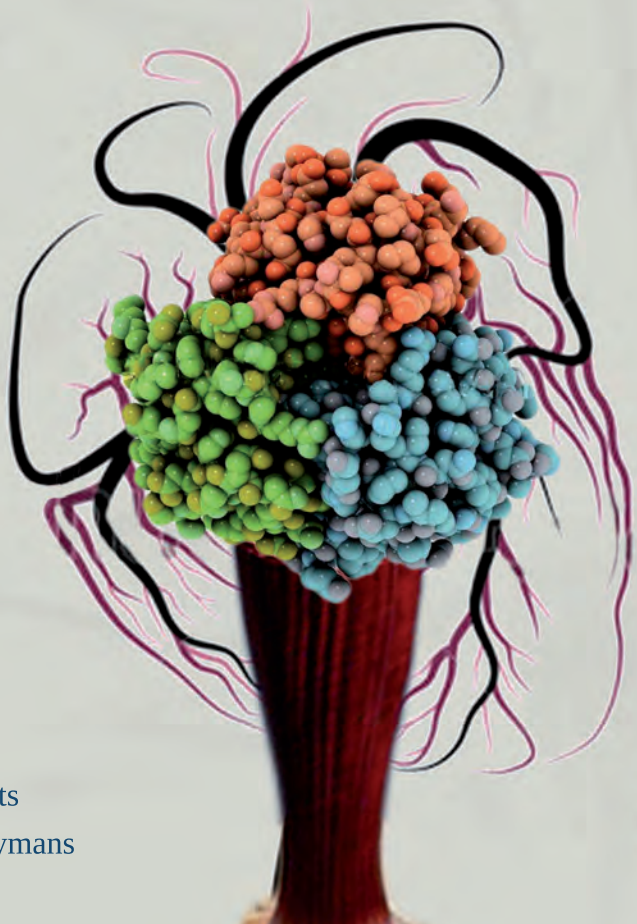
ADIPONECTIN: POTENTIAL THERAPEUTIC TARGET FOR THE TREATMENT OF MUSCLE WASTING IN CHRONIC HEART FAILURE

IN VITRO EXPLORATION

Proefschrift voorgelegd tot het behalen van de graad van doctor in de Medische Wetenschappen aan de Universiteit Antwerpen te verdedigen door

TAHNEE SENTE



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ADIPONECTIN: POTENTIAL THERAPEUTIC TARGET FOR THE TREATMENT OF MUSCLE WASTING IN CHRONIC HEART FAILURE: *IN VITRO* EXPLORATION

Adiponectine: potentieel therapeutisch target voor de behandeling van spieratrofie in chronisch hartfalen: *in vitro* exploratie

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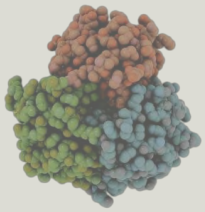


Preface

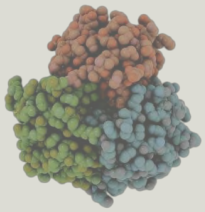
''Success is a journey, not a destination.
The doing is often more important than the outcome.''
***Arthur Robert Ashe** (1946 – 1993)*

This book is the result of my PhD thesis entitled '*Adiponectin: Potential therapeutic target for the treatment of muscle wasting in chronic heart failure',* and is written to obtain the degree of doctor in Medical Sciences at the University of Antwerp. The research described herein was conducted under the supervision of Prof. dr. CJ Vrints and Prof. dr. VY Hoymans at the department of Cardiology and the Laboratory for Cellular and Molecular Cardiology, Universal Hospital Antwerp (UZA), between August 2012 and September 2016.

I dedicate my PhD thesis to the memory of Prof. dr. Viviane Conraads who passed away in December 2013, during the second year of my PhD.

[Thank you for teaching me that success is a journey and not a destination]

Tahnee Sente
September 2016



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Chapter 1

Adiponectin and skeletal muscle wasting in heart failure

Partly adapted from:

Sente T, Gevaert A, Berendoncks AM, Vrints CJ, Hoymans VY. The evolving role of adiponectin as an additive biomarker in HFrEF. Heart Fail Rev. Jul. 2016

Sente T, Van Berendoncks AM, Hoymans VY, Vrints CJ. Adiponectin resistance in skeletal muscle: Pathophysiological implications in chronic heart failure. J Cachexia Sarcopenia Muscle. Oct. 2015.

Heart failure (HF) is a growing health problem. Despite improved management and outcome, the number of patients with HF is expected to keep rising in the following years. Among HF patients, about half have preserved left ventricular systolic function, which is also known as diastolic HF or HF with preserved ejection fraction (HFpEF), whereas the other half of the patients display a reduced ejection fraction (HFrEF). In recent years, knowledge of HF pathophysiology has shifted significantly. Whereas HF was previously considered to be a pure cardiac entity, it is now regarded as a complex multisystem syndrome which also affects peripheral organs. Abnormalities of skeletal muscle, including disturbed energy metabolism and muscle wasting, are common features in HF. The adipocytokine adiponectin was postulated to be centrally involved in HF-associated skeletal muscle changes. The objective of this **chapter** is to provide an overview on the role of adiponectin in HF, both in HFpEF and HFrEF. Particular emphasis will be given to the regulation of adiponectin at the level of the skeletal muscle and its metabolic relevance in HF.

HEART FAILURE: A MULTISYSTEM SYNDROME

Heart failure (HF) is a complex syndrome initiated by a structural or functional disorder impairing the ability of the heart to respond to physiological requirements. HF patients are characterized by shortness of breath, termed dyspnoea and by fatigue, in particular during exercise. Although HF may evolve from a variety of conditions, the main causes are hypertension, dilated cardiomyopathy and coronary heart disease (CHD) (1). HF is a major epidemiological burden in the industrialized world and continues to be a growing problem, mostly because of the increase in average life expectancy and the improved survival of patients with CHD. HF currently affects about 26 million people worldwide and is the most common diagnosis among patients who are 65 years of age and older (2). Moreover, HF is the leading cause of death and disability in the Western world and is associated with high rates of short term mortality (10-20%) (3). Despite improvements in therapy, many patients with HF still face poor prognosis. The overall five-year survival rate is only 30-40% (2).

In the last two decades, a paradigm shift in the pathophysiology of HF has taken place. HF is no longer considered as a single organ disease but as a complex multisystem syndrome involving diverse hemodynamic, neuro-hormonal and metabolic alterations (4). Overstimulation of the sympathetic nervous system and of the renine-angiotensin-aldosterone system (RAAS) together with a chronic inflammatory state and an increased ergoreflex activity are amongst the main features related to this disease (5-7). However, beyond the traditional concept that HF is a systemic disease, it is increasingly appreciated that peripheral manifestations such as endothelial dysfunction, ventilator inefficiency and alterations in peripheral skeletal muscles also contribute to disease progression and outcome (**Fig. 1**). It is now also well-established that among HF patients, about half have preserved left ventricular (LV) systolic function, which is also known as diastolic HF or HF with preserved ejection fraction (HFpEF), whereas the other half of the patients display a reduced ejection fraction (HFrEF) (8, 9). In addition to comorbid illnesses, patients with HFpEF and HFrEF likely have multiple pathophysiological mechanisms in common (10-12).

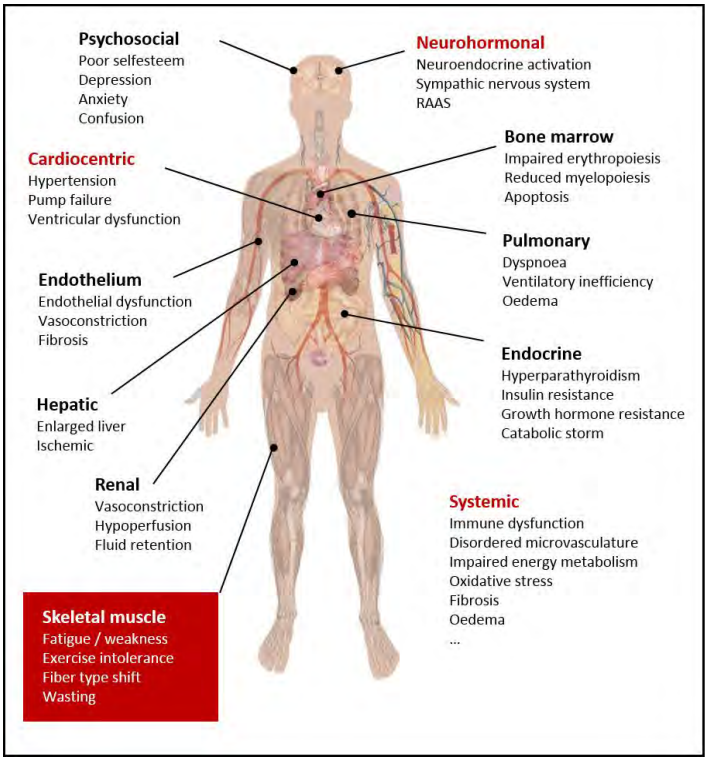

Figure 1. Heart failure: not a single organ disease but a multisystem syndrome

SKELETAL MUSCLE ALTERATIONS IN HEART FAILURE

Heart failure related myopathy

Exercise intolerance, resulting from dyspnea and fatigue is one of the most disabling symptoms of chronic HF. However, the origin of reduced exercise performance cannot directly be linked to hemodynamic or neurohormonal disturbances. In an attempt to identify the main underlying cause of exercise intolerance, several studies have focused on the contribution of peripheral skeletal muscle dysfunction. Evidence has shown that impaired exercise performance is indeed to a large extent caused by intrinsic alterations in the skeletal muscles of chronic HF patients, including HFrEF and HFpEF. These skeletal muscle abnormalities occur already early in the progression of the disease and are an integral part of the pathophysiology of HF. They are marked by several qualitative and quantitative changes in histological, biochemical and functional properties. Changes include a chronic shift towards the fast glycolytic type II anaerobic fibers, a disturbed intracellular phosphate

metabolism, fibrosis and an anabolic/catabolic imbalance (6, 13, 14). In addition, abnormalities of fibre type distribution and fibre size together with reduced maximal muscle isometric strength have been observed in patients suffering from chronic HF (15). Patients with chronic HF also demonstrate a reduction in the number of capillaries per muscle fibre, impaired muscle oxidative capacity and reduced peak oxygen uptake (14, 16). The disturbed muscle function is further characterized by an abnormal intracellular phosphate metabolism with an early acidosis and a significant depletion of phosphocreatine (5). Furthermore, patients with chronic HF have altered mitochondrial volume and cristae, and decreased enzyme activity in both cardiac and skeletal muscle, which argues for a generalized metabolic myopathy in HF (5, 14, 17).

Loss of skeletal muscle mass in the setting of chronic HF has attracted considerable research interest over the last years and has been extensively reviewed by several authors (6, 13, 18, 19). Skeletal muscle wasting is recognized in approximately 70% of chronic HFrEF patients (19, 20) and is a critical component of cachexia (6, 21). Cachexia typically manifests in chronic diseases such as HF and is associated with poor outcome (7, 22, 23). Although widespread abnormalities of peripheral skeletal muscles have thus far been described in chronic HF and represent a complex interplay of diverse anabolic/catabolic factors and signalling pathways, the exact underlying pathophysiology is still unclear.

Metabolic failure in heart failure

It is now well-recognized that skeletal muscle energy deficiency plays a main role within HF pathophysiology. Chronic HF patients are typified by a hyperadrenergic state leading to increased protein breakdown and turnover, and a disturbed skeletal muscle glucose uptake and use, partly due to high levels of the catabolic factor glucagon (24). A shift in substrate metabolism away from fatty acid β-oxidation (FAO) towards glucose metabolism was indicated, marked by a decreased oxidative capacity and high circulating levels of free fatty acids (FFA) (25). These latter inhibit cardioprotective glycolysis and impair insulin signalling. Hormonal imbalances, including insulin and growth hormone resistance, have also been reported as common features in HF (5, 26). Chronic HF is also typified by an altered myocardial oxidative metabolism resulting in a reduced ability to generate adenosine-5'-triphosphate (ATP). In addition, emerging evidence suggest that disturbances in energy substrate metabolism influence contractile dysfunction and ventricular remodeling in chronic HF patients (27). Hence, the cardiac metabolic vicious cycle also plays a crucial role in

peripheral myocytes (28). Therefore, restoration of the energy metabolism can be an attractive novel strategy for the prevention and treatment of chronic HF.

During the past decade, considerable progress has been made to identify the molecular basis of the metabolic deregulation and the energy deprived status of the insulin resistant skeletal muscle present in chronic HF patients. Recently, adipokines, in particular the adipocytokine adiponectin has attracted considerable attention because of its insulin-sensitizing properties and its fundamental role in energy metabolism. Moreover, adiponectin was postulated to be centrally involved in HFrEF-associated metabolic failure and skeletal muscle wasting (21).

ADIPONECTIN

Adiponectin and its accompanying receptors

Structure

Adiponectin was first described in 1995 by Scherer et al. (29). These authors discovered a novel adipocyte complement-related protein of 30 kDa, alternately named Acrp30, by cDNA cloning from 3T3-L1 adipocytes. Adiponectin, mapped to chromosome 3q27, contains 244 amino acids (247 amino acids for mouse orthologous) composed of four distinct domains: an amino-terminal signal sequence, a non-conserved variable region, a collagen-like domain (cAd) and a C-terminal globular domain (gAd) (29). The globular region is homologous to the complement 1q (C1q) family and shows a strong similarity with tumor necrosis factor-alpha (TNF-α), suggesting that there might be an evolutionary link between adiponectin and TNF-α (30). Adiponectin is one of the most abundant proteins in human plasma (31, 32). Circulating levels of adiponectin range from 5 to 30 μg/ml in healthy individuals, accounting for approximately 0.05% of total blood proteins. In the circulation, the 30-kDa monomers of adiponectin assemble into several polymeric forms including trimeric low molecular weight (LMW; ~67 kDa), hexameric medium molecular weight (MMW; ~180 kDa) and oligomeric high molecular weight (HMW; ≥300 kDa, e.g.; dodecamers and octadecamers) forms (33). The combination of these forms is often referred to as full-length adiponectin. LMW adiponectin is most predominant in circulation. Proteolytic cleavage of adiponectin may also occur, so that a smaller, globular fragment is found in plasma (34). It remains controversial whether or not these different forms of adiponectin have different biological activities (35,

36). Ample data, however, do suggest that the HMW hexamer is of higher clinical significance, and that it has a more prominent role in cardioprotection (37-39).

