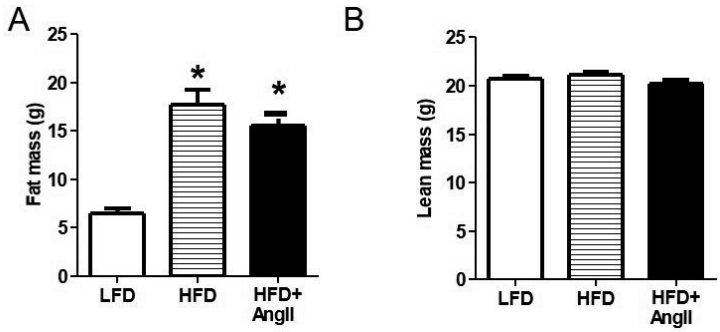
Supplemental figure 6. Cardiac biomarker protein levels. (A) Cardiac proteins levels after 8 weeks tLAD and pLAD and sham control group. **(B)** Cardiac proteins levels after 4 and 8 weeks TAC and sham control group. **(C)** Cardiac proteins levels after 16 weeks HFD with or without 4 weeks AngII infusion and control (LFD) group). From top to bottom, ANP, Gal-3, GDF-15 and TIMP-1. ANP cardiac levels were determined by Western blot and are relative to the respective sham group. All other cardiac levels were determined by ELISA and are absolute levels. N=4-8. *P<0.05 versus respective groups. #P<0.05 versus tLAD, TAC4 or HFD group.



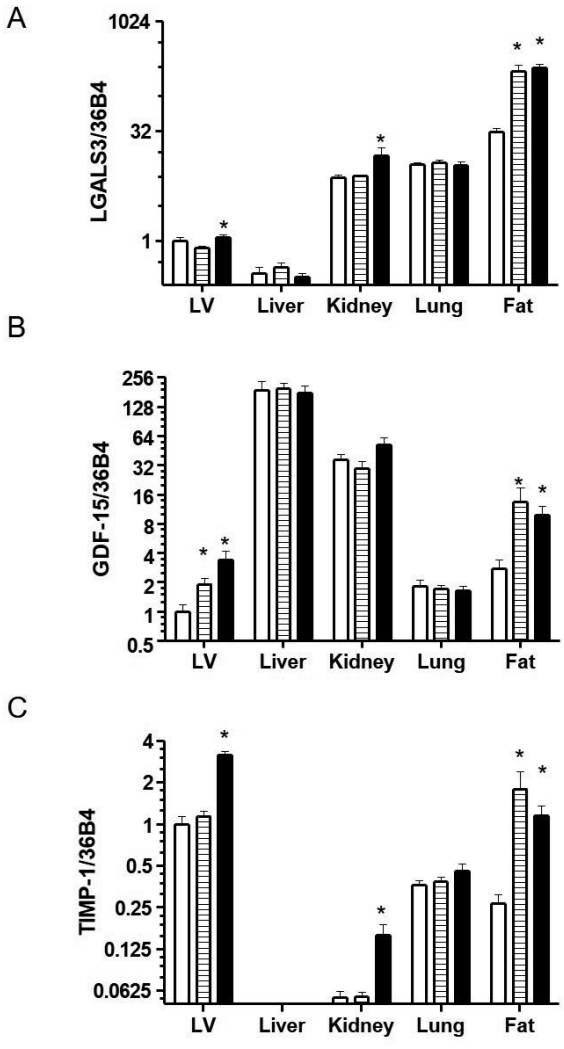
Supplemental figure 7. Cardiac fibrosis in the TAC groups. Quantification based on Masson Trichrome staining of mid-ventricular slices of LV tissue. N=7-10. *P<0.05 versus control group.



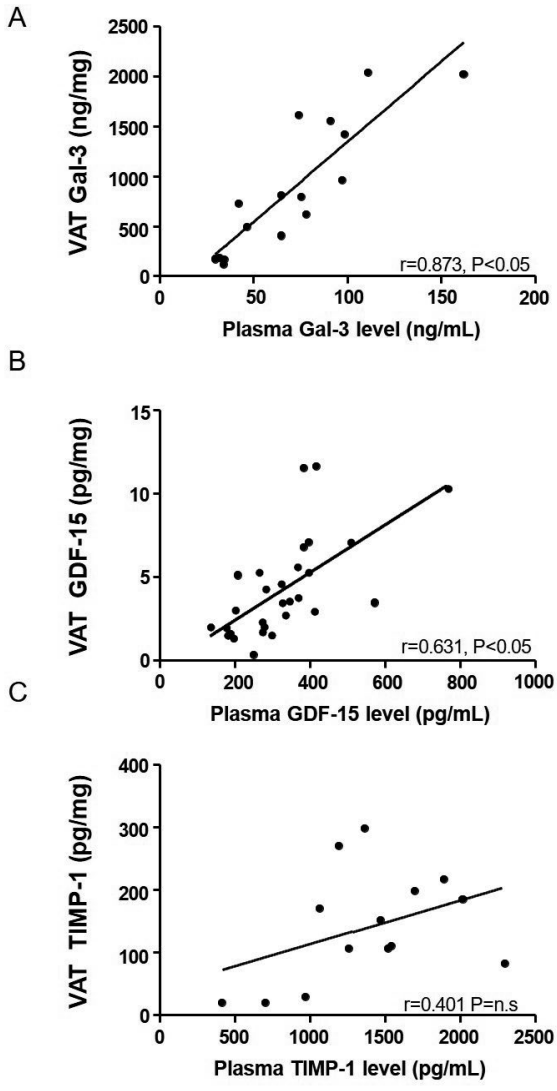
Supplemental figure 8. Biomarker gene expression in different organs after 8 weeks tLAD and pLAD. **(A-D)** Gene expression levels in LV, liver, kidney and lung **(A)** NPPA, **(B)** LGALS3, **(C)** GDF-15, **(D)** TIMP-1. Gene expression changes were corrected for 36B4 and are shown as fold changes relative to the LV levels in the respective sham group. N=6-10. Bars represent means. Error bars represent SEM. *P<0.05 versus control group. #P<0.05 versus tLAD.



Supplemental figure 9. Fat and lean mass, in obese/hypertensive/ mice. (A) Quantification of fat mass after diet intervention and with or without AngII infusion. **(B)** Quantification of lean mass. N=11-13 per group. Bars represent means. Error bars represent SEM. *P<0.05 versus control, #P<0.05 versus HFD.



Supplemental figure 10. Biomarker gene expression in different organs in hypertensive/obese mice. (A-C) Gene expression in left ventricle (LV), liver, kidney lung and Fat (VAT) tissue. Data at 16 weeks after start of diet intervention and 4 weeks after start AngII infusion. **(A)** LGALS3, **(B)** GDF-15, (**C**) TIMP-1. Gene expression was corrected for 36B4 gene expression and is presented as fold change, hereby using LV gene expression of the respective gene in the control (LFD) group as the reference group. N=7-13 per group. Bars represent means. Error bars represent standard error of the mean. Points on graph represent individual measurements. *P<0.05 versus control group.



Supplemental figure 11. Correlation between biomarker VAT protein levels and plasma levels. VAT and plasma levels of the indicated biomarkers were determined by ELISA. **(A)** Gal-3, **(B)** GDF-15, **(C)** TIMP-1. Points in the graph represent individual measurements of the combined experiments. N=20-39. Spearman's correlation test was performed

Chapter 6

Tissue plasma-biomarker expression in Ren2 hypertensive heart failure rats

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Manuscript in preparation

ABSTRACT

Background: Despite elaborate clinical investigations of potential plasma heart failure biomarkers their relation with cardiac function and remodeling has remained vague. Recently, we showed in multiple mouse models that levels of these biomarkers are not directly related to cardiac function, except for natriuretic peptides. To investigate how the expression of these biomarkers is affected by hypertension induced heart failure we used the Ren2 rat model overexpressing the mouse renin gene.

Methods: Ren2 and age matched Sprague-Dawley (SD) rats were sacrificed between 14-16 weeks of age when most Ren2 animals showed signs of fatigue and breathlessness (humane endpoints). Echocardiography was used to assess cardiac structural and functional remodeling and invasive pressure catheter measurements were performed to record hemodynamic parameters. Atrial natriuretic peptide (NPPA, ANP), Galectin-3 (LGALS3, Gal-3) TIMP-1 and GDF-15 gene and protein expression were investigated in multiple organs and tissues.

Results: Significant cardiac dilatation was observed in Ren2 rats at 14-16 weeks, resulting in a significantly reduced ejection fraction and fractional shortening and decreased contractility (dP/dT_{max}) and relaxation (dP/dT_{min}). Heart, kidney, liver and lung weights were all higher in Ren2 rats. NPPA expression was restricted to the heart and strongly induced in the Ren2 group. In contrast, LGALS3, GDF-15 and TIMP-1 were expressed in multiple tissues and GDF-15 expression was almost 55 fold higher in kidney as compared to cardiac tissue. In the Ren2 group expression of these genes was elevated in the heart, but also in kidney and liver (Gal-3). Gal-3 protein level was also significantly elevated in liver and cardiac tissue of the Ren2 group, whereas ANP protein level was solely elevated in cardiac tissue. Unfortunately, plasma Gal-3 levels could not be determined in rats, but plasma ANP, GDF15 and TIMP1 levels were all significantly elevated.

Conclusions: In summary, our study shows that expression of Gal-3, GDF-15 and TIMP-1 in Ren2 rats is elevated in multiple organs, including heart and kidney. Potential elevated plasma levels appear therefore to be a reflection of stress in multiple organs by this systemic disease in these animals.

Introduction

Heart failure plasma biomarkers have received huge interest in the last decade because of their promise to further stratify patient populations and to provide further insight in ongoing pathological processes. These biomarkers may aid in guiding heart failure management and patient tailored therapies. Till now, natriuretic peptides, including B-type natriuretic peptide (BNP) and the N-terminal domain of the prohormone (NT-proBNP), can be considered as the gold standard plasma biomarkers in the diagnosis of HF [1, 2]. These peptides as well as A-type natriuretic peptide (ANP) are secreted by cardiomyocytes and production and secretion are enhanced upon cardiomyocyte stretch [3]. Elevated plasma levels are therefore considered as an indication of elevated cardiac wall stress and indicative that therapy to unload the heart is required. Natriuretic peptides can therefore aid in therapy guidance for HF patients and provide prognostic information. There is a lasting promise that other potential heart failure plasma biomarkers may provide information on other pathological processes in the heart, like cell death, fibrosis and inflammation [4, 5]. However, despite intensive investigations and abundant correlative clinical studies the value of most other putative heart failure biomarkers has remained elusive [6, 7].

The American College of Cardiology (ACC)/AHA heart failure guidelines recognize the plasma proteins Galectin-3 (Gal-3) and soluble Suppression of Tumorigenicity 2 (sST2) as emerging biomarkers of myocardial fibrosis that are predictive for hospitalization and death in patients with HF and may have additive prognostic value beyond natriuretic peptides [1]. Numerous correlative clinical HF studies have shown a correlation between plasma Gal-3 protein levels and prognosis, both for patients with HF with reduced ejection fraction (HFpEF) and for patients with reduced ejection fraction (HFrEF) [8-11]. Galectin-3 appears to have multiple functions and play a prominent role in driving fibrosis and it has been linked to fibrotic diseases in several organs [12, 13]. In rats Gal-3 promoted cardiac fibrosis and subsequent heart failure development, while inhibition or genetic ablation of Gal-3 in rats and mice resulted in attenuation and partial reversal of cardiac remodeling upon pathological stimuli [14, 15]. sST2 is secreted into the circulation in response to cardiac stress and exhibits pro-hypertrophic and fibrotic effects by severing as a decoy receptor for IL33. However, like Gal-3, sST2 plasma concentrations are also increased in various other diseases, including fibrotic and inflammatory diseases and is not disease specific [16-18]. A number of other plasma proteins involved in extracellular matrix remodeling have been shown to be elevated in HF patients, including Tissue Inhibitor of Metalloproteinases (TIMP-1) [19]. Another potential HF biomarker that has received a lot of attention is Growth Differentiation Factor 15 (GDF-15). Plasma levels of GDF-15 has been reported to be associated with inflammation and cell apoptosis in cardiac and

extra-cardiac disease [20-23]. GDF-15 plasma levels are associated with severe outcome, and its levels are able to provide incremental value to clinical HF risk factors [24].

In recent mouse studies we provided evidence that Gal-3, GDF-15 and TIMP-1 were elevated in cardiac tissue after myocardial infarction, but plasma levels were not elevated despite strong reductions in EF (below 20%) [25]. Elevated levels were observed in certain HF mouse models, like cardiac pressure overload or after a high fat diet, but this appeared to be mostly a reflection of production by other stressed organs/tissues [25]. The potential use of these novel HF biomarkers is therefore still elusive and the lack of understanding of dynamic expression in the heart and in other organs currently hampers potential clinical use.

To further investigate the expression of HF biomarkers in the heart and other tissues we employed herein a transgenic Ren2 hypertensive rat model. Hypertension is a key driver of heart failure development, but has systemic effects also in other tissues, particular the kidneys and hence might also affect expression of putative HF biomarkers in these tissues. Cardiac function and remodeling and expression of ANP, Gal-3, GDF-15 and TIMP1 were therefore investigated in this rat model.

Materials and Methods

Animal experimental protocol

Animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethical Committee of the University of Groningen. (permit number: DEC6954A). Animal experiments were performed in 10–16-week-old male homozygous TGR (mREN2)27 rats (n = 13). Age- and gender-matched Sprague Dawley (SD) rats (n = 8) served as controls. REN2 rats overexpress the murine Ren-2d gene that causes hypertension and progressive HF [26-28]. Male Sprague-Dawley and age matched Ren2 rats were housed on a 12/12 hours day/night cycle in a controlled environment and ad libitum access to water and chow.

Cardiac function assessment

Echocardiography was performed to assess functional parameters in vivo at 10 weeks and at the end of the experiment. Briefly, M-mode and 2D transthoracic measurements were obtained under anesthesia (Vivid 7, 14-MHz linear array transducer; GE Healthcare, Chalfont St. Giles, UK). Rats were maintained on a heated pad, and a topical depilation agent was used to remove chest hair. From the parasternal short axis view, M-mode tracings were recorded to measure left ventricular (LV) inner diameters in systole and diastole (LVIDs/d in mm) and calculate percent fractional shortening (%FS) and ejection fraction (%EF). The thickness of the LV

posterior wall in diastole (LVPWd in mm) and intraventricular septal wall thickness (IVSd in mm) were also determined.

PV-loop measurements

Prior to sacrifice, heamodynamics were recorded by aortic and LV cathetherization. During this procedure, mice were anesthetized with 2% isoflurane/oxygen and catheterization was performed with a (Mikro-Tip pressure catheter 1.4F, Transonic Scisense, Transonic Europe, The Netherlands) The catheter tip was inserted via the left carotid artery and pressures in the aorta and LV were monitored. Parameters of cardiac function were recorded, including maximal LV pressure (LV Pmax), minimal LV pressure (LV Pmin), dP/dTmax (an indicator for maximal LV contraction capacity), dP/dTmin (an indicator for maximal LV relaxation capacity), maximal aortic pressure (aorta Pmax) and heart frequency (HF). Thereafter, the catheter was removed and animals were sacrificed and tissues and organs were collected for molecular analysis.

Enzyme-linked immunosorbent assay (ELISA)

The following commercial enzyme-linked immunosorbent assays (ELISA) were used to determine protein levels in plasma: NT-proANP (BI-20892, BIOMEDICA, Austria); GDF-15 (MGD150, R&D, USA); and TIMP1 (DY580, R&D, USA). All used reagents and buffers were supplied in the kit and were prepared for analysis as described in the manual. Plasma samples were thawed, mixed and diluted 20 times with dilution buffer. Next, standard, samples and controls were transferred to antibody coated ELISA plates and plates were processed according to the manufacturer instructions. Tetramethylbenzidine (TMB) was used as a substrate in the final peroxidase reaction and absorbance of samples, standards and controls was measured at 450nm using a plate reader (Synergy H1 microplate reader, Biotek, Vermont, USA). Plasma MPO levels were calculated using GEN5 software (GEN5 version 2.04, Biotek, Vermont, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Ribonucleic acid (RNA) was extracted from powdered tissues using Trizol reagent (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA). cDNA was synthesized using QuantiTect Reverse Transcriptional kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. Relative gene expression was determined by quantitative real time PCR (qRT-PCR) on the Bio-Rad CFX384 real time system (Bio-Rad, Veenendaal, the Netherlands) using ABsolute QPCR SYBR Green mix (Thermo Scientific, Landsmeer, the Netherlands). Gene expressions were corrected for reference gene values (*36B4*), and expressed relative to the control group. Primers used for RT-PCR are shown in supplemental table 1.

Western blot

Protein was isolated with RIPA buffer (50 mM Tris pH 8.0, 1% nonidet P40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl) supplemented with 40 ul/ml phosphatase inhibitor cocktail 1 (Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands), 10 ul/ml protease inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN, USA) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Roche Diagnostics Corp., Indianapolis, IN, USA). Protein concentrations were determined with a DC protein assay kit (Bio-Rad, Veenendaal, the Netherlands). Equal amounts of proteins were separated by SDS-PAGE and proteins were transferred onto PVDF membranes. The following antibodies were used: ANP (ab91250); Gal-3 (MA1-940); glyceraldehyde-3-phosphate dehydrogenase (10R-G109A, Fitzgerald, USA). Signals were visualized with ECL and analyzed with densitometry (ImageQuant LAS4000, GE Healthcare Europe, Diegem, Belgium).

Statistical analysis

All values are presented as means ± standard errors of the mean (SEM). Student's paired two-tailed *t*-test was used for two-group comparisons. For non-normally distributed data or data without homogeneity of variance non-parametric tests were performed. In this case Mann-Whitney tests were used for two group comparisons. $P < 0.05$ was considered to be significant. SPSS software (PASW Statistics 22) was used for statistical analyses.