Of note, adiponectin levels are generally two to three times higher in females than in males (29, 40). This sexual dimorphism has been attributed to the inhibiting effect of testosterone on the secretion of adiponectin and the higher body fat percentage observed in women (41, 42). However, although primarily secreted by adipocytes, adiponectin is also produced by non-adipose cell types such as hepatocytes, osteoblasts, cardiomyocytes and skeletal muscle cells (43-45).

Function

Adiponectin is able to exert insulin-sensitizing, anti-atherogenic, anti-diabetic, anti-ischemic and anti-inflammatory properties (46-50). Adiponectin also promotes endothelial function and modulates a wide range of metabolic processes, thereby regulating energy homeostasis (51, 52). Adiponectin mainly performs its actions through interaction with AdipoR1 and AdipoR2, which were cloned for the first time in 2003 by Yamauchi et al. (46, 53, 54). These receptors share a significant molecular homology, with an internal N-terminus and an external C-terminus, but differ in the root-mean square deviations (i.e.; bond lengths and angles). They contain seven transmembrane domains, similar to the G-protein-coupled cell surface receptors (GPCRs), are capable of binding globular and full-length adiponectin and therein, will stimulate the main downstream effector adenosine monophosphate activated protein kinase (AMPK) (53, 55) (56). The high-affinity receptor AdipoR1 is ubiquitously expressed in endothelial cells and skeletal muscle fibers where it regulates glucose and lipid metabolism through AMPK, whereas AdipoR2, which serves as a moderate-affinity receptor, is mainly expressed in the liver and is involved in activation of peroxisome proliferator-activated receptor alpha (PPARα), leading to increased insulin sensitivity (46, 54). Further, AdipoR1 has been postulated to play an important role in adiponectin signalling of macrophages (57). Activated AdipoR1 and AdipoR2 improve fatty acid oxidation (FAO) and glucose uptake, increase mitochondrial biogenesis and lactate production, reduce hepatic gluconeogenesis and suppress oxidative stress, inflammation and several important metabolic risk factors (e.g.; diabetes mellitus type 2) for HF (58, 59). AdipoR1 and AdipoR2 interact directly with adaptor protein containing a pleckstrin homology domain, a phosphotyrosine binding domain and a leucine zipper motif 1 (APPL1) (60, 61). APPL1 mediates the adiponectin-signalling cascade, transmitting signals from both adiponectin receptors to their downstream targets by

interacting with the N-terminal intracellular region. Signalling via APPL1 is necessary for adiponectin to exerts its anti-inflammatory and cytoprotective effects on endothelial cells (62). Apart from AdipoR1 and AdipoR2, a third adiponectin receptor, T-cadherin has been identified. T-cadherin exclusively binds with HMW adiponectin and acts as a co-receptor to facilitate adiponectin signalling (63). T-cadherin is highly expressed in the heart and the vasculature, and to a lesser extent in skeletal muscle and liver (64).

Biological actions of adiponectin in the heart and vasculature

Adiponectin is able to induce a wide range of myocardial and vascular protective effects (32, 48, 65-69). Experimental studies using adiponectin knock-out (KO) mice subjected to transverse aortic constriction (TAC) to induce pressure overload have demonstrated a protective role for adiponectin against cardiac hypertrophy, adverse LV remodeling and systolic dysfunction (70-73). In addition, treatment of apolipoprotein E–deficient (apoE−/−) mice with recombinant adenovirus expressing human adiponectin could suppress the development of atherosclerotic lesions (74). Also, adiponectin deficiency in mice resulted into enhanced thrombus formation and platelet aggregation upon carotid arterial injury, whereas adenovirus-mediated over-expression of adiponectin attenuated these processes (75). Moreover, in preclinical pig and mouse models of ischemia/reperfusion injury, adiponectin treatment reduced infarct size and improved cardiac function, (76, 77). However, from these preclinical (animal and *in vitro*) studies it became increasingly evident that the signaling effects of adiponectin diverge with its sites of action.

In the vasculature, adiponectin exerts anti-atherosclerotic effects by inhibiting vascular smooth muscle cell proliferation and migration, suppressing macrophage to foam cell formation, contributing to plaque stability and by reducing inflammation (50, 74, 78-81). In endothelial cells, adiponectin diminishes the generation of reactive oxygen species (ROS), stimulates the formation of nitric oxide (NO) and limits the production of pro-inflammatory cytokines and chemokines, including TNF-α and interleukin (IL)-8 (82, 83). Its anti-oxidant activity and effects on endothelial function (e.g.; vasodilatation, increasing NO bioavailability) occur to be mediated by AMPK and cAMP-dependent protein kinase A (PKA) (82, 83). Studies have further demonstrated that adiponectin is able to promote endothelial repair and angiogenesis (84). In the myocardium, adiponectin reduces apoptosis, fibrosis, and oxidative stress but also promotes cell survival by attenuating the expression of inducible NO synthase (iNOS) and nicotinamide adenine dinucleotide phosphate (NADPH)

oxidase (46, 66). In addition, in vitro and animal experiments demonstrated that not only elevated circulatory levels of adiponectin, but also locally produced adiponectin protects against ischemia-reperfusion injury via AMPK and cyclooxigenase (COX)-2-dependent mechanisms (85). AdipoR1 and AdipoR2 are both expressed in the myocardium. Yet, an experimental study in rats suggested that binding to T-cadherin is de facto critical for adiponectin-mediated cardioprotection (64). In particular, in mice deficient in T-cadherin adiponectin failed to interact with cardiac tissue, leading to increased hypertrophy and worsening of ischemia-reperfusion injury (64). In this regard, Joshi et al demonstrated how T-cadherin is essential for the protection of vascular endothelial cells against apoptosis resulting from oxidative stress (86).

The signalling mechanisms of adiponectin, however, are not yet entirely clear. Moreover, several recent studies uncovered that the elevation of serum adiponectin levels is an independent predictor of mortality and morbidity in HFrEF patients (87-90).

ADIPONECTIN: THE *JANUS* MOLECULE OF HEART FAILURE

Circulating adiponectin levels correlate inversely with multiple metabolic diseases and related disorders associated with insulin resistance. Adiponectin levels are decreased in obesity and type-2 diabetes and negatively associated with the metabolic syndrome (91, 92). Low levels of adiponectin have been linked with the presence of common cardiovascular risk factors including smoking and hypertension (93-96). Moreover, adiponectin deficiency resulted into increased myocardial damage, cardiac hypertrophy and HF (89, 93, 97, 98). In addition, a strong correlation between myocardial infarction and hypoadiponectinaemia was documented in a number of studies (99-101). Adiponectin levels in patients with coronary artery diseases (CAD) seem to be diminished if compared to healthy subjects and are strongly predictive of incident cardiovascular events (102). Hence, whereas the role of adiponectin within CAD and myocardial infarction is quite straightforward, its function in HF seems to be more complicated. The variation of adiponectin level in the setting of HF, including HFrEF, HFpEF and end-stage HF is summarized in **figure 2**.

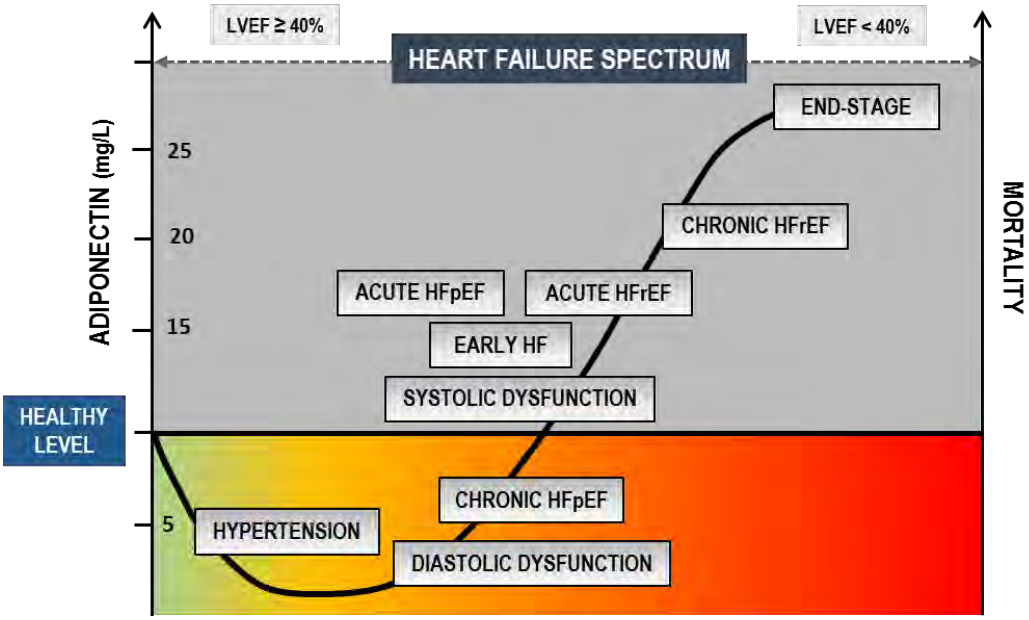

Figure 2. Adiponectin levels within the heart failure spectrum

Serum adiponectin levels are related to the severity of HF and increase with HF progression. Adiponectin concentrations are decreased in patients with hypertension, diastolic dysfunction and chronic HFpEF if compared to healthy controls (**horizontal bar**). With the advent of systolic dysfunction, adiponectin concentrations slightly rise. In acute HF (HFpEF and HFrEF) and chronic HFrEF, adiponectin concentrations are substantially increased, whereas in patients with end-stage HF, high levels of adiponectin assumedly further deteriorate cardiac function and increase the risk of death. *Abbreviations: HF; Heart failure, HFpEF; Heart failure with preserved ejection fraction, HFrEF; Heart failure with reduced ejection fraction, LVEF; Left ventricular ejection fraction.*

Heart failure with reduced ejection fraction

Since 2005, adiponectin has been extensively studied in the setting of chronic HFrEF (**Table 1**). Elevated serum levels of total adiponectin were repeatedly reported in these patients (24, 97, 103-107) (**Fig. 2**). Baldasseroni et al. demonstrated that patients with CAD and overt HFrEF have marked increased circulating levels of total adiponectin compared to patients with CAD and no HF symptoms (108). In addition, circulating adiponectin levels were higher in patients with CAD and HF as opposed to asymptomatic HF patients with normal or reduced left ventricular ejection fraction (LVEF) (108, 109). Also, patients with acute HFrEF were shown to have elevated levels of circulating adiponectin (**Fig. 2; Table 1**). A cohort study by Ohara et al. reported increased plasma levels of total and HMW adiponectin in patients with acute HFrEF (non-ischemic, non-valvular origin) compared to patients with supraventricular

arrhythmia without HF (110). In chronic HFrEF, high levels of total adiponectin were shown to confer poor prognosis and predict all-cause mortality, independent of cardiovascular risk factors including age, systolic blood pressure, LVEF <25%, HF duration and creatinine clearance (104). This association between high total adiponectin and mortality risk was more pronounced in elderly patients suffering from HFrEF having a normal body mass index (BMI; 21–25 kg/m^2) than in aged HFrEF patients with a BMI value that is too low or too high (<21 or >25 kg/m^2) (104, 105). Moreover, in a large, cross-sectional community based study of elderly patients (>70 years of age, n= 954), and by using an in-house time resolved immunofluorometric assay, adiponectin concentrations were inversely associated with LVEF in men, but not in women (111). The population-based Cardiovascular Health study of 5553 older adults (≥ 65y) demonstrated an increased risk of all-cause mortality for elderly HFrEF subjects with adiponectin levels above 12.4 mg/L (112). In addition, total adiponectin in circulation was associated with the severity of HF, as assessed by New York Heart Classification (NYHA) class (88-90, 107, 113). Likewise, Wannamethee and colleagues investigated the association of adiponectin with all-cause and cardiovascular disease (CVD) mortality in a prospective study of 4046 men aged 60-79 years, participating in the British Regional Heart Study (114). Moreover, in patients with chronic HFrEF, elevated levels of total adiponectin were associated with higher cardiovascular mortality and morbidity (115, 116). Increased levels of adiponectin in chronic HFrEF thus seem to parallel a worsening prognosis (**Fig. 2; Table 1**). In addition, with regard to acute HFrEF, a high value of the HMW to total adiponectin ratio at hospital admission, and also a larger decrease in this ratio from admission to discharge, has been related to improved prognosis (110). Total and HMW levels of adiponectin had no significant impact on patient prognosis in acute HF. Also, a strong positive correlation between total adiponectin and brain natriuretic peptide (BNP) or N-Terminal (NT)-proBNP levels has been demonstrated in patients with HFrEF (88, 89, 117, 118).

These findings are in contrast with several *in vitro* studies supporting a beneficial role for adiponectin in HF (77, 119). The relation of high plasma adiponectin with advanced disease state, symptomatic status and metabolic impairment in patients with HF, however, seems to be robust (24, 120). Because adiponectin is credited with multiple beneficial effects on cardiac hypertrophy and failure, its seemingly negative impact on the survival of patients with chronic HFrEF has been designated *'the adiponectin paradox'* (121). Up to now, the

underlying pathophysiologic mechanisms that explain the negative connotation of increased adiponectin concentration in chronic HFrEF patients remain unclear.