Table 1. Hemodynamic parameters

Hemodynamics	N=8	N=13
HR, bpm	308,3 ± 12,9	340,3 ± 4,5*
SBP, mmHg	107,3 ± 2,3	146,7 ± 5,6*
DBP, mmHg	76,00 ± 2,31	93,77 ± 4,29*
LVP_{max} , mmHg	109,6 ± 2,2	148,2 ± 5,8*
LVESP, mmHg	106,6 ± 2,8	147,9 ± 5,8*
LVEDP, mmHg	9,93 ± 1,22	17,12 ± 2,02*
$dP/dT_{max}/P_{max}$, 1/s	61,33 ± 1,42	50,34 ± 1,21*
$dP/dT_{min}/P_{min}$, -1/s	66,77 ± 2,84	54,25 ± 2,79*

Data are presented as means ± standard error of the mean. HR=Heart rate, SBP=Systolic blood pressure, DBP=Diastolic blood pressure, LVP_{max}=Maximal left ventricular pressure, LVESP=Left ventricular end systolic pressure, LVEDP=Left ventricular end diastolic pressure, dP/dT_{max} =Measure for left ventricular (LV) contraction capacity, here corrected for LV maximal pressure. dP/dt_{min}=Measure for LV relaxation capacity, here corrected for LV maximal pressure. Tau=Measure for LV relaxation capacity. n= 8-13, * $P<0.05$ as compared to SD group.

Results

Echocardiographic measurements reveal structural and functional alterations

Echocardiographic measurements were performed in Ren2 rats and control SD rats to explore cardiac dimension and function at 10 and 14-16 weeks of age. As depicted in figure 1, cardiac function was still conserved in Ren2 rats at 10 weeks. However, Interventricular septal wall thickness at end-diastole (IVSd) was already significantly increased in Ren2 rats and a similar trend was observed for the left ventricular posterior wall (LVPWd) (Figure 1A-B). At an age of 14-16 weeks signs of heart failure development (fatigue, shortness of breath) became evident in Ren2 rats in agreement with previous reports [26, 29, 30]. At this stage echocardiographic measurements were performed again. This revealed remarkable changes in cardiac function and structure in the Ren2 rats. Cardiac dilatation had occurred as shown by the strong increase in left ventricular diameter at end-diastole (LVIDd) and end-systole (LVIDs) (Figure 1C-D). This was accompanied by a strong reduction in percent ejection fraction (EF) and fractional shortening (FS) (Figure 1E-F). Both IVSd and LVPWd were significantly higher in the Ren2 group at this stage (Figure 1A-B). Cardiac hypertrophy was also confirmed by a significantly larger heart weights (LV and atria corrected by tibia length) in the Ren2 group (Figure 2A-C). Importantly, we also observed significantly increased lung, kidney and liver organ weights in the Ren2 groups, indicating that other organs were also affected in this animal model (Figure 2D-F).

Pressure catheter measurements reveal systolic and diastolic dysfunction.

Before sacrifice pressure catheter measurements were performed. The arterial and LV systolic and diastolic blood pressure were significantly higher in Ren2 rats as compared to SD rats (Table 1). Corrected dP/dT_{max} and dP/dT_{min} values were significantly lower in Ren2 rats, indicating that both cardiac contraction and relaxation were impaired (Table 1).

Tissue gene expression

Gene expression analysis of NPPA, LGALS3, GDF-15 and TIMP1 revealed significant higher expression levels in LV tissue of the Ren2 group (Figure 3). NPPA even increased 66 fold in the LV of the Ren2 group, whereas only a 4.6 fold increase was observed in the RV of the Ren 2 group (Figure 3A). In other tissues NPPA expression was below detection level. The other genes showed much smaller changes in LV expression levels between the SD and Ren2 group, with the strongest change shown for GDF-15 (almost 4 fold elevation in Ren2) (Figure 3B-D). Whereas NPPA expression was confined to the heart (LV and RV shown), the other genes were also expressed in other tissues tested, including lung, kidney and liver. GDF-15 expression was even ~50 fold higher in kidney as compared to heart and was elevated to ~100 fold higher

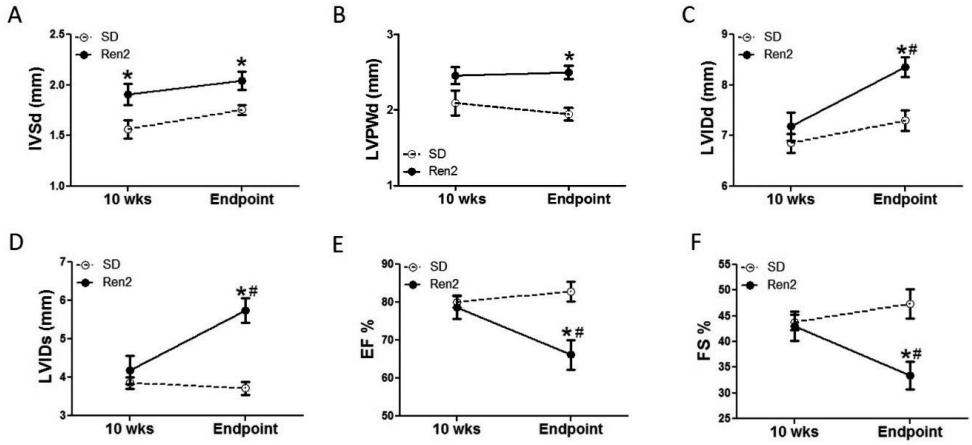

Figure 1. Measurement of cardiac function by echocardiography in Ren2 rats. (A) Interventricular septum thickness in diastole. **(B)** Left ventricular posterior wall thickness in diastole. **(C)** Left ventricular internal diameter in diastole. **(D)** Left ventricular internal diameter in systole. **(E)** Assessment of percent ejection fraction. **(F)** Assessment of percent fractional shortening. n=8-13, * P<0.05 as compared to SD group; # P<0.05 as compared to 10 wks.

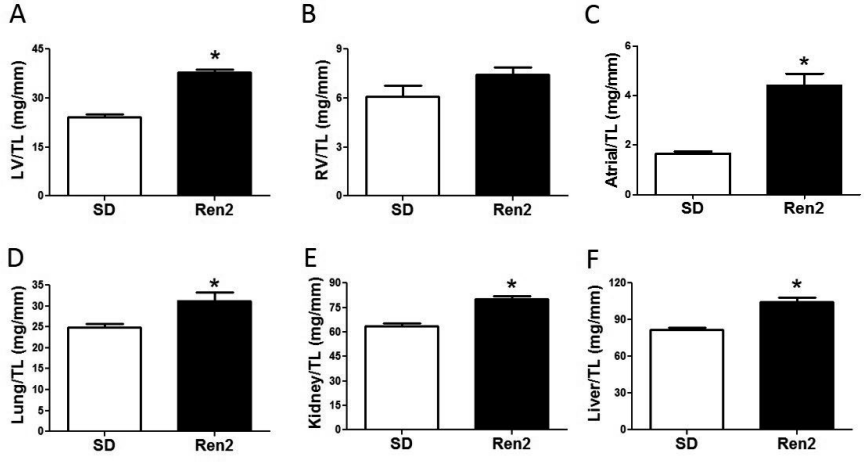

Figure 2. Organs weight in SD and Ren2 rats at sacrifice. (A) Left ventricle; **(B)** Right ventricle; **(C)** Atrial; **(D)** Lung; **(E)** kidney; **(F)** Liver. Weights were corrected for tibia length. n=8-13, * P<0.05 as compared to SD group.

levels in Ren-2 kidneys as compared to SD hearts. All genes showed increased expression in kidney tissue of the Ren-2 group and Gal-3 was also elevated in liver tissue of the Ren-2 group.

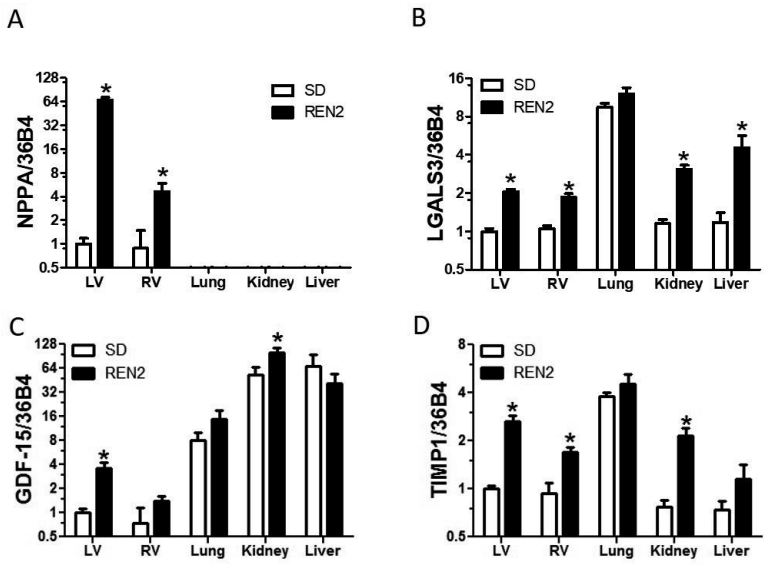

Figure 3. Biomarker gene expression in multiple organs in SD and Ren2 rats. (A-D) Relative mRNA expression of biomarkers in each organ at sacrifice. SD, open bars, Ren2, black bars. All expression was normalized to 36B4 and expressed as fold change. n=8-13, * P<0.05 as compared to SD group.