An overview of the different studies on adiponectin in patients with HFrEF is provided in **table 1**.

Heart failure with preserved ejection fraction

In contrast to patients with acute and chronic HFrEF, chronic HFpEF patients have low levels of circulating adiponectin (**Fig. 2**). Negi. et al. demonstrated that a diminished level of total adiponectin in chronic HFpEF is related to the severity of diastolic dysfunction, independent of age, BMI and the presence of hypertension or diabetes (122). Also, in patients with known or suspected CAD referred for cardiac catheterization, low total adiponectin has been associated with LV diastolic dysfunction (123). Mice deficient in adiponectin were prone to develop chronic HFpEF (124). In particular, lack of adiponectin in mice with aldosterone-induced HFpEF exacerbated LV hypertrophy, hypertension, diastolic dysfunction and HF. Yet, adiponectin over-expression in aldosterone-infused mice led to improved LV hypertrophy and diastolic function, pointing to adiponectin's therapeutic potential (12). In addition, Fujioka et al. investigated the impact of adiponectin on endothelin-1 induced hypertrophy of cultured cardiomyocytes and showed that total adiponectin suppressed the endothelin-1 induced increase in cell surface in a dose-dependent manner (125). Further, and although definite evidence is currently absent, preliminary clinical data suggest that circulating adiponectin levels are increased in patients with acute HFpEF; a recent prospective cohort study by Tang et al. demonstrated that higher plasma adiponectin levels are directly associated with the severity of diastolic dysfunction (**Fig. 2**) (126). Higher levels of adiponectin in HFpEF may be attributed, at least partially, to a rise in BNP correlating with the stage of diastolic dysfunction (127). Circulating BNP is within a normal range in asymptomatic patients having a mild degree of diastolic dysfunction (impaired relaxation), moderately elevated in patients with pseudonormal diastolic function, and highest among those with a restrictive filling pattern and acute HFpEF (128, 129).

So even though preclinical studies demonstrate that adiponectin is protective against hallmarks of HFpEF such as hypertension, LV hypertrophy and diastolic dysfunction, knowledge on the pathophysiological significance of adiponectin in HFpEF is still in its infancy and more comprehensive studies are awaited.

From hypertension to diastolic and systolic heart failure

Up to now, few studies have investigated the dynamics in circulating adiponectin levels during the transition from hypertension to onset of cardiac dysfunction and ultimately HFrEF (**Fig. 2**). Fu and colleagues examined whether total serum adiponectin is associated with onset of HF from hypertension through LV hypertrophy in spontaneously hypertensive rats (130). The adiponectin level in rats decreased with increasing hypertension during the first three months, and continued to decline when diastolic dysfunction became overt. At month 15, levels started to rise again prior to the appearance of systolic dysfunction at month 18. In another study, community-based and cross-sectional (n=1414), serum adiponectin concentration was negatively associated with LV mass index in subjects at low risk of LV hypertrophy, but a positive association was shown in subjects who were at a high risk of developing LV hypertrophy (115). These results indicate that the relationship of LV mass index with adiponectin may vary with the risk and the severity of LV hypertrophy. Interactions between risk factors including age, sex, and diabetes mellitus type 2 may also affect adiponectin concentration in both patients with HFrEF and HFpEF, making it difficult to ascertain the role of circulating adiponectin in the development and progression of HF. Prospective longitudinal studies may help to clarify these variations in circulating adiponectin from normal cardiac activity toward progressive impairments in myocardial function and ultimately, HF.

End-stage heart failure

Patients with a more severe stage of HF have higher concentrations of adiponectin compared to HF patients with rather mild symptoms (97). Although no study results are available yet, it is, therefore, not unlikely that in patients with end-stage HF adiponectin levels are utterly high. Progression to end-stage HF is associated with weight loss and wasting, long-term systemic inflammation and renal failure. Weight loss and renal failure have been correlated with high blood levels of adiponectin in patients with ischemic heart disease (IHD) (104, 131, 132). In addition, patients with end-stage renal disease have been associated with a doubling of circulating adiponectin concentrations (133). Therefore, it is conceivable that the co-morbid conditions which are present in end-stage HF contribute to an increase in circulating adiponectin (**Fig. 2**) (134). Consistent with this hypothesis, high body mass and hence rather low circulating adiponectin might favour outcome in patients with end-stage HF.

BNP levels, which closely relate with the severity and prognosis of HF, have been linked with adiponectin in a cross-sectional study consisting of 90 patients with HFrEF (89). Evidence supports that adiponectin is involved in the same cardio-regulatory pathway as BNP (111). Therefore, adiponectin levels might rise in a fashion similar to that of BNP also in end-stage HF (135, 136).

Adiponectin and additional determinants

A small, but growing, literature suggests that adiponectin levels vary according to a patient's metabolic status. Low plasma concentrations of adiponectin precede the onset of type-2 diabetes (94). HF patients with diabetes have lower adiponectin levels compared to patients without diabetes (109). This also applies to HF patients with or without the metabolic syndrome (92). In addition, it has been shown that respiratory comorbidities (e.g.; chronic obstructive pulmonary disease; COPD), renal dysfunction (e.g.; chronic kidney disease; CKD), anaemia and arthritis (e.g.; osteoarthritis), common co-morbidities in patients with HFrEF, may affect adiponectin levels (137-140). In addition, circulating adiponectin levels occur to be higher in women, and also increase with age (40, 138). A distinct response in plasma adiponectin was also shown at early and late stages of the disease and according to the extent of HF. As stated above, increased adiponectin levels in chronic HFrEF were correlated with worsening of functional capacity (i.e., NYHA) and cardiac performance (i.e., LVEF) (89, 104). In this regard, and although well-tolerated by the majority of patients, evidence has indicated that β-blockers may affect adiponectin concentrations (141). As stated by Antonopoulos et al., the net effect of adiponectin in circulation likely depends on a complex interplay of the underlying disease state and the nature of the inflammatory stimuli (142).

SKELETAL MUSCLE WASTING: IS ADIPONECTIN THE REAL CULPRIT?

A key adipocytokine in skeletal muscle metabolism

In search for the molecular basis of the energy-deprived status in HF patients, attention was focused on adiponectin. The skeletal muscle is a major contributor to whole-body energy expenditure, being responsible for ~80% of insulin-stimulated glucose disposal and, therefore, represents an important site of action for adiponectin. Indeed, as mentioned before, adiponectin is involved in the regulation of skeletal muscle metabolism via its interaction with the G-protein-coupled adiponectin receptors AdipoR1 and AdipoR2 (46). Disruption of these

receptors abrogates adiponectin binding and its metabolic actions. Recent characterization of mice lacking AdipoR1 and AdipoR2 confirmed important roles for these receptors in the maintenance of metabolic homeostasis (54). In particular, simultaneous disruption of both receptors abrogated adiponectin binding resulting into glucose intolerance and insulin resistance. Adiponectin also promotes glucose uptake by skeletal muscles and increases FAO by activation of multiple factors including AMPK, peroxisome proliferator-activated receptor α (PPARα) and p38 mitogen-activated protein kinase (MAPK) (59). AMPK is the master regulator in glucose utilization and FAO, whereas the nuclear transcription factor PPARα regulates the expression of several genes involved in lipid metabolism, ultimately preventing insulin resistance. Adiponectin further stimulates mitochondrial biogenesis and increases the oxidative capacity in skeletal muscle (143, 144). In this regard, Liao and his colleagues (71) reported energetic defects in HF in relation to adiponectin deficiency. More recently, increased circulating adiponectin concentrations were associated with metabolic impairments and a hyper-catabolic state in patients with HF (145, 146).

All these data stress that adiponectin exercises an important impact on skeletal muscle energy metabolism in HF (**Fig. 3**). The pathophysiological mechanisms underlying skeletal muscle energy alterations in HF, however, remain to be fully established.

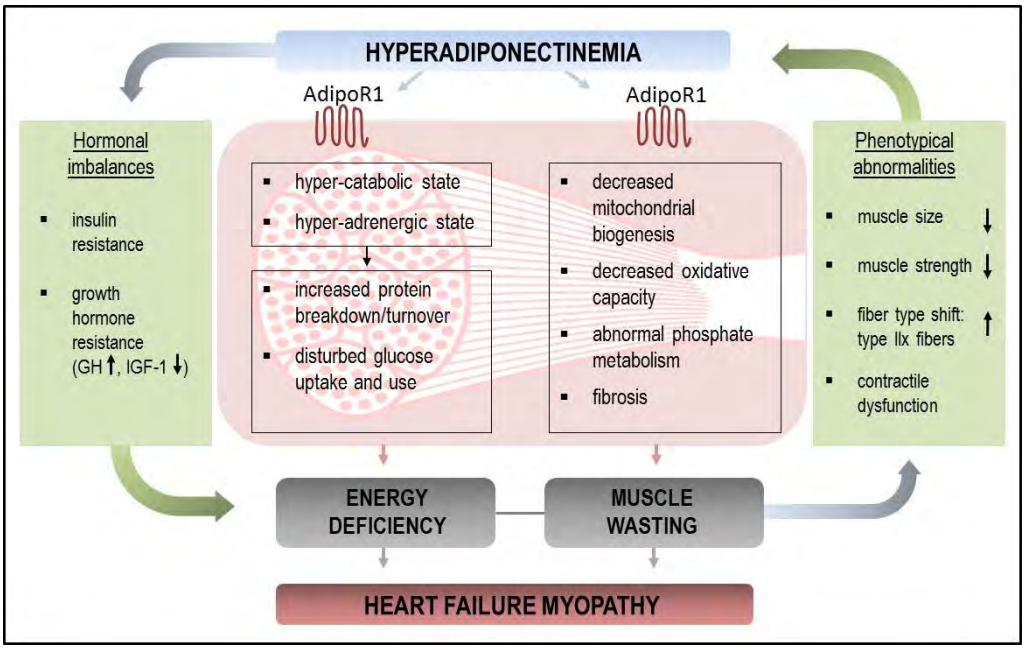

Figure 3. Adiponectin and heart failure-associated myopathy

Skeletal muscle hyperadiponectinemia contributes to energetic impairments, structural and functional alterations of muscle fibers and hormonal imbalances, eventually culminating into a state of HF-associated myopathy. *Abbreviations: GH: Growth hormone; IGF-1: Insulin like growth factor-1.*

Adiponectin as a marker of skeletal muscle wasting

The majority of patients with HF develop skeletal muscle wasting. Muscle wasting is recognized as a critical component of cachexia contributing to skeletal muscle weakness and fatigue (6, 21). In a recent case-control study by Mc Entegart and co-workers, patients with HFrEF and cachexia had remarkably higher adiponectin concentrations in comparison to patients without cachexia, irrespective of their BMI (145, 147). Latest research further revealed that circulating levels of adiponectin gradually increase with decreasing muscle fiber size and muscle strength, even in non-cachectic HFrEF patients (148, 149). Furthermore, absence of adiponectin expression in skeletal muscle of adiponectin-null knockout mice was associated with phenotypical muscle abnormalities and contractile dysfunction, including an increase in type II fibre size and a decreased peak contractile force if compared with wild-type littermates (45). Clinical research also revealed a '*fibre type shift'* in chronic HF patients, characterized by a decreased proportion of slow oxidative (fatigue-resistant) type I fibres, compensated for by an increased proportion of fast glycolytic (fatiguable) type IIx fibres

(150). This shift in muscle fibre type distribution occurs similar between HFrEF and HFpEF patients (150-152). In addition, reduced muscle oxidative capacity and abnormal intracellular phosphate metabolism were documented in HF patients (**Fig. 3**).

In summary, hyperadiponectinemia seems to parallel with alterations in the structural and functional characteristics of skeletal muscle fibres in HFrEF patients, and might even act as a marker of cachexia (21, 147). Adiponectin might be centrally involved in impaired metabolic signalling, forming a potential link between muscle wasting, disease progression and poor prognosis in HFrEF patients (145) (**Fig. 3**). The exact molecular basis by which adiponectin affects skeletal (and cardiac) muscle tissue in HF is still not completely clarified. Available data will be addressed in the next chapter (**Chapter 2**).

Supplementary: Table 1. Circulating adiponectin concentrations in patients with HFrEF

Author, Year	Design	Study Population	Number (Females)	Mean age in years	Follow-up in years	ADN form	Test (Company)	Main findings
Sente, 2016	Cross-sectional	Chronic HFrEF and healthy controls	10 (3) 10 (3)	57.4 53.9	N.A.	Total	ELISA (R&D Systems)	▪ ADN increased in chronic HFrEF vs. controls
Huang, 2016	Cross-sectional	1) DCM I (NYHA II) 2) DCM II (NYHA III) 3) DCM III (NYHA IV) 4) Healthy controls	22 (8) 45 (13) 33 (7) 45 (20)	58 54 52 52	N.A.	Total	ELISA (R&D Systems)	▪ ADN increases with severity (NYHA class) of DCM
Loncar, 2013	Cross-sectional	Chronic HFrEF and healthy controls	73 (0) 20 (0)	68 67	N.A.	Total	RIA (Linco Diagnostics)	▪ ADN increased in chronic HFrEF vs. controls
Bozcali, 2013	Cross-sectional	Chronic HFrEF and healthy controls	30 (-) 30 (-)	-	N.A.	Total	ELISA (-)	▪ ADN increased in chronic HFrEF vs. controls ▪ ADN positively associated with LV end-diastolic and end-systolic diameters
Sokhanvar, 2013	Cross-sectional	Chronic HFrEF	96 (41)	62	1	Total	ELISA (Biovendor)	▪ ADN increased in chronic HFrEF vs. controls ▪ ADN negatively correlated with LVEF ▪ ADN positively associated NYHA class
Szabó, 2014	Retrospective	Chronic HFrEF and healthy controls	111 (11) 36 (11)	65 60	4.4	Total	In-house immuno-fluorometric assay (N.A.)	▪ ADN increases with increasing HFrEF severity (NYHA class) ▪ ADN>13.5 mg/l increased mortality risk ▪ High ADN associated with poor functional capacity (low VO_2, high VE/VCO_2 slope)
Daniels, 2013	Cross-sectional	Chronic HFrEF and controls	30 (0) 34 (0)	67.4 64.3	N.A.	Total	N.A.	▪ ADN increased in chronic HFrEF vs. controls

Author, Year	Design	Study Population	Number (Females)	Mean age in years	Follow-up in years	ADN form	Test (Company)	Main findings
Djoussé, 2013	Prospective	Chronic HFrEF	1574 (N.R.)	58.7	22	Total	ELISA (R&D Systems)	▪ ADN is invers associated with HF risk in a non-linear fashion
Tang, 2013	Prospective	Chronic HFrEF	139 (32)	58	3.2	Total	ELISA (R&D Systems)	▪ ADN predicts increased risk of adverse clinical events (all-cause mortality, cardiac transplantation) independent of known cardiac risk factors ▪ High ADN associated with worse LV diastolic dysfunction and RV dysfunction
Kizer, 2012	Prospective	1) No CVD, HF, AF 2) CVD but no HF/AF 3) HF/AF	3272 (2061) 1030 (474) 383 (180)	74.4 75.6 77.0	11.8	Total HMW	ELISA (Millipore)	▪ 1a) ADN (total, HMW) invers associated with all-cause and CV mortality up to 12.4 mg/L ▪ 1b) ADN above 12.4 mg/L is associated with either outcome ▪ 2) ADN (total, HMW) not associated with all-cause and CV death ▪ 3) ADN (total, HMW) associated with all-cause, but not CV mortality
Wu, 2012	Prospective	Chronic HFrEF	108 (25)	62	2.1	Total	Radio-immunoassay (Linco Research)	▪ ADN associated with all-cause mortality
Yin, 2012	Prospective	Chronic HFrEF	96 (26)	53	0.8	Total	ELISA (R&D Systems)	▪ ADN is associated with death, heart transplantation, hospitalization and LVEF ▪ ADN increased with severity of HFrEF (NYHA)
Won, 2012	Cross-sectional	Ambulatory HFrEF patients	128 (48)	61.8	N.A.	Total	ELISA (Linco Research)	▪ ADN decreased in HFrEF patients with MetS ▪ ADN is associated with diastolic dysfunction in HFrEF patients with MetS
Tengiz, 2013	Cross-sectional	Chronic HFrEF and healthy controls	57 (15) 20 (9)	63.6 60.8	N.A.	Total	ELISA (Biovendor)	▪ Cut-off: 8.79 µg/ml ▪ ADN correlates positively with LV end-diastolic and end-systolic diameter ▪ ADN correlates negatively with LV fractional shortening and LVEF

Author, Year	Design	Study Population	Number (Females)	Mean age in years	Follow-up in years	ADN form	Test (Company)	Main findings
Baldasseroni, 2012	Cross-sectional	1) CAD 2) CAD with LV systolic dysfunction 3) CAD with HFrEF	46 (13) 24 (8) 37 (4)	65.7 67.3 68.9	N.A.	Total	ELISA (Linco Research)	▪ ADN rises in overt HFrEF
Schulze, 2011	Prospective	1) Acute decompensated HF (ADHF) 2) Chronic HFrEF 3) No HFrEF	44 (11) 26 (5) 21 (8)	61 63 57	8.4	Total	ELISA (Phoenix Pharmaceutical)	▪ High ADN associated with ADHF ▪ Clinical recovery in ADHF associated with a decrease in ADN close to concentrations as seen in stable HF
Ohara, 2011	Prospective	Acute HFrEF (non-ischemic and non-valvular origin) and patients admitted for the treatment of supraventricular arrhythmia served as controls	20 (3) 10 (2)	63 45	0.8	Total HMW HMWR	ELISA (Fujirebio)	▪ Higher HMWR at admission and larger decrease following treatment predicts a better prognosis
Matsumoto, 2010	Retrospective	ADHF; patients without HF [illegible] were admitted for coronary angiography served as controls	95 (34) 10 (3)	70 70	3.3	Total	ELISA (Otsuka Pharmaceutical)	▪ ADN increased in ADHF ▪ Higher event-free survival rate associated with largest decrease in ADN in response to treatment

Author, Year	Design	Study Population	Number (Females)	Mean age in years	Follow-up in years	ADN form	Test (Company)	Main findings
Van Berendoncks, 2011	Prospective	Chronic HFrEF	73 (21)	58.5	7	Total	ELISA (R&D Systems)	▪ Cut-off: 15.2 mg/L ▪ High ADN predicts poor outcome
Biolo, 2010	Retrospective	Chronic HFrEF	99 (26)	60	N.A.	Total	ELISA (Otsuka Pharmaceutical)	▪ Beta-blocker therapy correlates with lower ADN levels in chronic HFrEF
Kitaoka, 2010	Retrospective	HCM	106 (37)	63	N.A.	Total	ELISA (Otsuka Pharmaceutical)	▪ ADN associated with impaired LV systolic function
Ho, 2009	Prospective	Chronic HFrEF	81 (23)	61	0.7	Total	Radio-immunoassay (Linco Research)	▪ ADN associated with all-cause mortality
Dieplinger, 2009	Prospective	Acute destabilized HFrEF	137 (9)	77	1	Total	ELISA (Biovendor)	▪ ADN increased in acute destabilized HFrEF
Laoutaris, 2010	Retrospective	Chronic HFrEF	57	57.2	N.A.	Total	N.A.	▪ ADN associated with impaired exercise capacity (peak VO_2, 6MWT) ▪ ADN as an index of HF severity (BNP, LVEF, NYHA class)
Haugen, 2008	Prospective	Severe HFrEF (NYHA III-IV) and healthy controls	92 (26) 70 (22)	77.3 75.2	1	Total	ELISA (Otsuka Pharmaceutical)	▪ ADN increased in the very elderly (>70y) HFrEF patients ▪ ADN higher in non-ischemic vs. ischemic origin ▪ High ADN associated with increased all-cause mortality
Tamura, 2007	Prospective	Chronic HFrEF and healthy controls	54 (12) 55 (16)	63.2 59	1.8	Total	ELISA (Otsuka Pharmaceutical)	▪ Increase in ADN associated with higher mortality in ischemic vs. non-ischemic HFrEF

Author, Year	Design	Study Population	Number (Females)	Mean age in years	Follow -up in years	ADN form	Test (Company)	Main findings
Tsutamoto, 2007	Prospective	Chronic HFrEF	449 (84)	62.2	2.7	Total	ELISA (Otsuka Pharmaceutical)	▪ Total ADN is more useful for assessing mortality risk than HMW ADN ▪ High total ADN is an independent prognostic
Nakamura, 2006	Retrospective	Chronic HFrEF and healthy controls	90 (26) 20 (7)	61 56.8	N.A.	Total	ELISA (Otsuka Pharmaceutical)	▪ ADN increases according to the severity of HFrEF (NYHA class)
George, 2006	Prospective	Chronic HFrEF and healthy controls	175 (40) N.R.	71.1 N.R.	2	Total	Radioimmuno-assay (Linco Research)	▪ High ADN levels in chronic HFrEF ▪ High ADN associated with increased morbidity (NYHA class, BNP, age, renal failure) ▪ High ADN independent predictor of total mortality ▪ ADN lower in patients with ischemic CDM
Kistorp, 2005	Prospective	Chronic HFrEF	195 (86)	69.3	2.6	Total	ELISA (R&D Systems)	▪ High ADN is a predictor of mortality, independent of risk markers of HFrEF severity

Abbreviations: ADHF; Acute decompensated heart failure, AF; Atrial fibrillation, ADN; Adiponectin, BNP; Brain natriuretic peptide, CAD; Coronary artery disease, CHD; Coronary heart disease, CV; cardiovascular, CVD; Cardiovascular disease, DCM; Dilated cardiomyopathy, ELISA; Enzyme-linked immunosorbent assay, HCM; Hypertrophic cardiomyopathy, HF; Heart failure, HFrEF; Heart failure with reduced ejection fraction, HMW; High molecular weight, HMWR; HMW ratio, IHD; Ischemic heart disease, LV; Left ventricular, LVEF; Left ventricular ejection fraction, MetS; Metabolic Syndrome, 6MWT; 6-minute walk test, NYHA; New York Heart Association, RV; Right ventricular.

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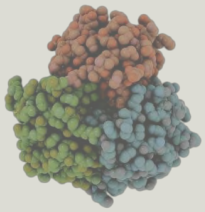

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Chapter 2

Skeletal muscle adiponectin resistance in heart failure

Partly adapted from

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Heart failure (HF) is associated with multiple metabolic disturbances of which many act negatively on skeletal muscle metabolism and wasting. Over the past few years, adiponectin emerged as an increasingly important player in the complex metabolic dysregulation related to HF. In HF patients with reduced left ventricular ejection fraction (HFrEF), circulating adiponectin levels appear to increase in proportion to the severity of the disease. In addition, these patients were shown to develop myocardial and skeletal muscle adiponectin resistance. In this **chapter**, we provide insights into the concept of adiponectin resistance in HF. The latest advancements in the field on mechanisms underlying HF-associated adiponectin resistance in skeletal and myocardial muscle will be discussed.

ADIPONECTIN RESISTANCE IN HEART FAILURE

Skeletal muscle myopathy constitutes an important mechanism that participates in the pathogenesis of heart failure (HF). To date, mounting evidence indicates a major role for adiponectin in skeletal muscle alterations among patients with chronic HF (1). As described in **chapter 1**, circulating adiponectin levels in chronic HF patients with reduced left ventricular ejection fraction (HFrEF) are increased in proportion to the severity of the disease (2, 3). Recently the concept of functional skeletal muscle adiponectin resistance has been suggested to explain compensatory elevated levels of adiponectin in chronic HFrEF.

Adiponectin resistance at the level of the skeletal muscle

Little is known about the regulation of adiponectin at the molecular level in skeletal muscle of patients with HFrEF. Therefore, our group recently investigated the local adiponectin system in skeletal muscle biopsies from chronic HFrEF patients (4). In agreement with increased circulating adiponectin concentrations, a 5-fold increase in the expression of adiponectin mRNA and protein were observed in patients with overt HFrEF. Despite these increases in circulating and muscle adiponectin, the mRNA and protein expression levels of the main skeletal muscle adiponectin receptor, AdipoR1 were decreased. Skeletal muscle metabolic deficiency in these patients was corroborated by results showing a deactivated adenosine monophosphate activated protein kinase (AMPK)/peroxisome proliferator-activated receptor α (PPARα) pathway, decreased phosphorylated AMPK and a concordant down-regulation of several target enzymes involved in the metabolism of free fatty acids (FFA) and glucose, including hexokinase 2 (HK2) and acyl-CoA dehydrogenase C-4 to C-12 straight chain (ACADM). Our results, therefore, signified the presence of a functional adiponectin resistance at the level of the skeletal muscle in HFrEF patients (4). Until now, data regarding skeletal muscle adiponectin signaling in HFpEF is not yet available.

Myocardial adiponectin resistance

Adiponectin and its receptors are also expressed by myocardial tissue. In the myocardium of patients with HFrEF, adiponectin expression is increased, whereas the level of AdipoR1 is reduced (5). Interestingly, mechanical unloading of the failing human heart through implantation of a ventricular assist device resulted into a reduction of circulating adiponectin and an increase in the level of AdipoR1 mRNA. These results suggest that myocardial adiponectin resistance in this population of chronic HF patients can be reversed (5). Kreth et al. recently investigated the myocardial adiponectin signaling in end-stage HFrEF patients (6). Besides high circulating adiponectin levels, myocardial adiponectin expression was low if compared to healthy controls, whereas myocardial AdipoR1 and AdipoR2 mRNA were increased. As such, the myocardial adiponectin pathway seems to be up-regulated in these patients with end-stage HF. The question remains as to what happens between early and end-stage HF. Systemic release of cellular adiponectin might be triggered in the progression to end-stage HF as a last resort, although this hypothesis needs to be tested by further investigation.

Adiponectin resistance and insulin resistance

Hormonal imbalances, including growth hormone resistance and insulin resistance have been previously reported as intrinsic features of chronic HF leading to both increased morbidity and mortality (7). Insulin resistance progresses in parallel with the severity of chronic HF and is considered instrumental in the development of skeletal muscle wasting (8). In particular, insulin sensitivity is worse in cachectic HFrEF patients (9). Adiponectin, by binding on its receptors, regulates insulin-sensitizing effects at the level of the skeletal muscle partly via activation of AMPK, PPAR-α and presumably other yet unknown signaling pathways (10, 11). In a cross-sectional study of 461 men Ingelsson et al. found higher circulating adiponectin concentrations with increasing skeletal muscle capillary density and in individuals with a higher proportion of slow type I muscle fibers (12). Taking into account that higher capillary density and more type I muscle fibers are associated with increased

insulin sensitivity, adiponectin indeed mediates insulin's actions (**Fig. 1**). In this regard, Stefan and colleagues demonstrated that plasma adiponectin concentrations in healthy humans are positively associated with *in vivo* insulin-stimulated glucose disposal, whereby low adiponectin concentrations were observed in parallel with reduced insulin sensitivity (13). In the case of HF, however, adiponectin levels have not been convincingly associated with insulin sensitivity. Moreover, adiponectin concentrations are elevated in chronic HFrEF despite a profound resistance to insulin, suggesting a disconnection between raised circulating adiponectin and improvement of insulin sensitivity in chronic HFrEF (14).