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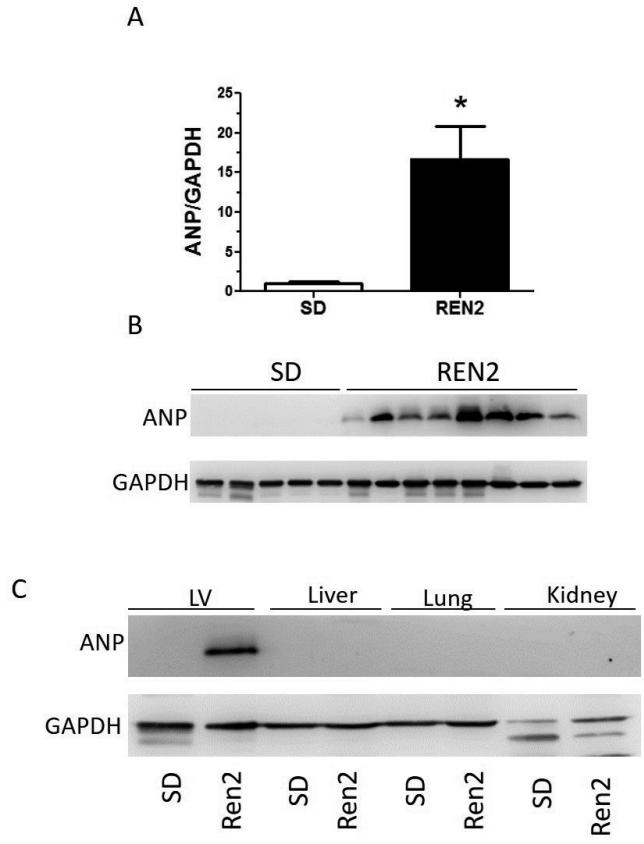

Figure 4. ANP protein levels in SD and Ren2 rats. (**A**) Quantification of ANP levels in Western blots (corrected for glyceraldehyde phosphate dehydrogenase GAPDH levels). (**B**) Representative Western blot showing ANP and GAPDH protein levels in left ventricle in SD and Ren2 rats. (**C**) Representative Western blots for ANP in the indicated organs of SD and Ren2 rats. n=8-13, * P<0.05 as compared to SD group.

Investigation of protein levels

Using specific antibodies against ANP and Gal-3 we could determine their protein levels also in rat tissues. ANP protein levels were low in LV tissue of SD rats, but were strongly elevated in LV tissue samples of the Ren2 group (Figure 4A-B). No ANP could be detected in other tissues investigated of either SD or Ren2 group (Figure 4C). In contrast to ANP, Gal-3 protein could also be detected in liver and lung tissue, but in kidney Gal-3 was barely visible, despite similar gene expression levels between LV, kidney and liver (Figure 5A and Figure 3B). Importantly, lung protein levels were much higher as compared to LV protein levels and this reflects the gene expression data. Gal-3 protein levels were significantly elevated in LV tissues of the Ren-2 group, albeit not as dramatic as for ANP (Figure 5B). Although we could not

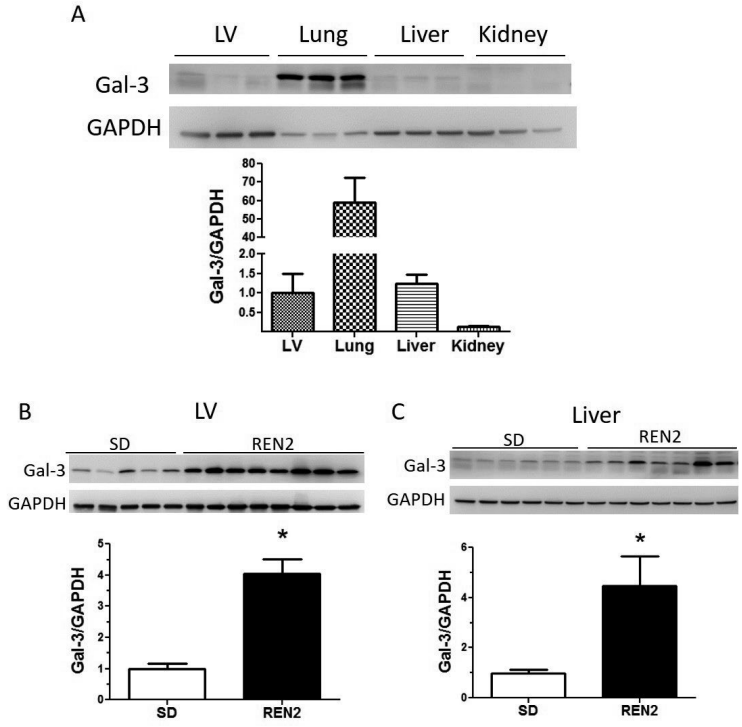

Figure 5. Gal-3 protein levels in SD and Ren2 rats. (**A**) Western blot analysis of Gal-3 and GAPDH protein levels in the indicated organs. Bar diagram shows quantification of ANP levels corrected for GAPDH. (**B**) Western blot analysis of Gal-3 and GAPDH protein levels in LV in SD and Ren2 rats. Quantification shown in lower panel. (**C**) Western blot analysis of Gal-3 and GAPDH protein levels in liver SD and Ren2 Rats. Quantification shown in lower panel. n=8-13, * P<0.05 as compared to SD group.

detect increased kidney Gal-3 levels in the Ren-2 group, which could be a detection limit issue, in liver Gal-3 was significantly elevated in the Ren-2 group in accordance with the gene expression data (Figure 5C).

Plasma levels

ANP, GDF-15 and TIMP-1 plasma levels were all significantly elevated in the Ren-2 group (Figure 6). Surprisingly, NT-proANP only showed a limited increase of 1.6 fold, which is much lower as compared to the increased cardiac gene and protein expression levels (Figure 6). Unfortunately, we were not able to detect rat Gal-3 protein levels in plasma with the currently available ELISA detection kits.

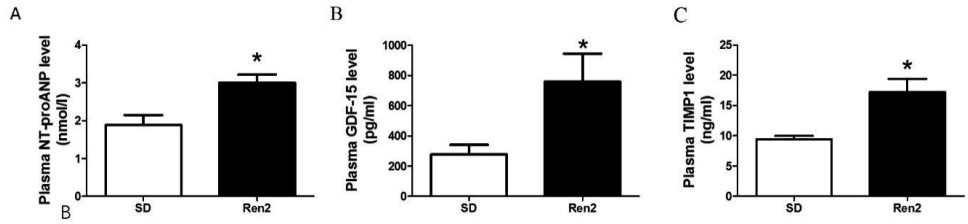

Figure 6. Plasma levels of biomarkers in SD and Ren2 Rats. (A) NT-proANP, **(B)** GDF-15 and **(C)** TIMP1. n= 8-12, * P<0.05 as compared to SD group.

Discussion

Our investigations show that, except for ANP, other emerging HF biomarkers are also expressed in other tissues. Moreover, the expression in other tissues is often much higher as compared to cardiac expression and is also dynamically controlled. It will therefore be difficult to directly link plasma levels of these biomarkers to cardiac pathology. Their elevated plasma levels also reflect pathological stress or injury in other tissues and elevated levels most likely indicate multiple organ involvement. This explains why these biomarkers have strong predictive value for hospitalization and mortality in general.

Novel HF biomarkers could be useful in providing incremental information to current clinical risk factors and natriuretic peptides for prognosis and risk stratification of HF patients [31, 32]. However, except for natriuretic peptides and troponins, all the emerging novel HF biomarkers appear to be non-cardiac specific. We recently provided evidence, using mouse studies, that plasma levels of Gal-3, GDF-15 and TIMP-1 did not, or only minimally, alter upon myocardial injury and/or stress, despite elevated cardiac expression [25]. This indicates that cardiac tissue minimally contributes to the plasma levels of these biomarkers. The advantage of this mouse model was the absence of stress and changed expressions in other organs and hence provided the ability to investigate solely the cardiac contribution. Although on one hand this is good, one could also argue that this does not fully recapitulate the human situation. Herein, we used a rat model of hypertension that develops overt heart failure with clinical like symptoms. In this model, cardiac hypertrophy and functional loss is very evident, but also other organs are affected (based on organ weight increase). Upregulation of all biomarkers occurred in the heart, but also in kidney, an organ that is often affected in heart failure and by hypertension. Elevation of these biomarkers in plasma was also observed (unfortunately we could not detect Gal-3 in rat plasma), but since elevated expression was also observed in the kidney this plasma elevation appears to reflect stress in the kidney as well. In this respect it is also important to mention that GDF-15 gene expression was ~100 fold higher in Ren2 kidney as compared to baseline (SD)

ventricular expression, indicating that the kidney may contribute much stronger to the plasma levels. Previously, we showed that plasma levels of Gal-3, TIMP-1 and GDF-15 in mice increased upon involvement of other issues, particularly lungs in a mouse TAC model and adipose tissue in a high fat mouse model. Collectively, this shows that expression of these biomarkers is responsive to stress in multiple organs and depending on etiology different organs and tissues may contribute to their plasma levels.

The current HF biomarker investigations involve predominantly clinical association studies. These studies are now shifting from single or a few biomarkers to multi-marker panels. Such panels, together with unsupervised cluster analysis may generate distinct endotype classifications that show different responses to HF therapy [33]. To take full advantage of such multi-marker screening platforms, it will in the end be pivotal to have in depth information about single organ contribution of each separate biomarker. Without this information it will be difficult, if not impossible to fully delineate the biological information from such platforms. This study is one of the first attempts to provide information from a limited number of biomarkers using animal studies. As shown here, these studies are still hampered by proper reagents, as exemplified by the absence of proper rat Gal-3 plasma assays. To further expand in this direction it will be important to invest in platforms that also allow determination of these substances in animal models.