Impaired insulin sensitivity might also represent a causal link between adiponectin resistance and skeletal muscle wasting in HF (8). As such, López Teros et al. studied insulin levels and skeletal muscle mass in a cohort study of 147 older adults during an average follow-up period of 4.6 years and demonstrated that hyperinsulinemia is a significant risk marker of appendicular skeletal muscle wasting at older age (15). Still, it should be noted that Mullen et al. found evidence for adiponectin resistance to emerge prior to the initiation of skeletal muscle insulin resistance in high-fat fed rats (16). The interplay between insulin and adiponectin resistance is discussed in more detail below.

ADIPONECTIN RESISTANCE - THE WHYS AND WHEREFORES

The hypothesis on adiponectin resistance in HFrEF patients has garnered considerable attention in the past five years, but has also been the subject of controversy. Adiponectin in target tissues is regulated physiologically by several circulating factors among which are hormones, chemokines, growth factors and cytokines. Crosstalk between these various signaling molecules and the adiponectin pathway constantly tunes adiponectin expression. Although primarily secreted by white adipose tissue, adiponectin is also produced by non-adipose tissues and cell types such as hepatocytes, osteoblasts and even skeletal muscle fibres (17-19). Cardiomyocytes are also capable of synthesizing adiponectin, advocating the existence of a local adiponectin system at the myocardium (20). Whether the synthesis,

expression and release of adiponectin by non-adipose cells and tissues contribute to the increased circulating adiponectin concentrations in HFrEF, is still unclear. A recent study, however, demonstrated that adiponectin production by cardiomyocytes in end-stage HFrEF patients is low, not being significantly different between HF and healthy myocardium, making its participation to higher blood levels unlikely (6). Further, as summarized by Yamauchi and colleagues, growing evidence supports a role for adiponectin resistance in the development and progression of HFrEF, not only by promoting metabolic dysfunction, but also by affecting peripheral organs and tissues, i.e.; the endothelium, liver and skeletal muscle, and as such, contributes to the HF syndrome (21).

The state of adiponectin resistance in HFrEF fits well with the metabolic alterations in skeletal muscle, such as increased FFA availability, ultimately leading to skeletal muscle wasting (**Fig. 1**). Increasing our knowledge on the regulation of adiponectin and its downstream pathways in skeletal muscle of HF patients, and with respect to the failing heart, might therefore contribute to a greater understanding of the pathophysiology of HF.

PATHOPHYSIOLOGY OF ADIPONECTIN RESISTANCE

Since the initial observation by Van Berendoncks et al., many efforts were done to identify the causes underlying adiponectin resistance in chronic HFrEF (22). Although not yet completely resolved, adiponectin resistance seems to develop by multiple molecular and cellular interaction mechanisms between inflammatory and metabolic pathways. Study results will be discussed in the next paragraphs and are summarized in **figure 1**.

New insights originating from AdipoR1

Adiponectin resistance, initially described in obesity and conditions associated with insulin resistance, is thought to be partly due to down-regulation of the adiponectin receptor AdipoR1 (16, 23). This hypothesis is supported by a number of studies in humans and rodents and laboratory work with *in vitro* cell lines (23-29). Molecular cloning and knock-down of

AdipoR1 have improved understanding of the physiological and pathological actions of AdipoR1 and its role in adiponectin resistance. Yamauchi et al. evaluated the effects of adenovirus-mediated disruption of AdipoR1 in the skeletal muscle of Lepr$^{-/-}$ mice (29, 30). AdipoR1 null mice are glucose intolerant and insulin resistant due to mitochondrial defects. Targeted disruption of AdipoR1 also resulted into the abrogation of adiponectin dependent AMPK/Sirtuin 1 (SIRT1) activation.

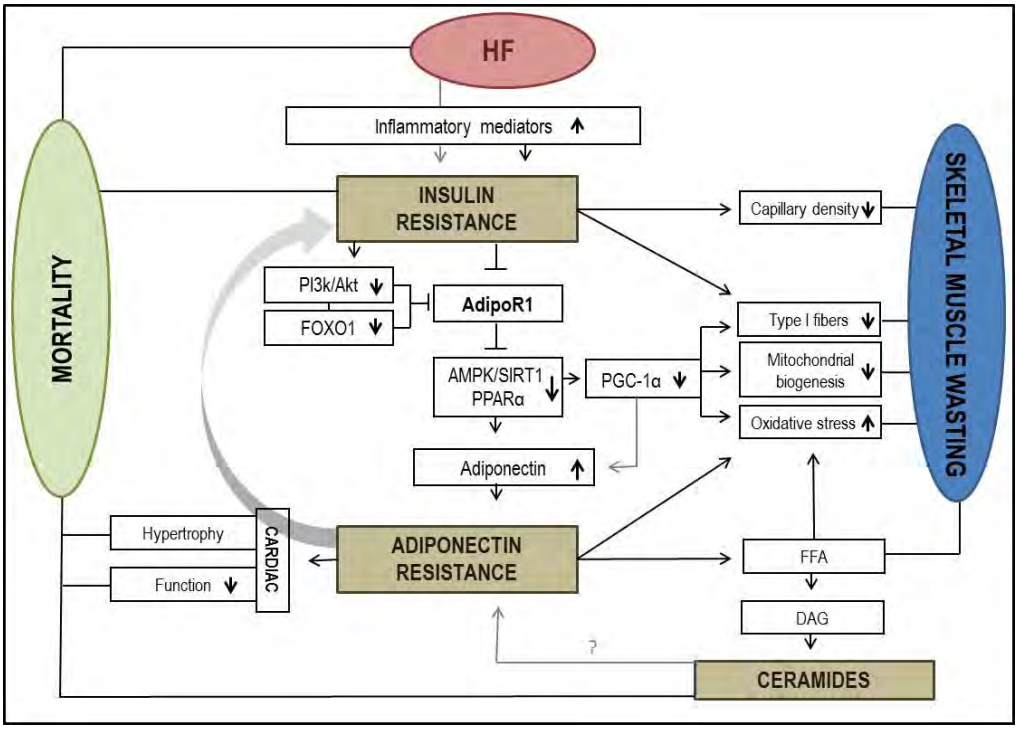

Figure 1. Hypothesis on skeletal muscle adiponectin resistance in HF

In HF, insulin resistance is associated with decreased levels of PI3K/Akt/FOXO1 and a reduced expression of the adiponectin receptor AdipoR1. Skeletal muscle metabolic deficiency in CHF is corroborated by a deactivated AMPK/SIRT1/PPARα pathway and down-regulation of PGC-1α, ultimately progressing to a state of skeletal muscle adiponectin resistance. This adiponectin resistance contributes to lipotoxicity, and further worsens insulin resistance. All these facets infer a disease process that eventually fulminates into skeletal muscle wasting. *Abbreviations: HF: Heart failure; PI3K: Phosphatidylinositol 3-kinase; Foxo1: Forkhead box protein O1; AMPK: adenosine monophosphate activated protein kinase; SIRT1: Sirtuin 1; PPAR-alpha: peroxisome proliferator-activated receptor alpha; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; FFA: Free fatty acids; DAG: diacylglycerol.*

Furthermore, adiponectin deficiency accelerated the transition from cardiac hypertrophy to HF and was causally related to a decreased cardiovascular function (31). Evidence in support of a concomitant reduction in receptor protein levels came from an additional study in obese mice demonstrating a lowered AdipoR1 protein content in skeletal muscle of insulin-resistant animals (28). Kadowaki and colleagues examined the regulation of AdipoR1 and AdipoR2 under insulin-resistant conditions (28). mRNA levels of both receptors decreased in response to increases in insulin. In line, in a recent study by Cui et al., myocardial AdipoR1 expression and activation of AMPK were reduced in insulin-treated rats (24). In addition, an impaired AdipoR1 mediated effect was observed in insulin treated primary cultures of rat cardiomyocytes and C_2C_{12} myoblasts (32).

Combined, these data indicate that changes in adiponectin via AdipoR1 relate to insulin signaling in chronic HFrEF patients (**Fig. 1**). The recently developed orally active synthetic small molecule AdipoR agonists, which specifically bind to and activate both AdipoR1 and AdipoR2, have generated promising results with regard to insulin sensitivity and mitochondrial capacity in muscles from *db/db* mice (33). Treatment with AdipoRon increased AdipoR1/AdipoR2, AMPK and PPARα mRNA expressions as well as the mRNA levels of genes involved in fatty acid β-oxidation (FAO), thereby ameliorating insulin resistance and glucose intolerance. Moreover, AdipoRon induced an up-regulated expression of peroxisome proliferator-activated receptor γ coactivator1 α (PGC-1α), contributing to increased mitochondrial biogenesis. It remains, however, interesting to investigate whether restoration of AdipoR1 expression will fully abrogate adiponectin resistance and lead to improvements in the metabolic and functional capacity of HF patients.

Rethinking the role of PGC-1α

HF is characterized by a disrupted mitochondrial function and biogenesis in which the production, transfer and consumption of high-energy phosphatases and oxidative capacity are reduced (**Fig. 1**). PGC-1α is responsible for the co-activation of transcription factors, including PPARs and ERRs (estrogen related receptors), and thereby controls mitochondrial

biogenesis and several antioxidant mechanisms (30, 34, 35). PGC-1α becomes up-regulated in skeletal muscle in response to Ca^{2+}/calmodulin-dependent protein kinase (CaMK). Interestingly, adiponectin stimulates CaMK via activation of AdipoR1 and AMPK/SIRT1, which would lead to the increased expression of PGC-1α. However, PGC-1α is down-regulated in the skeletal muscle of chronic HFrEF patients (35, 36). In this respect, it has been speculated that PGC-1α might play a crucial role in the processes of skeletal muscle wasting and adiponectin resistance in chronic HF (**Fig. 1**). Muscle-specific AdipoR1 knockout (KO) mice have been able to abolish an adiponectin-related increase in CaMK and AMPK/SIRT1, but induced a decrease in the expression and deacetylation of PGC-1α. Subsequently, a decline in mitochondrial content and activity, a decrease in the number of oxidative type I fibers, and ultimately, a reduction in oxidative stress-detoxifying enzymes associated with insulin resistance were found in the skeletal muscle (30). Adiponectin over-expression led to increased muscle mitochondrial biogenesis through up-regulation of PGC-1α (37). These findings suggest that in chronic HFrEF, impaired AdipoR1 levels might well be causally related to a decrease in PGC-1α and a disturbed mitochondrial activity of the skeletal muscle (**Fig. 1**).

The ceramide hypothesis

Increased levels of the toxic lipid intermediates, ceramide and diacylglycerol (DAG) in the failing human myocardium and in skeletal muscle are established features of chronic HFrEF (**Fig. 1**). Recent studies have questioned the primacy of adiponectin to altered sphingolipid ceramide profiles in skeletal muscle. Data from animal studies is provided showing that ceramidase activity is affected by the actions of adiponectin (38). Adiponectin lowers ceramide levels via activation of ceramidase, which actively metabolizes ceramides to sphingolipids in skeletal muscle with subsequent enhanced insulin sensitivity and mitochondrial biogenesis. In addition, an inverse correlation between circulating adiponectin and skeletal muscle ceramide content was demonstrated in healthy men (39). Ceramide signaling in chronic HFrEF further mainly depends upon adiponectin activation of both

AdipoR1 and AdipoR2. Moreover, over-expression of AdipoR1 and AdipoR2 in primary neonatal ventricular cardiomyocytes reduced ceramide levels and improved insulin sensitivity (40).

Lipotoxicity in chronic HFrEF is a consequence of impaired FAO and storage leading to increased levels of FFA, which are in turn converted into DAG and ceramides (41). These lipotoxic metabolites accumulate in insulin responsive tissues, including skeletal muscle. Whether this lipotoxic state contributes to adiponectin-resistance in chronic HFrEF needs to be proven. Recently, attention was drawn to the role of fibroblast growth factor 21 (FGF21) in mediating the effect of adiponectin on energy expenditure, including ceramide metabolism. FGF21 KO mice were shown to have reduced circulating adiponectin along with increased circulating ceramide levels, thereby impairing insulin sensitivity (42). The interaction between FGF21–adiponectin–ceramide seems a major axis for the control of energy consumption and the action of insulin (42, 43).

Nevertheless, the question remains whether a disturbed adiponectin-sphingolipid signaling is involved in the etiology of adiponectin resistance in chronic HFrEF. Very recently, Yu et al. demonstrated a stepwise increase in plasma ceramides in chronic HFrEF patients along with increased adiponectin levels in relation to the severity of HF (44). In addition, an increased diaphragm ceramide content was noted in rats with chronic HF following coronary artery ligation and associated with disturbed energy metabolism and diaphragm dysfunction (45). All these data highlight a main role for ceramides in the pathophysiology of HFrEF (**Fig. 1**).