We like to mention that gene expression may be different between species and hence changes observed in mice or rat not necessarily reflect the situation in humans. Nevertheless, GDF-15, TIMP-1 and Gal-3 plasma levels have been shown to be affected by multiple diseases affecting different organs in humans. This clearly supports our view that these biomarkers do not only reflect stress in the heart, but also in other organs. Since heart failure is a systemic disease that affects other organs and is associated with many co-morbidities it is highly likely that changed plasma levels in HF patients are a complex reflection of multi-organs involvement.

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Supplementary Table 1. Oligonucleotide pairs used for qPCR

Gene	5'-3' forward	3'-5' reverse
NPPA	ATGGGCTCCTTCTCCATCAC	TCTACCGGCATCTTCTCCTC
LGALS3	CCCGCTTCAATGAGAACAAC	ACCGCAACCTTGAAGTGGTC
GDF-15	TGACCCAGCTGTCCGGATAC	GTGCACGCGGTAGGCTTC
TIMP1	AGAGCCTCTGTGGATATGTC	CTCAGATTATGCCAGGGAAC
36B4	GTTGCCTCAGTGCCTCACTC	GCAGCCGCAAATGCAGATGG

Chapter 7

Summary, Discussion and Future perspectives

Summary, discussion and future perspectives

Due to the ageing population, the increase of lifestyle associated syndromes (e.g. diabetes and obesity) and better survival of patients with precipitating cardiovascular events, the number of heart failure (HF) patients is on the rise. The diagnostic strategies for HF are based on several approaches, including enquiry of medical history of the patient, a physical exam, and cardiac imaging. HF is, a very heterogeneous disorder that is also accompanied and/or affected by multiple co-morbidities, such as obesity, diabetics and hypertension, among others. The exact HF etiology is therefore difficult to extract from clinical evaluations only and for many decades, the diagnosis of HF was a garden variety of cardiac diseases with one common denominator, a decreased cardiac output. In the last decade, clinicians and researchers came to the realization that more precise categorization of HF patients will be essential for optimal HF patient management – in order to provide therapeutic granularity it will be imperative to improve on phenotyping. As it stands, HF is mainly divided in two categories according to values of left ventricular ejection fraction, which is either classified as HF with reduced ejection fraction or HF with preserved ejection fraction. It should be mentioned that the exact cut-off points for HFrEF and HFpEF have varied largely between studies, but EF<40% is now mostly denoted as HFrEF and EF≥50% as HFpEF [1]. These patient groups have about equal population sizes, but whereas therapeutic treatment regimens have dramatically increased outcome for HFrEF patients, so far no proven therapy is available for patients with HFpEF. More recently, a third class was introduced with mid-range ejection fraction (HFmrEF; 40%≤EF≤49%), which was also incorporated in the 2016 ESC HF guidelines [2]. There are several reasons why the distinction based on LVEF is far from ideal, e.g. the symptoms, severity of neurohormonal activation, underlying etiologies, presence of co-morbidities, and response to treatment: all these crucial denominators of HF are largely independent from LVEF.

HF plasma biomarkers, in particular the natriuretic peptides (NPs) have substantially improved diagnosis HF patients. The cardiac specific plasma NPs, have been incorporated into routine clinical practice of HF patients for their incremental values in diagnostic and prognostic purposes [1, 2]. Since the levels of these cardiac specific biomarkers can be influenced by several non-cardiac diseases like sepsis, kidney disease and obesity [3-5], these biomarkers are still not ideal. Moreover, whereas the NPs indicate pathological wall stress in the heart, other plasma biomarkers may be indicative for other pathological processes, like cardiac fibrosis and inflammation. The emerging novel HF biomarkers Galectin-3 (Gal-3), Growth Differentiation Factor-15 (GDF-15) and soluble suppression of tumorigenicity 2 (sST2), amongst others, have been extensively studied in clinical trials, because they could provide additional value for prognosis and disease management.[6] Of these, Gal-3 and sST2 have been included in the

American College of Cardiology (ACC)/AHA HF guidelines as markers of myocardial fibrosis, with a class IIb recommendation, to be considered for additional risk stratification of HF patients [2, 7]. These novel HF biomarkers have been shown to represent common pathophysiological processes not only in the heart but also in other stressed or injured organs and tissues [8]. Therefore, their plasma levels can also be affected by other diseases and potential HF co-morbidities. This may explain why these purported HF biomarkers have still not found their way into daily clinical practice, in contrast to NPs. In addition to these proteins, circulating microRNAs (miRNAs) have been reported to be associated with HF severity [9-11], but, so far, none of them have been included in AHA and/or ESC guidelines. Further studies will be required to provide insight in the usefulness of these molecules as HF biomarkers.

Besides the potential role in for prognosis and HF management, these biomarkers may also constitute interesting therapeutic targets. As an example, the positive vasodilatory and cardiac unloading effects of NPs has recently resulted in the development of molecules targeting the degradation of NPs. Sacubitril, a neprilysin inhibitor prodrug, is a component of the successful HF drug, entresto, which has shown to improve HF patient outcome by limiting degradation of NPs via inhibition of neprilysin peptidase [12]. Gal-3 is another potential drug target as genetical and pharmacological inhibition of Gal-3 exhibited protective effects by reducing cardiac fibrosis and subsequent cardiac remodeling in murine HF models [13, 14]. Myeloperoxidase (MPO), a specific marker of neutrophil activation, has been shown to be elevated in blood plasma of chronic HF patients, and the increased levels were associated with HF severity [15, 16]. Genetic deletion of MPO in mice protected against cardiac remodeling post-MI and suppressed atrial fibrosis in AngII infused mice, suggesting that MPO could be an attractive therapeutic target for cardiac diseases [17-20]. Additionally, several miRNAs have been shown to be implicated in cardiac diseases [21]. Circulating miR-328 levels were significantly upregulated in myocardial infarction (MI) and atrial fibrillation and transgenic overexpression of miR-328 contributed to fibrosis associated adverse cardiac electrical remodeling [22-24]. Whether miR-328 is involved in the regulation of cardiac fibrotic responses upon MI remains unknown.

The aim of this thesis (**chapter 1**) was to investigate the potential of therapeutic targeting of certain HF biomarkers and to investigate the cardiac and non-cardiac contribution of HF biomarkers in cardiac disease by using mouse and rat HF models.

In **chapter 2**, we review current clinical and experimental studies regarding the diagnostic and prognostic role of the most relevant and potential new HF biomarkers. We address deficiencies in current biomarker studies and discuss the non-cardiac specific nature of most of these novel

HF biomarkers. These novel HF biomarkers are associated with specific processes, like fibrosis and inflammation, and hence could provide us with information on the fibrotic or inflammatory state of the heart. These are, however, generic processes which can also be activated in other organs and tissues upon stress or injury, and hence plasma levels do not necessarily reflect the cardiac status. Since HF is a systemic disease and is also associated with multiple co-morbidities this may hamper the interpretation of elevated plasma biomarker levels. Therefore, it is still unclear whether altered plasma biomarker levels represent solely cardiac production and can be directly associated with the degree of cardiac remodeling. Clinical association studies will not provide sufficient information to solve these issues, because cardiac samples are often not available and full body biomarker profiling will not be realistic or may be impossible. We finally address some of the common issues and propose to investigate these elusive biomarkers more comprehensively in HF animal models.

In **chapter 3**, we studied the role of miR-328 in cardiac fibrosis and the underlying mechanisms. In patients with atrial fibrillation and after myocardial infarction circulating levels of miR-328 have been shown to be strongly elevated. Here we showed that in mice miR-328 expression was strongly upregulated in the border zone of the infarcted myocardium. Expression of pro-fibrotic TGF-β1 was concomitantly, increased whereas anti-fibrotic TGF-βRIII was decreased in the infarcted myocardium. In vitro, miR-328 transfection of primary mouse cardiac fibroblasts resulted in enhanced collagen production via due to miR-328 mediated TGF-βRIII downregulation. Importantly, application of specific miR-328 antisense by an antagomir remarkable reduced collagen content and cardiac fibrosis in *in vivo* and in *vitro* studies. Thus, we identified a novel pathway in which miR-328 directly targeted and reduced TGF-βRIII levels, which in turn stimulated TGF-β1 mediated cardiac fibrosis. These effects were abrogated by specific antagomir of miR-328. MiR-328 may therefore represent a promising therapeutic target for treatment of cardiac fibrotic diseases in ischemic heart disease. Since circulating miR-328 levels are decreased in HF (in contrast to MI), the situation in HF may be more complicated and this requires further investigation.

In **chapter 4**, we conduct a pharmacological study to investigate whether a novel myeloperoxidase (MPO) inhibitor, AZM198, could prevent cardiac adverse remodeling in an in vivo mouse model of pressure overload (TAC) after 4 and 8 weeks. We found that treatment with this MPO inhibitor temporally delayed cardiac hypertrophy and preserved cardiac function at 4 weeks. This inhibitor, however, failed to halt deterioration of cardiac remodeling and no differences could be observed anymore at 8 weeks post-TAC. Although previous studies showed that genetic inhibition of MPO could suppress fibrotic responses in MI [19] and AngII treated mice [20]. We did not observe anti-fibrotic effects with this inhibitor in our

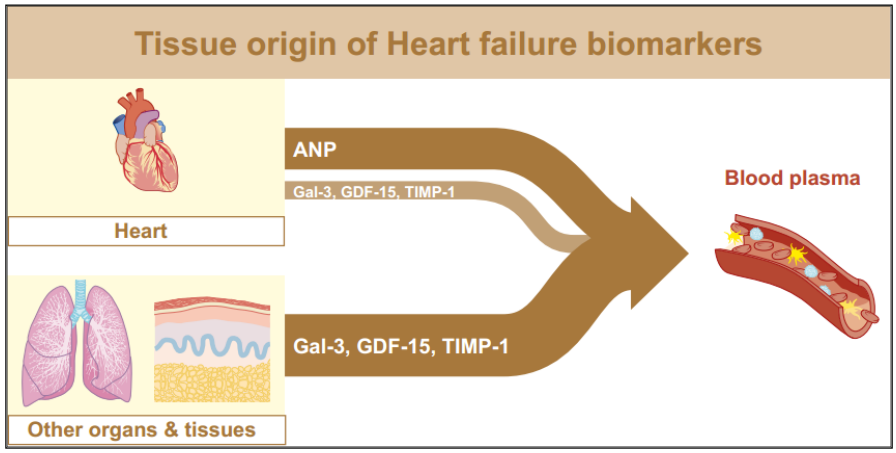

Figure 1. Tissue origin of heart failure biomarkers. Schematic depiction of organ/tissue to blood plasma biomarkers levels. Included organs/tissues: heart, lungs, kidney, livers, and visceral adipose tissue (VAT).

mouse model. Importantly, MPO plasma levels were not increased in these mice, despite strongly reduced cardiac function, challenging its potential function as a cardiac biomarker and target.

In **chapter 5**, we describe an elaborate mouse plasma biomarker study involving three different mouse HF models. In particular, we included two models of HFrEF, namely a transverse aortic constriction (TAC) and a myocardial infarction (MI) model and one model with HFpEF characteristics generated by a high fat diet (HFD) and angiotensin II (AngII) infusion (obesity/hypertension). We subsequently investigated HF biomarkers Atrial natriuretic peptide (ANP), Gal-3, GDF-15 and TIMP-1 at three different levels: i) organ gene expression, ii) organ protein quantities and iii) plasma protein levels, all in relation to cardiac function and structure. Surprisingly, in contrast to the established HF biomarker ANP, plasma levels of the purported HF biomarkers (Gal-3, GDF-15, TIMP-1) did not show a direct association with cardiac function. All biomarkers were elevated in cardiac tissue in these diseased hearts and this paralleled in most cases cardiac protein levels, but this did not affect plasma pools. The increased circulating levels of GDF-15 and TIMP1 correlated with lung weight after TAC, suggesting that TAC mediated congestion may be responsible for their increased plasma levels. In contrast, high fat diet strongly elevated plasma levels of these biomarkers, most likely as a result of elevated production in adipose tissue. Our study suggested that rather than being specific for indices of cardiac remodeling, these biomarkers reflect health status beyond cardiac function. They also reflect stress in other organs, either as a consequence of the failing heart and/or as a consequence of other underlying comorbidities, like the metabolic syndrome (Figure 1).

In **chapter 6**, we extend these observations by investigating cardiac expression and plasma levels of these biomarkers in a transgenic rat model with hypertension (Ren2). Ren2 rats showed reduced ejection fraction and impaired hemodynamic parameters. Cardiac hypertrophy and dilatation were observed in these rats, but also other organs showed an increased weight in these systemic hypertensive rats. Expression of all investigated biomarkers was elevated in the heart, but importantly also in the kidney. ANP, GDF-15 and TIMP1 plasma levels were all elevated in Ren2 rats, and in the case of ANP this could only be cardiac derived. However, the elevated plasma levels of the other biomarkers could not be solely linked to the heart and elevated kidney expression could also have a significant contribution. This clearly displays, how difficult it will be to link the plasma levels to specific indices of cardiac function or remodeling, as other tissues will obscure plasma levels in this complex syndrome. Unfortunately, we were not able to determine rat Gal-3 plasma levels, as a result of inappropriate reagents, an issue which was also discussed in Chapter 2 and which hampers biomarker research in animal models.

In summary, we provided mechanistic insights of miR-328 in promoting cardiac fibrosis and the potentials as a therapeutic target for treatment of cardiac fibrotic disease by a specific antagomir. MPO inhibition, only temporally delayed but did not inhibit cardiac remodeling upon pressure overload indicating that under this condition it does not play a substantial role in cardiac remodeling. This is in agreement with a lack of elevation of MPO levels post-TAC, which is opposite to what has been found in post-MI mouse studies. Thus, the role of MPO in cardiac remodeling may be dependent on the etiology and this requires further investigation. Our comprehensive HF animal models in mouse and in rat revealed that only ANP plasma levels can indicated specific indices for cardiac remodeling, but other alleged novel biomarkers may also reflect stress in other organs, either as a consequence of the failing heart and/or as a consequence of other underlying comorbidities.

Future perspectives

Heart failure is a heterogeneous complex syndrome and further patient stratification allowing patient tailored therapy is an important goal. We are at the brink of personalized medicine, but at current, we lack tools for proper pathophysiological stratification and to provide patients with personalized treatment. Although sub-division based on LVEF has some merits, it will on its own not be sufficient to adequately categorize the heterogeneous HF population. Moreover, we also need to understand the underlying biology, in order to generate “personalized” therapy for HF patients and in particular to identify treatment opportunities for HFpEF patients. Plasma biomarkers may have the potential to allow further stratification and may themselves be potential targets for tailored therapy.

Our animal studies do, however, show that this will require further exploration. Specifically, the multi-organ expression of these biomarkers prevents to directly link plasma levels to one organ or tissue, and the heart in particular. Nevertheless, our studies also showed that not all biomarkers are equally expressed in all organs and are also not equally affected by stress in different organs. Therefore, we would need a topological and spatiotemporal human organ/tissue map with expression (and specific plasma contributions derived from these local productions) of a large number of plasma biomarkers in relation to specific stress conditions. With that in hand we could generate a biomarker profile that allows us to extract more specific information about pathological processes in specific organs. A recent study has demonstrated that a multi-biomarker panel together with unsupervised cluster analysis may identify distinct endotypes with apparent different outcomes and response to therapy [25]. A better understanding of organ plasma biomarker contribution under different disease states will be extremely helpful in the interpretation of such complex multi-biomarker panels and may also provide a more rational analysis and explanation regarding the underlying biology. Although this will be a daunting task, it is most likely the only way forward in biomarker research and without doubt such a map will be very valuable and not only for cardiac disease.

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Samenvatting, discussie en toekomstperspectieven

Door de verouderende populatie, de toename van de prevalentie van met levensstijl geassocieerde aandoeningen (diabetes en obesitas) en steeds betere overleving van patiënten met een cardiovasculaire voorgeschiedenis neemt het aantal patiënten met hartfalen toe. Het diagnosticeren van hartfalen is voornamelijk gebaseerd op de medische voorgeschiedenis van de patiënt, het lichamelijk onderzoek, en cardiale beeldvormingstechnieken. Hartfalen is een zeer heterogene aandoening die vaak samengaat met en/of beïnvloed wordt door co-morbiditeiten zoals obesitas, diabetes en hypertensie. Het is moeilijk om alleen op basis van een klinische evaluatie van een patiënt met hartfalen de exacte etiologie en pathologie van het ziektebeeld vast te stellen. Decennia lang bestond de diagnose van hartfalen daarom uit een variëteit van cardiovasculaire aandoeningen met één gemeenschappelijke factor: een verlaagde cardiale output. In de afgelopen jaren zijn clinici en onderzoekers zich gaan realiseren dat een meer specifieke indeling van patiënten met hartfalen noodzakelijk is voor optimale behandeling en om patiëntspecifieke therapie mogelijk te maken. Om dit doel te bereiken zal het noodzakelijk zijn de fenotypering van de patiënt te verbeteren. Op dit moment wordt hartfalen voornamelijk ingedeeld in twee categorieën. Aan de hand van de ejectiefractie (EF) van de linker kamer wordt een onderscheid gemaakt tussen hartfalen met verlaagde (reduced) ejectiefractie (HFrEF) en behouden (preserved) ejectiefractie (HFpEF). Het is belangrijk hierbij te noemen dat er aanzienlijke variatie is geweest in de grenswaarde voor HFrEF en HFpEF in verschillende klinische studies, maar momenteel wordt een EF <40% meestal geclassificeerd als HFrEF en een EF >50% als HFpEF [1]. Deze twee patiëntengroepen hebben ongeveer eenzelfde groepsgrootte, maar waar met behulp van behandelingen de situatie van HFrEF-patiënten aanzienlijk hebben verbeterd is er tot nu toe geen bewezen behandelingsmethode voor patiënten met HFpEF. Recentelijk is er een derde klasse toegevoegd gekenmerkt door een gemiddelde (mid-range) ejectiefractie (HFmrEF; 40%<EF<50%) en deze klasse is, net zoals HFrEF en HFpEF, opgenomen in de hartfalenrichtlijn van de Europese Society of Cardiology (ESC) van 2016 [2]. Veel cruciale bepalende factoren van hartfalen, zoals de symptomen, mate van neurohormonale activatie, onderliggende etiologie, aanwezigheid van co-morbiditeiten, en behandelingseffecten, zijn voor een groot deel onafhankelijk van de EF van de linker kamer. De indeling op alleen EF is dan ook verre van ideaal en betere patiëntstratificatie is daarom zeer gewenst.