Connecting the chain of events: A disturbed insulin - AdipoR1 axis

The association between insulin and adiponectin was reported for the first time in an adipocyte-specific insulin KO mouse model, demonstrating that hyperadiponectinemia can result from a lack of insulin signaling in adipose tissue (46). In addition, Lin et al. and Kim et al. both investigated the interactions between adiponectin and insulin in mice with muscle-specific insulin resistance and showed that insulin resistance may lead to hyperadiponectinemia and even adiponectin resistance (23, 25). Evidence was also provided

that phosphatidylinositol 3-kinase (PI3K)/Akt mediates the insulin-inhibited expression of AdipoR1 in skeletal muscle (28, 47). Upon binding to its cell surface receptor, tyrosine kinase IGF-1, insulin will trigger downstream signaling cascades including PI3K/Akt (47). Akt then regulates gene transcription through inactivation of forkhead box protein O1 (FOXO1). As such, the PI3K/Akt/FOXO1 axis has a central role in energy metabolism and signal transduction of insulin, governing insulin sensitivity (**Fig. 1**). In the study of Cui et al., FOXO1 silencing inhibited AdipoR1 expression and the activation of AMPK, supporting the hypothesis that insulin induces a decrease in skeletal muscle AdipoR1 expression and function in a PI3K/Akt and FOXO1 dependent fashion (24). Moreover, the PI3K/Akt pathway is deregulated in muscle wasting, and hence, in chronic HFrEF (**Fig. 1**). As Akt is chronically activated, FOXO1 becomes inactive leading to decreased levels of AdipoR1. Interestingly, mice deficient in cardiac and skeletal muscle insulin as well as the IGF-1 receptor demonstrated FOXO1 activation, resulting from Akt inactivation, and eventually died from HF within the first month (48, 49). Furthermore, a study by Sun et al. in C_2C_{12} myoblasts revealed an insulin-responsive element region in the AdipoR1 promoter, nuclear inhibitory protein, which is involved in the negative regulation of AdipoR1 by insulin (27). This region also seems to contain a regulatory site for FOXO1, again providing proof that FOXO1 plays a role in insulin mediated AdipoR1 expression. FOXO1 may bind directly to the AdipoR1 receptor and ultimately, suppress AdipoR1. In this regard, over-expression of AdipoR1 will ameliorate insulin sensitivity, mediated by increased phosphorylation and expression of several intermediates in the PI3K/Akt signaling pathway (50).

Altogether, these observations portray an important connection between insulin and AdipoR1. Consequently, elevated insulin levels may result into a decreased expression of AdipoR1, leading to diminished binding of adiponectin, a reduction in PGC-1α and an increase in sphingolipid ceramides, ultimately progressing to adiponectin resistance (**Fig. 1**). This in turn could cause a reduction of the insulin-sensitizing effects of adiponectin, and start a vicious cycle (39). In this regard, the group of Khawaja et al. recently reported the restoration of adiponectin signaling in HFrEF patients who underwent a ventricular assist device

implantation (51). At baseline, these patients had decreased levels of insulin together with increased expression of AdipoR1 and genes involved in FAO metabolism. The authors hypothesized that the improvements in adiponectin resistance were, at least partly, due to diminished insulin resistance (5, 51). On the contrary, Staiger et al. demonstrated a strong positive correlation between AdipoR1 expression and insulin secretion, whereas insulin treatment did not modulate AdipoR1 in myotubes cultures *in vitro* (52). Therefore, the insulin-AdipoR1 interaction is far from being obvious and further research is needed.

OTHER MECHANISMS UNDERLYING ADIPONECTIN RESISTANCE: WHAT ONE SHOULD KEEP IN MIND

The interaction between insulin, AdipoR1 and PGC-1α appears to be critical in the development of adiponectin resistance in HFrEF. However, its etiology may be more complex and multifactorial. Adipose tissue abnormalities, oxidative stress, increased inflammatory mediators, a shift in energy metabolism in the failing heart and deregulated natriuretic peptides might all be implicated as mediating factors in the process of adiponectin resistance in the setting of chronic HFrEF.

Inflammation and oxidative stress

HF patients have raised circulating levels of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, and several chemokines such as IL-8. This increase in inflammatory mediators is accompanied by a corresponding increase in adiponectin levels in chronic HFrEF patients. It has been firmly established that this increase in adiponectin renders protective effects by its anti-inflammatory actions (53). In a study by Khan et al., the decrease in systemic and myocardial inflammation after ventricular assist device implantation in patients with severe HF and reduced ejection fraction went along with a reduction in adiponectin levels (5). *In vitro* experiments have shown that addition of inflammatory stimuli to cultured cardiomyocytes suppresses AdipoR1 and PPARα expression levels (54-56). Furthermore, a significant negative correlation between plasma adiponectin

and plasma oxidized low-density lipoprotein (oxLDL), a marker of oxidative stress, was found in patients with chronic HFrEF (57). In patients with the metabolic syndrome, lower levels of adiponectin were associated with adverse oxidative stress profiles, and thus, negatively correlated with increased production of reactive oxygen species (ROS) and measures of lipid peroxidation (58, 59). Consequently, the increase in adiponectin concentrations in chronic HFrEF could represent a protective compensatory mechanism in an attempt to overcome the pro-inflammatory and oxidative stress conditions that characterize the disease, and as such, may have a role in the initiation and maintenance of adiponectin resistance (60).

Natriuretic peptides

Cardiac secretion of natriuretic peptides has been associated with increased ventricular filling pressure whereas circulating brain natriuretic peptide (BNP) levels correlated with the prognosis of HF (60). In addition, plasma levels of BNP have been positively associated with adiponectin levels in CHD and chronic HFrEF patients (3, 60, 61). Natriuretic peptides promoted adiponectin secretion and synthesis, mainly via their lipid mobilizing effect (53, 61). Both natriuretic peptides atrial natriuretic peptide (ANP) and BNP augmented the production of adiponectin in primary cultured human adipocytes via a cyclic GMP-dependent protein kinase (PKG) signaling pathway. Moreover, administration of recombinant ANP in patients with HFrEF, increased their plasma adiponectin levels (61). Accordingly, natriuretic peptides may add to a state of adiponectin resistance in chronic HFrEF.

Metabolic deregulation

It is often argued that circulatory and skeletal muscle adiponectin levels increase in an attempt to compensate for the shift in energy metabolism in the failing heart (62). Interestingly, mechanical unloading of the failing heart induced the normalization of metabolic deregulation and mitochondrial function, and went along with a decrease in circulating adiponectin (63). For this reason, adiponectin resistance has been put forward as a counter-regulatory response to disturbed metabolic energy metabolism in patients with chronic HFrEF (64).

Endocytosis of AdipoR1

Surface receptor activity can be decreased due to endocytotic uptake and subsequent lysosomal degradation of proteins (65). It has been demonstrated that exposure of high concentrations of adiponectin to hepatocytes, epithelial cells and HeLa cells leads to the internalization of AdipoR1 (66-68). This endocytosis of AdipoR1 is probably clathrin-dependent (66). Blocking of AdipoR1 internalization enhances adiponectin-stimulated phosphorylation of AMPK. Whether the down-regulation of AdipoR1 in chronic HFrEF is the result of receptor-mediated endocytosis needs further research.

Adipose tissue inflammation

Recent studies have questioned the primacy of abnormalities in adipose tissue in chronic HF as a possible mechanism of adiponectin resistance (5, 69). Adipocytes in chronic HF are not only decreased in number, but also much smaller in size compared to adipocytes from healthy subjects. Furthermore, patients with chronic HF develop adipose tissue inflammation with increased macrophage infiltration (5). To date, it is not known if these alterations in adipose tissue contribute to adiponectin resistance in chronic HFrEF.

CONCLUSION

Skeletal muscle energy deficiency and adiponectin resistance are two important features of chronic HFrEF. Emerging clinical and experimental evidence indicates that the initiation of adiponectin resistance is multi-factorial, integrating abnormalities emanating from insulin signaling, mitochondrial biogenesis and ceramide metabolism. Further studies, however, are needed to fully elucidate and understand the mechanism of adiponectin resistance in HFrEF. Hereof, we believe that the use of primary skeletal muscle cells from HFrEF patients is valuable to investigate more precisely by how adiponectin resistance in chronic HFrEF interrelates with muscle wasting. In the end, these new insights may open up new avenues in the treatment of patients with HF.

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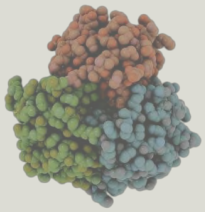


Chapter 3

Aims and outline of the thesis

AIMS OF THE THESIS

The contribution of skeletal muscle myopathy to the phenotype of patients with heart failure (HF) has become generally accepted. Skeletal muscle abnormalities in patients with HF occur already early in the disease process and are marked by qualitative and quantitative changes in biochemical, histological and functional properties. Energetic deprivation has been attributed a key role in the pathophysiological cascade of events. Skeletal muscle energy deficiency and adiponectin resistance are considered instrumental in the pathophysiology of muscle wasting in HF with reduced ejection fraction (HFrEF). The general objectives of this thesis are to study skeletal muscle growth and phenotype, the role of adiponectin and the mechanisms of adiponectin resistance in HFrEF. Our hypothesis is that adiponectin and its receptors represent potential therapeutic targets to combat muscle wasting in HFrEF.

The diagram below illustrates the topics addressed during the course of this thesis and their corresponding chapters.

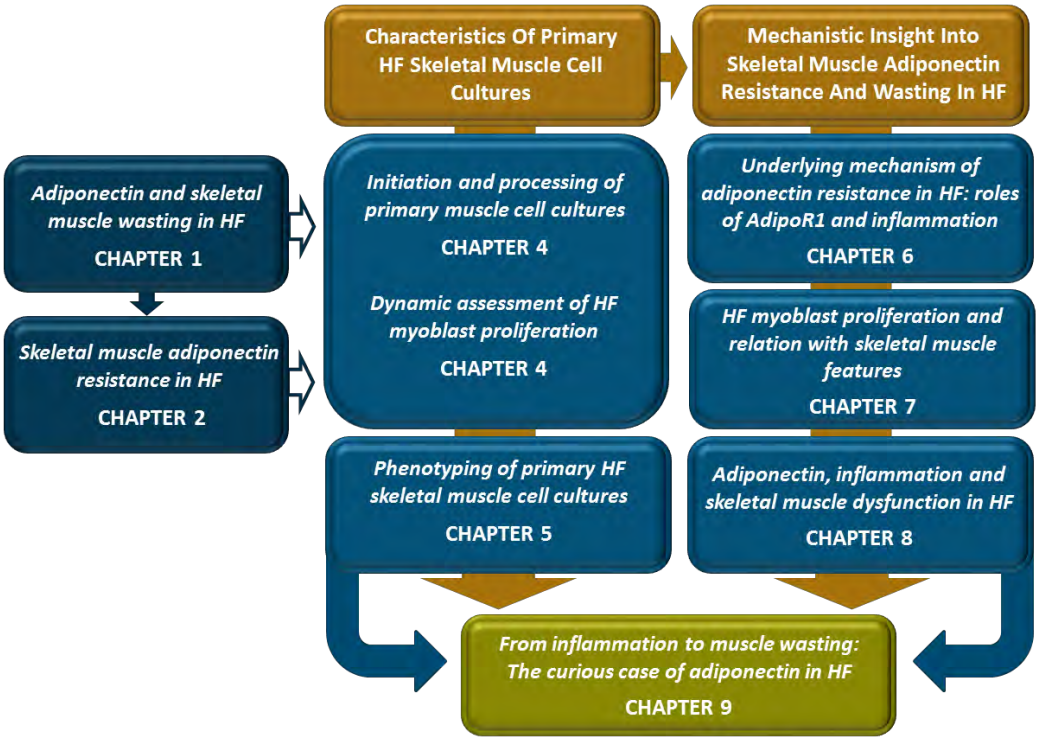


Figure 1. Overview of the objectives outlined in this thesis

OUTLINE OF THE THESIS

Peripheral muscle changes, including impaired muscle energy metabolism, are a common finding in HF. The insulin-sensitizing adipocytokine adiponectin is of particular interest because of its fundamental role in skeletal muscle energy homeostasis. The underlying pathophysiological relevance of adiponectin in HF, and its contribution in the disturbed skeletal muscle metabolism is still unclear. This thesis intends to improve knowledge on the complex role of adiponectin in HF, and especially in HFrEF. In order to be able to explore the differential regulation of adiponectin at the level of the skeletal muscle in patients with HFrEF, we isolated satellite cells and established primary myoblasts and myotubes cultures from human muscle biopsy (*m. vastus lateralis)*. In the first part of this thesis, in **chapters 4 and 5**, we report the development and the characterization of primary HFrEF muscle cell cultures. The second part of this thesis, **chapters 6 to 8**, provides further mechanistic insight into skeletal muscle adiponectin resistance and wasting in HFrEF.

PART I. Characteristics of primary heart failure muscle cell cultures

Skeletal muscle biopsy collection, tissue sample handling, but also the processing and the analysis of primary cultured skeletal myoblasts and myotubes are critical issues. For molecular assessment, the extracted cellular RNA needs to be of sufficient quality to ensure it will not compromise the outcomes of downstream applications, nor distort the expression patterns of genes that are associated with the disease condition. Furthermore, a most accurate, quantitative analysis of muscle cell proliferation is of value in order to support further investigation of biological pathways contributing to muscle wasting in HF. Therefore, in **chapter 4**, we describe the development of a protocol for the cultivation of pure populations of primary skeletal HFrEF myoblasts under standardized conditions. In addition, we report a method for the extraction of high-quality total RNA from primary cultured cells and we evaluate the xCELLigence real-time cellular analysis (RTCA) system as a means to monitor myoblast proliferation in real-time.

In **chapter 5**, we characterize primary skeletal muscle cells from HFrEF origin in relation to healthy donor cell cultures. *In vitro* myoblasts- and myotubes cultures were efficiently isolated and expanded in a controlled environment and analyzed for phenotypic and proliferative properties, which included morphology, myogenic differentiation capacity,

viability and senescence. In such manner, we introduce the primary culture of skeletal muscle cells from individual HF patients as a useful and supportive model to further unravel the driving factors behind muscle loss in this patient population.

PART II: Mechanistic insight into muscle adiponectin resistance and wasting in heart failure

In **chapter 6**, we first examine whether primary cultures from the skeletal muscle of HFrEF patients do preserve the principal characteristics of adiponectin resistance *in vivo*. Since both adiponectin receptor deregulation and inflammation have been implicated in adiponectin resistance and alterations of skeletal muscle in HFrEF, we also evaluate in this chapter the significance of adiponectin receptor 1 (AdipoR1) deficiency and inflammation (TNF-α) on adiponectin signalling, proliferative capacity, myogenic differentiation and mitochondrial biogenesis of primary human skeletal muscle cells.