Biomarkers van hartfalen aanwezig in bloedplasma, met name de natriuretische peptiden (NPs), hebben substantieel bijgedragen aan de verbetering van het stellen van de diagnose hartfalen. Aangezien deze hartspecifieke plasma NPs de diagnostische en prognostische voorspellingen ten aanzien van hartfalen sterk hebben verbeterd, is het gebruik van deze markers nu standaard

in de diagnostiek en behandeling van hartfalenpatiënten [1, 2]. Omdat de niveaus van deze biomarkers in het bloed worden beïnvloed door meerdere niet-cardiale aandoeningen, zoals sepsis, nierziekte en obesitas, zijn deze biomarkers nog steeds niet ideaal [3-5]. Daarnaast geven deze biomarkers slechts een indicatie van pathologische wandstress in het hart, maar worden andere pathologische processen verantwoordelijk voor het ontstaan van hartfalen niet weerspiegeld in de niveaus van deze markers. Biomarkers die pathologische processen zoals fibrose (verbindweefseling) en ontsteking in het hart kunnen representeren, zouden extra inzicht kunnen verschaffen over het zieke hart. Verschillende nieuwe biomarkers van hartfalen, waaronder Galectin-3 (Gal-3), Growth Differentiation Factor-15 (GDF-15) en soluble Suppression of Tumorigenicity 2 (sST2), zijn uitgebreid onderzocht in klinische studies omdat ze toegevoegde prognostische waarde zouden kunnen hebben en kunnen bijdragen aan de behandeling van hartfalen [6]. Van deze drie zijn Gal-3 en sST2 ook genoemd in de HF-richtlijnen van de American College of Cardiology / American Heart Association (ACC/AHA) als mogelijke markers van myocardiale fibrose, met een klasse IIb aanbeveling, en kunnen overwogen worden voor extra risicostratificatie van patiënten met hartfalen [2, 7]. Deze nieuwe biomarkers van hartfalen geven algemene pathologische processen weer (fibrose) die niet alleen in het hart voorkomen, maar ook in andere beschadigde organen of weefsels die stress ondervinden [8]. De plasmaniveaus van deze biomarkers kunnen daardoor ook door andere ziektes en aandoeningen, en dus ook door co-morbiditeiten van hartfalen worden beïnvloed. Dit kan verklaren waarom deze veronderstelde biomarkers van hartfalen nog steeds geen plaats hebben gekregen in de dagelijkse klinische praktijk, in tegenstelling tot de NPs. Naast eiwitten zijn er ook circulerende microRNAs (miRNAs) gevonden die geassocieerd zijn met de ernst van hartfalen [9-11], maar tot nu toe zijn geen van deze miRNAs toegevoegd aan de richtlijnen van de ACC/AHA en/of de ESC. Verder onderzoek zal noodzakelijk zijn om inzicht te geven in de bruikbaarheid van deze moleculen als biomarkers van hartfalen.

Naast de mogelijke rol voor de prognose en behandelingsmogelijkheden, kunnen biomarkers ook interessante therapeutische targets zijn. De positieve vasodilatatie-effecten van NPs hebben bijvoorbeeld geleid tot de ontwikkeling van medicijnen die de afbraak van NPs tegen gaan. Sacubitril, een neprilysin-inhibitor pro-drug, is een onderdeel van het succesvolle hartfalenmedicijn Entresto, die de uitkomst van patiënten met hartfalen verbetert door de afbraak van NPs tegen te gaan [12]. Genetische en farmacologische inhibitie van Gal-3 resulteert in verminderde fibrose en zogenaamde remodeling van het hart in muismodellen van hartfalen en is daarom een ander voorbeeld van een mogelijk aangrijpingspunt voor nieuwe medicamenten [13, 14]. Myeloperoxidase (MPO), een specifieke marker van activatie van neutrofielen, is verhoogd in bloedplasma van patiënten met chronisch hartfalen en de

verhoogde niveaus zijn gecorreleerd aan de ernst van hartfalen [15, 16]. Genetische deletie van MPO in muizen is beschermend tegen cardiale remodeling na een myocardinfarct en verminderde tevens fibrose in de hartboezems in muizen die angiotensine-II kregen toegediend. Dit suggereert dat MPO-inhibitie mogelijk interessante mogelijkheden biedt als nieuwe therapeutische interventie van hartspierziektes [17-20]. Daarnaast zouden miRNAs die geassocieerd zijn met hartspierziektes als mogelijke targets kunnen worden gezien [21]. De niveaus van circulerend miR-328 zijn significant verhoogd na een myocardinfarct en bij boezemfibrilleren en transgene overexpressie van miR-328 in muizen veroorzaakt fibrose-geassocieerde geleidingsstoornissen in het hart [22-24]. Of miR-328 direct betrokken is bij de regulatie van cardiale fibrose na een myocardinfarct is tot nu toe onbekend.

Het doel van dit proefschrift (**hoofdstuk 1**) was tweeledig, namelijk onderzoeken of bepaalde biomarkers van hartfalen als mogelijke therapeutische targets konden dienen en om de cardiale en niet-cardiale contributie van biomarkers van hartfalen te onderzoeken in muis- en ratmodellen van hartfalen.

In **hoofdstuk 2**, bespreken we de huidige klinische en experimentele studies die betrekking hebben op de diagnostische en prognostische rol van de potentieel meest relevante biomarkers van hartfalen. We adresseren deficiënties in huidige biomarkerstudies en bediscussiëren de niet-cardiale origine van de meeste nieuwe biomarkers van hartfalen. Deze nieuwe biomarkers zijn geassocieerd met specifieke processen, zoals fibrose en ontsteking, en kunnen daarom, in theorie, informatie geven over de mate van fibrose en ontsteking in het hart. Dit zijn echter algemene processen die ook geactiveerd kunnen worden in andere beschadigde organen of weefsels die stress ondervinden en de niveaus in het plasma zijn daarom niet noodzakelijkerwijs een directe reflectie van de status van deze processen in het hart. Omdat hartfalen een systemische ziekte is en ook geassocieerd is met vele co-morbiditeiten kan dit de interpretatie van verhoogde niveaus van biomarkers in het plasma verder bemoeilijken. Het is daarom nog steeds onduidelijk of veranderingen in niveaus van biomarkers in het plasma de cardiale productie correct weergeven en geassocieerd zijn met de ernst van hartfalen. Klinische associatiestudies zullen deze vraagstukken niet gaan beantwoorden, omdat hartbiopten meestal niet beschikbaar zijn en het genereren van volledige biomarkerprofielen in weefsels van de mens waarschijnlijk niet realistisch is. We stellen in dit overzichtsartikel daarom voor om deze leemtes in onze kennis aan te vullen door middel van uitgebreide weefselanalyse van biomarkers in diermodellen van hartfalen

In **hoofdstuk 3**, hebben we de rol van miR-328 in cardiale fibrose bestudeerd en de onderliggende processen blootgelegd. In patiënten met boezemfibrilleren of met een

myocardinfarct zijn de circulerende miR-328 niveaus sterk verhoogd. We laten hier zien dat in muizen miR-328 expressie sterk verhoogd is in de borderzone van het myocardinfarct. Dit ging gepaard met de expressie van het pro-fibrotisch eiwit TGF-β1, terwijl de anti-fibrotische TGF-βRIII receptorexpressie verlaagd was. Transfectie van primaire cardiale muisfibroblasten *in vitro* resulteerde in verhoogde collageenproductie als gevolg van miR-328-gemedieerde TGF-βRIII suppressie. Toedienen van specifiek miR-328 antisense in de vorm van een antagomir resulteerde in een opzienbarende verlaging van de hoeveelheid collageen en cardiale fibrose in *in vivo* en *in vitro* muisstudies. We hebben daarom een nieuwe signaaltransductieroute geïdentificeerd, waarin miR-328 op een direct wijze TGF-βRIII-niveaus reduceert en daardoor TGF-β1-gemedieerde cardiale fibrose stimuleert. Deze effecten werden voorkomen door specifieke antagomirs tegen miR-328. MiR-328 kan daarom een interessant target zijn voor de behandeling van fibrotische cardiale aandoeningen in ischemisch hartziektes. Omdat circulerende miR-328-niveaus verlaagd zijn bij hartfalen (in tegenstelling tot myocardinfarct), zal de situatie in hartfalen gecompliceerder zijn en dit vereist verder onderzoek.