A major consequence of HF is the reduction in skeletal muscle mass resulting in progressive muscle weakness, degeneration and loss of muscle strength. Weakness of the skeletal muscles is largely dependent on the quantity of skeletal muscle mass and the quality of contractile proteins. Satellite cell dysfunction is considered a major underlying factor contributing to muscle wasting. In **chapter 4** and **chapter 5** we demonstrate that the proliferative capacity of primary myoblasts isolated from patients with HF is impaired. We extent these *in vitro* finding by evaluating whether primary myoblast proliferation relates with *in vivo* parameters of aerobic capacity and skeletal muscle mass and strength. The results of this study are presented in **chapter 7**.

The results obtained from the different studies and the corresponding assumptions were evaluated in a clinical study. We assessed the relationship between inflammation (systemic and local), adiponectin concentrations (circulating and skeletal muscle) and *in vivo* parameters of exercise capacity and quadriceps mass, strength and quality of HFrEF patients, and these findings are described in **chapter 8**.

A general discussion arising from the obtained results of this thesis and in light of recent literature, as well as future perspectives of ongoing research are provided in **chapter 9**.

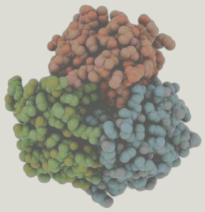
A summary of all findings from this thesis is available in **chapter 10**.

ORGANISZATION OF THE STUDIES

HFrEF patients were recruited from the Cardiac Rehabilitation Centre and the Heart Failure Clinic of the Antwerp University Hospital (head: Prof Dr C Vrints). Inclusion of patients took place throughout the entire time span of the different research projects starting in October 2012 until December 2015 (**Chapters 4 to 7**). Healthy control subjects were gathered from family members, acquaintances and by poster advertisement on a voluntary, non-profit base. They were enrolled between 08/2011 and 09/2011 and between 04/2013 and 07/2013 (**Chapters 4 to 7**). For the clinical study (**chapter 8**), HFrEF patients were included during different time periods, starting from January 2008 until December 2015, with ongoing recruitment. All the studies comply with the principles outlined in the Declaration of Helsinki and were approved by the local Ethics Committee of the Antwerp University Hospital (committee for medical ethics Antwerp University Hospital - University of Antwerp). Written informed consent was obtained from all patient and control participants prior to enrolment.

Clinical examination, blood sampling, anthropometric measurements, cardiopulmonary exercise testing (CPET) and skeletal muscle biopsy procedures were performed at the Cardiac Rehabilitation Centre of the Antwerp University Hospital. Routine biochemistry tests were done at the Laboratory of Clinical Biology; Dual-energy X-ray absorptiometry (DEXA) and computed tomography (CT) scans were conducted in cooperation with the Department of Nuclear Medicine (Antwerp University Hospital).

Laboratory tests were performed at the Laboratory of Cellular and Molecular Cardiology (flow cytometry, cell cultures, gene silencing experiments) and the Laboratory of Experimental Hematology (molecular testing), which are both part of the Centre for Cellular and Regenerative Therapy (CCRG; Antwerp University Hospital and University of Antwerp) as well as at the Laboratory of Clinical Biology (Antwerp University Hospital). Immunocytochemistry, immunohistochemistry and western blotting were carried out at the Laboratory of Pharmacology (University of Antwerp). xCELLigence measurements were performed at the Centre for Oncological Research (CORE; University of Antwerp). Meso-scale discovery experiments were conducted at the Flemish Institute for Technological Research (VITO).



Chapter 4

Initiation, processing and proliferation of primary skeletal muscle cell cultures

ABSTRACT

Skeletal muscle wasting is a common finding with adverse effects in heart failure (HF). Skeletal muscle biopsies, and especially primary cultures of skeletal muscle myoblasts, are important supportive tools to study myopathology in HF. The processing of myoblasts from primary cultures using molecular methods, however, is a critical issue. The extracted cellular RNA needs to be of sufficient quality to ensure it will not compromise the outcomes of downstream applications. In addition, accurate measurement of myoblast proliferation is helpful in investigating the biological pathways contributing to muscle wasting in HF. We optimized a myoblast primary culture from the skeletal muscle of HF patients with reduced ejection fraction (HFrEF). In addition, we created a procedure for isolating high-quality total RNA from primary myoblasts. For this purpose, phenol-based approaches with and without subsequent RNA cleanup were evaluated. Furthermore, dynamic label-free, high-resolution assessments of myoblast proliferation were assessed using an xCELLigence real-time cellular analysis (RTCA) system and compared with a conventional 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay.

Keywords

Skeletal muscle biopsy, primary muscle cell culture, RNA isolation, myoblast proliferation, xCELLigence

I. INITIATION OF PRIMARY SKELETAL MUSCLE CELL CULTURES FROM PATIENTS WITH HEART FAILURE

Scientific background

In chronic heart failure (HF), patients develop central dysfunctions, but also systemic illness such as generalized peripheral muscle abnormalities (**chapter 1)**. Important clinical manifestations in HF patients with reduced ejection fraction (HFrEF), including reduced exercise capacity and poor quality of life, have been related to changes in skeletal muscle structure and function (1). Peripheral muscle wasting is commonly associated with chronic HFrEF and confers poor prognosis (2). Accordingly, in recent years myopathology has become a main topic of research and clinical interest to cardiologists and many other physicians. An important supportive tool to study myopathology is skeletal muscle biopsy, and more in specific, skeletal muscle cell culture. As shown in the timeline, the generation of immortalized muscle cell lines (e.g. C_2C_{12} mouse myoblasts) began more than four decades ago (**Fig. 1**). Interest in the use of primary human muscle cell cultures grew after it became apparent that in a uniform cell culture environment and by using standard culture conditions, an underlying defect in skeletal muscle could be retained *in vitro* (3). The *in vitro* culture of primary skeletal muscle myoblasts therefore may provide important experimental opportunities replacing or complementing those in the human skeletal muscular system.

At the start of this thesis in 2012, we established for the first time primary cell cultures from the skeletal muscle of patients with HFrEF. In **chapters 4 to 7**, we used these primary HFrEF muscle cell cultures to study skeletal muscle energy deficiency, including skeletal muscle adiponectin resistance and muscle wasting.

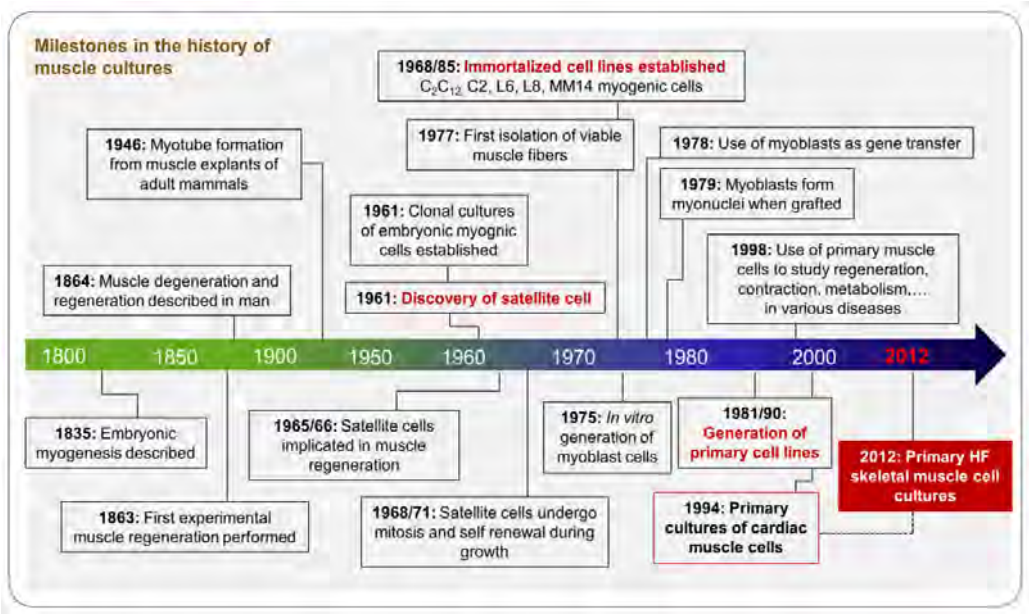


Figure 1. Timeline of events in the history of muscle cells

METHODS

Subjects

HFrEF patients included in the different studies were recruited from the Cardiac Rehabilitation Centre and the Heart Failure Clinic of the Antwerp University Hospital (UZA). In addition to the in- and exclusion criteria described in each **chapter**, special attention was paid to possible metabolic interference and muscle alterations. As such, patients with acute or chronic infections, allergies, cancer, inflammatory diseases, diabetes mellitus treated with thiazolidinediones (TZD) or fibrates, renal failure and musculoskeletal abnormalities were excluded. The control groups consisted of healthy subjects of similar age and gender, with a normal lipid profile, free of medication intake and without any significant medical history. In order to obtain a control group with activity levels that matched the reduced physical activity level of the heart failure population, only sedentary healthy subjects were included. All subjects gave written informed consent before participation. The study was conducted in accordance with the Declaration of Helsinki and ethical approval was obtained from the Local Ethics Committee (UZA - University of Antwerp).

Skeletal muscle biopsy collection

Muscle biopsy samples were taken from the distal *musculus vastus lateralis* under aseptic conditions and local anesthesia (Xylocaine 2%; AstraZeneca) using the Bergström needle muscle biopsy technique (4). The thigh was shaved and disinfected. Through a small superficial (0,5cm) skin incision 15 cm above the patella, muscle surface was exposed to allow the introduction of a Bergstrom's needle with vacuum assist. Vacuum was applied and a small muscle specimen was collected (**Fig. 2**). Muscle specimens were cleaned to remove any visible macroscopic fat residues, fibrous tissue and excess blood. Biopsies were either fixed with formaldehyde and subsequently embedded in paraffin or frozen in liquid nitrogen and stored at -80°C. A small part of the biopsy sample was captured in ice-cold dulbecco's phosphate buffered saline (PBS-D, Sigma-aldrich), transported immediately to the laboratory and processed for cell culture.

Primary skeletal muscle cell culture

Each biopsy sample was minced in ice cold PBS-D and enzymatically dissociated by a series of incubations in 0.05% trypsin/EDTA/collagenase. Satellite cell populations were collected after centrifugation (1300g) and transferred in Dulbecco's Modified Eagle Medium (DMEM, Lonza) with FCS. The resulting suspension of muscle satellite cells was suspended in skeletal muscle growth medium (SKGM; Lonza) supplemented with fetal calf serum (FCS, 15%), insulin (5ml), recombinant human epidermal growth factor (rhEGF, 0.5ml), bovine serum albumin (BSA, 5ml), fetuin bovine (5ml), dexamethasone (0.5ml) and penicillin/streptomycin and subsequently cultivated in collagen-coated culture flasks and incubated at 37°C. Then, pre-plating was used as a purification technique. Growth medium was refreshed every two days until cells reached about 70% confluence. Cultures were expanded for three passages.

Immunohistochemistry

Myogenic purity was tested by immunohistochemical staining using an antibody specific for desmin. Briefly, myoblasts were plated in collagen-coated glass chamber slides at a cell density of 5 x 10^3 cells and allowed to adhere for 24 h. Cultures were fixed in methanol and processed for immunohistochemistry using the primary monoclonal mouse anti-desmin antibody (1:100, Sigma-aldrich,). The secondary antibody used was biotinylated horse radish peroxidase (HRP) anti-mouse (1:200, Vector Laboratories). Before incubation, slides were blocked in horse serum to prevent non-specific staining. Nuclei were counterstained with

hematoxylin eosin. Negative controls consisted of human umbilical vein endothelial cells (HUVEC) and were exposed to all conditions except the primary antibody, were they were incubated with normal rabbit serum for the appropriate period of time. In addition, fusion index was calculated as the average number of nuclei belonging to desmin-positive cells containing at least three nuclei above the total nuclei (5). Images were analyzed using a phase contrast light microscope (Olympus BX40, Olympus Optical co) with a 3CCD color video camera (Sony corporation). Image acquisition was achieved using Universal Grab 6.1 software.

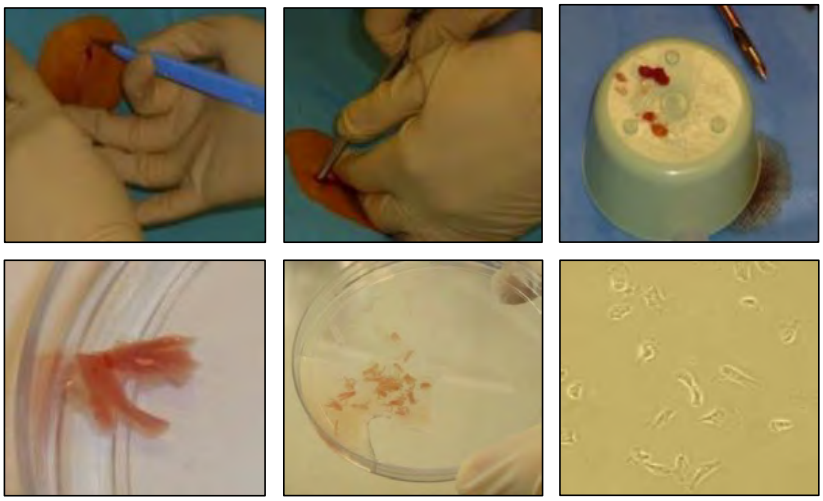


Figure 2. Isolation and cultivation of human primary muscle cell cultures

Muscle biopsy sample is obtained from the *m. vastus lateralis* using the Bergstrom needle technique. Muscle specimen is dissected free of any blood, connective tissue and fat residues and minced finely into small pieces. The isolated satellite cells will proliferate *in vitro* giving rise to myoblasts.