In **hoofdstuk 4**, hebben we een farmacologische studie beschreven waarin we onderzocht hebben of een nieuwe inhibitor van myeloperoxidase (MPO), AZM198, pathologische cardiale remodeling kon voorkomen in een *in vivo* muis model gecreëerd door 4 en 8 weken cardiale drukoverbelasting (zogenaamd TAC model). In dit model bleek dat behandeling met deze MPO-inhibitor een vertraging gaf in het ontwikkelen van cardiale hypertrofie met tijdelijk behoud van hartfunctie. Deze inhibitor was echter niet in staat om cardiale remodeling te stoppen en na 8 weken konden er geen verschillen meer worden gezien in cardiale remodeling en hartfunctie tussen de behandelde en onbehandelde groepen. Eerdere studies hebben laten zien dat genetische deletie van MPO in muizen cardiale fibrose na een myocardinfarct [19] en na infusie van angiotensine-II [20] kon verminderen in deze muizen. We hebben geen anti-fibrotische effecten waargenomen met deze inhibitor in ons muismodel. Het is van belang om op te merken dat plasmaniveaus van MPO ook niet waren verhoogd in muizen met cardiale drukbelasting, ondanks sterk verminderde hartfunctie. De bruikbaarheid van MPO als biomarker van hartfalen en als target voor medicamenten moet daarom eerst nog verder worden vastgesteld en zou afhankelijk kunnen zijn van de etiologie van hartfalen.

In **hoofdstuk 5,** beschrijven we een uitgebreide studie van biomarkers in het plasma van muizen uit drie verschillende modellen van hartfalen. Dit betreft twee HFrEF-modellen, namelijk een transverse aorta constrictie (TAC) en een myocardinfarct (MI) model en één model met karakteristieken van HFpEF, geïnduceerd door een hoog vet dieet (HFD) en Angiotensine-II (AngII) infusie (obesitas/hypertensie). We hebben vervolgens de biomarkers van hartfalen Atrial natriuretic peptide (ANP), Gal-3, GDF-15 and TIMP-1 op drie

verschillende niveaus onderzocht, te weten: i) genexpressie in organen, ii) eiwitgehaltes in organen en iii) eiwitniveaus in het plasma. Dit is tevens onderzocht in relatie met hartfunctie en mate van remodeling in het hart. In tegenstelling tot de bewezen biomarker van hartfalen ANP waren de plasmalevels van Gal-3, GDF-15, TIMP-1 verrassenderwijs niet geassocieerd met hartfunctie. Genexpressie van alle biomarkers was verhoogd in de aangedane harten en dit resulteerde in de meeste gevallen in verhoogde eiwitniveaus, maar dit had geen effect op de plasmalevels. De verhoogde plasmalevels van GDF15 en TIMP1 correleerden met longgewicht na TAC en dit suggereert dat TAC-geïnduceerde longcongestie mede verantwoordelijk was voor deze verhoogde plasmaniveaus. Hoog vet dieet gaf daarentegen een sterke verhoging in plasmaniveaus van deze biomarkers (Gal-3, GDF-15 en TIMP-1), wat hoogstwaarschijnlijk een gevolg is van de sterk verhoogde productie in vetweefsel. Deze studie impliceert dat deze biomarkers niet specifiek zijn voor indices van remodeling van het hart, maar een meer algemeen inzicht geven in de gezondheid van de patiënt. Deze markers zijn ook een maat voor stress in andere organen en dit kan zowel een consequentie zijn van hartfalen, maar ook een indirect gevolg zijn van andere co-morbiditeiten, zoals het metabool syndroom (Figuur 1).

In **hoofdstuk 6** hebben we onze observaties verder uitgebreid door cardiale expressie en plasmaniveaus van bovenstaande biomarkers in een transgeen ratmodel (Ren2) met hypertensie te onderzoeken. Ren2 ratten ontwikkelden hartfalen met een verlaagde EF en een sterk verslechterde hemodynamiek. Cardiale hypertrofie en dilatatie waren zichtbaar in deze ratten, maar ook andere organen waren aangedaan, zoals bleek door verhoogde orgaangewichten in deze hypertensieve ratten. Expressie van alle onderzochte biomarkers was verhoogd in de harten, maar niet minder belangrijk ook in de nieren. ANP, GDF-15 and TIMP1 plasmaniveaus waren allen verhoogd in Ren2 ratten en in het geval van ANP kon dit alleen aan de productie in het hart worden toegeschreven. De plasmaniveaus van de andere biomarkers konden echter niet specifiek aan het hart worden toegeschreven en verhoogde nierexpressie zou ook een significante bijdrage aan de verhoogde plasmaniveaus kunnen geven. Dit laat duidelijk zien hoe moeilijk het is om niveaus van biomarkers in het plasma specifiek toe te dichten aan indices van hartfunctie of remodeling, omdat andere weefsels de plasmaniveaus dynamisch kunnen “vervuilen” in dit complexe syndroom. Helaas, waren we niet in staat Gal-3 plasmaniveaus in ratten te bepalen door een gebrek aan geverifieerde reagentia, een probleem dat ook al in hoofdstuk 2 ter sprake kwam en het onderzoeken van biomarkers in dierstudies bemoeilijkt.

Samenvattend hebben we inzicht gegeven in de mechanismen van miR-328 gemedieerde hartfibrose en de mogelijkheid om antagomirs te gebruiken als mogelijk middel tegen cardiale fibrose. MPO-inhibitie liet slechts tijdelijke vertraging zien in het ontstaan van cardiale remodeling na drukbelasting van het hart, wat aantoont dat MPO geen essentiële factor is in

remodeling van het hart onder deze condities. Dit is in overeenstemming met de afwezigheid van verhoogde MPO-niveaus na TAC-inductie, wat daarom afwijkt in andere beschreven muismodellen van hartfalen. De rol van MPO in cardiale remodeling zou dus afhankelijk kunnen zijn van de etiologie van hartfalen en dit maakt verder onderzoek noodzakelijk. Onze uitgebreide dierstudies in muis- en ratmodellen van hartfalen lieten uiteindelijk zien dat alleen plasmaniveaus van ANP specifiek zijn voor indices van hartfunctie, en dat andere biomarkers ook stress in andere organen en weefsels weergeven.

Toekomstperspectieven.

Hartfalen is een heterogeen en complex syndroom en een verbetering van patiëntstratificatie kan wellicht helpen in patiëntspecifieke behandeling en verbeterde management van hartfalen. We zijn nu beland in een tijdperk waarbij “personalized medicine” een steeds belangrijkere rol gaat spelen, maar op dit moment missen we nog de instrumenten voor goede pathofysiologische stratificatie van de verschillende patiëntpopulaties die worden geschaard onder het ziektebeeld hartfalen. Alhoewel onderverdeling op basis van EF enige houvast geeft, is dit op zichzelf onvoldoende om tot een adequate indeling te komen van deze heterogene patiëntengroep. Bovendien moeten we ook de onderliggende biologie begrijpen om uiteindelijk patiëntspecifieke therapieën te kunnen ontwikkelen. Dit is met name belangrijk voor de patiënten met HFpEF waarvoor nog steeds geen bewezen behandelingsmethode bestaat. Biomarkers in plasma hebben de potentie om verdere stratificatie mogelijk te maken en zouden zelf ook potentiele targets kunnen zijn voor patiëntspecifieke therapie. Onze dierstudies laten echter zien dat dit gecompliceerder is dan gedacht en dat hiervoor verder onderzoek nodig is. Met name de expressie van biomarkers in meerdere organen bemoeilijkt het maken van directe associaties tussen verhoogde plasmaniveaus en schade of stress in een specifiek orgaan, zoals het hart. Desalniettemin, laat onze studie ook zien dat expressie van biomarkers in organen verschillend is en dat expressie niet gelijkelijk verandert in al deze organen onder stresscondities. Daarom zou het hebben van een topologische en tijdsafhankelijk overzicht van de humane orgaan- en weefselexpressie van deze biomarkers aanwezig in bloedplasma in relatie tot specifieke stresscondities een zeer behulpzaam instrument kunnen zijn. Met een dergelijk overzicht in de hand zouden er biomarkerprofielen kunnen worden opgesteld die ons in staat stellen om specifieke informatie over pathologische processen in specifieke organen te kunnen vaststellen. Een recente studie heeft laten zien dat een dergelijk multi-biomarkerpanel tezamen met unsupervised cluster analyse verschillende endotypes kan identificeren met verschillende uitkomst en verschillende reactie op behandeling [25]. Een beter inzicht in de bijdrage van verschillende organen aan de niveaus van biomarkers in plasma in verschillende ziektecondities zou zeer waardevol kunnen zijn in de interpretatie van zulke complexe multi-

biomarkerpanels en kan ook een rationele geven voor deze analyse en de onderliggende biologische processen duiden. Het betreft hier een omvangrijke taak, maar dit is een weg die bewandeld zal moeten worden om verdere voortgang binnen het biomarkeronderzoek te bewerkstelligen, en zonder twijfel zal een dergelijk overzicht niet alleen voor cardiologie, maar ook voor tal van andere specialismen, waardevol zijn.

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Weijie

BIOGRAPHY

Weijie Du was born in Neimongol, China. After graduating from high school in 2006, he entered the University via the University Entrance Examination. He moved from Tongliao to Harbin and studied pharmacy in Heilongjiang University of Traditional Chinese Medicine, and got a Bachelor of Science degree in 2010. In the same year he entered the graduate school at the Harbin Medical University. In the Department of Pharmacology at the Harbin Medical University, he investigated the novel players involved in cardiovascular diseases and the underlying mechanisms for his Master of Science degree under supervision by Prof. Hongli Shan and Prof. Yanjie Lu. In 2013 under the corporation between Harbin Medical University in China and Groningen University in the Netherlands he was selected as a sandwich PhD candidate. In October, 2013 he moved to Groningen, The Netherlands, to pursue a doctoral degree at the Department of Experimental Cardiology, University Medical Center Groningen. The outcome of his research is presented in this thesis.