RESULTS AND DISCUSSION

Characterization and purity of myoblast cultures

It has been shown that myoblast purity deteriorates as the number of passages increases and therefore, staining was carried out on myoblast cultures at passage 4, which are used for all further experiments outlined in the different **chapters** (6). Myoblast cultures of both HFrEF patients and healthy subjects were >90% desmin-positive, confirming the purity and specificity of each culture (**Fig. 3A, 3B**). Only a minor percentage of fibroblasts (<10%;⬆) was noticed. Negative controls displayed absence of signal (**Fig. 3C**). In addition, we

observed no difference in fusion index between healthy and HF myoblast cultures (3.33 ± 0.67% and 2.33 ± 0.33%, respectively). Moreover, 88.33 ± 2.04% myoblasts derived from healthy subjects and 90.00 ± 2.65% myoblasts from HFrEF patients were mononuclear, suggesting that both myoblast cultures were in the same initial myoblast stage.

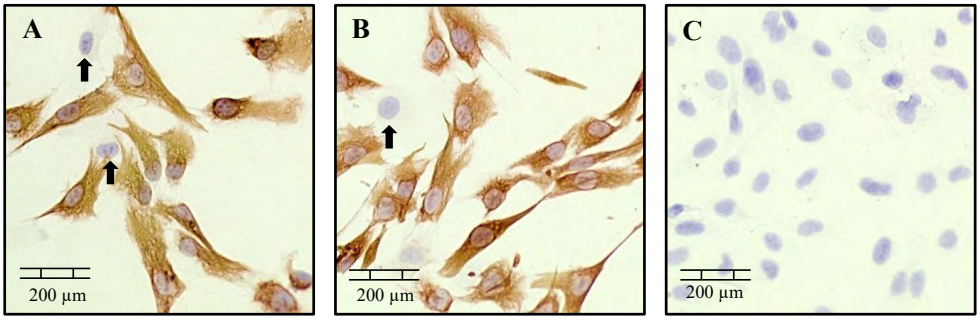


Figure 3. Assessment of skeletal muscle myoblast purity

Immunohistochemical staining of desmin/hematoxylin eosin within myoblast cultures obtained from HFrEF patients and healthy controls. Desmin-positive myoblasts (red) were surrounded by some desmin-negative fibroblasts (blue ⬆). **A**: myoblasts from a healthy subject; **B**: myoblasts derived from a HFrEF patient; **C**: HUVEC cells serving as a negative control. Magnification of 20x. Scale bar = 200µm.

It can be concluded that staining for desmin is essential to control for the purity, specificity and stage of myoblast cultures.

II. PROCESSING OF PRIMARY CULTURED SKELETAL MYOBLASTS: OPTIMIZED METHOD FOR RNA EXTRACTION

Scientific background

Primary myoblast cultures have been successfully established from skeletal muscle biopsies of healthy individuals as well as of patients with amyotrophic lateral sclerosis and duchenne muscular dystrophy (7, 8). At present, cell culture experiments have been studied using a variety of methods including enzyme histochemistry, immunocytochemistry and electron microscopy. The analysis of myoblasts from primary cultures using molecular methods, however, is a critical issue. Skeletal muscle biopsy collection, sample handling and cell culture processing may all affect RNA quality and consequently, distort the expression patterns of genes that are associated with the disease condition. Therefore, the rigorous assessment of RNA purity and integrity is essential before using the RNA samples in downstream applications. (9).

We generated an efficient protocol for high-quality total RNA extraction from primary skeletal myoblast cultures. Two phenol-based methods, that are PureZOL^{TM} and Qiazol along with RNA cleanup based on silica membrane technology, were employed for total RNA extraction. Various RNA quality parameters were determined using a NanoDrop 1000^{TM} spectrophotometer (Isogen Life Science) and an Agilent 2100 Bioanalyzer (Agilent Technologies), i.e. RNA integrity number (RIN) and 28S/18S rRNA ratio to assess RNA integrity, the A_{260}/A_{280} ratio to estimate protein contamination and the A_{260}/A_{230} ratio to demonstrate residual chemical contamination.

METHODS

Extraction of total RNA and quality assessment

Prior to total RNA extraction, growth medium was removed and myoblasts were washed with PBS-D. Subsequently, myoblast were harvested (0.05% trypsin/PBS-D) and transferred into PureZOL^{TM} RNA Isolation Reagent (Invitrogen). Total RNA was isolated from each primary myoblast culture in two different ways using either the PureZOL^{TM} Isolation Reagent technique (Bio-rad Laboratories, Nazareth, Belgium) whether or not followed by purification on a Qiagen spin column (RNA Cleanup kit, Qiagen), or the RNeasy Mini technique including QIAshredders (Qiagen) to homogenize cell lysates and RNA cleanup (RNA

Cleanup kit, Qiagen). All steps were performed according to the manufacturer's instructions. In addition, to remove contaminating genomic DNA, we performed an on-column DNAse treatment (DNase I treatment, Qiagen) of RNA samples in both protocols. The final total RNA fraction was obtained by eluting the sample in a final volume of 30 µl RNAse-free water (Qiagen). RNA concentration and purity (A_{260}/A_{280} and A_{260}/A_{230}) were estimated by ultraviolet absorbance using a NanoDrop 1000^{TM} spectrophotometer. The ratio of 28S/18S rRNA and RIN value were determined by microcapillary electrophoresis using an Agilent 2100 Bioanalyzer. RIN ranges samples from completely degraded (RIN 1) to intact RNA (RIN 10).

Statistics

Statistical significance of results was determined by the Mann-Whitney U test using the software statistical package for the social sciences (SPSS, IBM SPSS Statistics Inc, Version 20.0). A p-value < 0.05 was considered as statistically significant. All data are represented as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

We found profound differences concerning RNA quality and quantity between the two RNA extraction methods. RNA isolation using the PureZOL^{TM} RNA Isolation Reagent technique yielded rather low quality RNA. Neither the ratio of the 28S to 18S ribosomal units nor the RIN value could be assigned due to the presence of critical anomalies in the Inter and Fast regions of the sample electropherograms (**Fig. 4A**). In addition, the fluorescence intensity of the 28S rRNA was weak relative to that of the 18S rRNA. Such anomalous profiles may arise from contaminants remaining within the eluted RNA. Indeed, purity measurements on the NanoDrop revealed a low A_{260}/A_{230} ratio of 0.83 ± 0.13, pointing at residual chemical contamination from the extraction procedure. In contrast, the A_{260}/A_{280} ratio reached a value of 1.80 ± 0.09, which is in the ideal range of 1.7 to 2.0 indicating that there is no detectable protein contamination. The presence of chemical contaminants in RNA samples can interfere with downstream applications such as reverse transcriptase-polymerase chain reaction (RT-PCR), lowering their efficiency (10). Therefore, RNA purification needed further optimization.

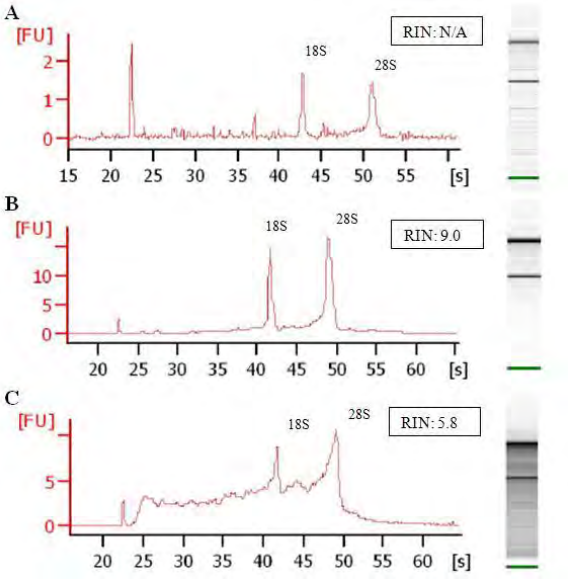


Figure 4. RNA integrity using an Agilent 2100 Bioanalyzer

Representative electropherograms and gel-like images of total RNA recovered from human primary cultured skeletal myoblasts using the PureZOL™ RNA Isolation Reagent technique **(A)**, the PureZOL™ RNA Isolation Reagent technique along with silica membrane technology for RNA cleanup **(B)** and the RNeasy Mini technique along with silica membrane technology for RNA cleanup **(C)**.

RNA extraction using the PureZOL™ RNA Isolation Reagent technique immediately followed by RNA cleanup with Qiagen silica membrane technology did provide pure and good quality RNA. RNA samples demonstrated a RIN value of 9.1 ± 0.21, an A_{260}/A_{280} ratio of 1.98 ± 0.03 and an improved A_{260}/A_{230} ratio of 1.13 ± 0.14. In addition, sharp peaks representing 28S and 18S rRNA with a ratio of 1.77 ± 0.12 confirmed successful extraction of high-integrity RNA (**Fig. 4B**). Hence, RNA cleanup had clearly increased sample quality. Moreover, the isolation of intact, high-quality RNA was not at the expense of a low RNA concentration (**Table 1**; p= 0.700 for PureZOL™ versus PureZOL™ plus RNA cleanup). RNA purification using the RNeasy Mini procedure, including Qiagen silica membrane technology for RNA cleanup, also resulted into pure RNA isolates with an A_{260}/A_{280} ratio of 2.01 ± 0.07 and an A_{260}/A_{230} ratio of 1.85 ± 0.06. In contrast, RIN values ranged from 5.0 to 6.0, whereas the 28S/18S rRNA ratios varied from 1.4 to 2.8 (**Table 1**). In addition, gel-like images displayed a smeared appearance whereas no well-defined distinct, sharp 18S and 28S rRNA peaks could be delineated in the electropherograms (**Fig. 4C**). Consequently, in our hands, myoblast samples were more susceptible to RNA degradation during the RNeasy Mini isolation procedure. Yet, the RNeasy Mini method enabled a significant increase in total RNA

yield (**Table 1**; p=0.048 for RNeasy Mini plus RNA cleanup versus PureZOL™ plus RNA cleanup). Finally, freeze/thawing of isolated RNA did not adversely affect the quality as is shown in **figure 5**.

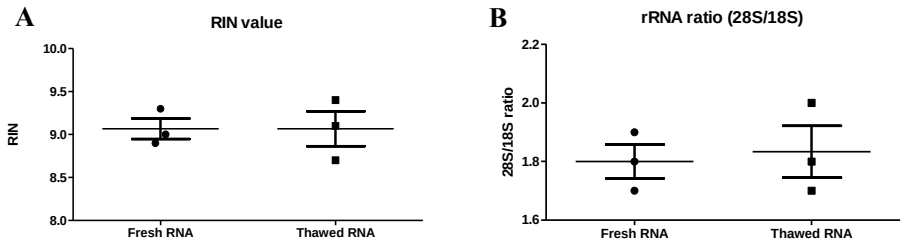


Figure 5. Impact of freeze/thawing on RNA quality

Using the PureZOL™ plus RNA cleanup method, a single freeze/thaw process had no significant effect on **(A)** RIN value (p= 1.000), and **(B)** 28S/18S rRNA ratio (p= 0.500). N=5 skeletal muscle myoblast cultures.

Table 1. Purity, integrity and yield of total RNA recovered from primary cultured skeletal myoblasts according to extraction method.

	Purity		**Integrity**		**Yield (ng/µl)**
	A_{260}/A_{280}	**A_{260}/A_{230}**	**28S/18S ratio**	**RIN**	
PureZOL™	1.80 ± 0.09	0.83 ± 0.13	N/A	N/A	132.2 ± 47.71
RNeasy Mini + RNA cleanup	2.01 ± 0.07	1.85 ± 0.06	2.27 ± 0.76	5.60 ± 0.53	344.57 ± 55.2
PureZOL™ + RNA cleanup	1.98 ± 0.03	1.13 ± 0.14	1.77 ± 0.12	9.1 ± 0.21	115.67 ± 19.6

The table summarizes RNA purity, integrity and yield of RNA samples isolated from human primary skeletal muscle myoblast cultures from five healthy subjects. RNA extraction based on PureZOLTM and silica membrane technology can be considered an efficient extraction method regarding high-quality total RNA from human skeletal myoblasts cultures. N=5 skeletal muscle myoblast cultures. *RIN: RNA integrity number.* Data are expressed as mean ± SEM.

In conclusion, the RNeasy Mini method, including NucleoSpin RNA cleanup, generated pure RNA samples, but these were prone to degradation. RNA extraction based on PureZOL™ and silica membrane technology proved to be an efficient method for the recovery of sufficient amounts of high-quality intact total RNA from primary cultures of human myoblasts derived from skeletal muscle biopsies.

III. DYNAMIC ASSESSMENT OF HEART FAILURE MYOBLAST PROLIFERATION USING AN IMPEDANCE-BASED METHOD

Scientific background

Accurate monitoring of myoblast proliferation is still challenging, yet critical to unravel and subsequently modulate the biological processes of muscle wasting. In the past, several methods have been developed to measure the proliferative potential of myoblasts. These approaches include direct cell counting, flow cytometric measurement of cellular DNA content and colorimetric testing of metabolic activity. A well-established technique to evaluate myoblast proliferation is the colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay. This assay was developed as a non-radioactive alternative technique to tritiated thymidine incorporation into DNA. Viable, proliferating cells with active metabolism convert MTT, a yellow tetrazolium salt, into a purple colored formazan product by intracellular oxidoreductase enzymes (11). In addition, MTT reduction occurs extramitochondrially and involves the pyridine nucleotide cofactor NAD(P)H (11, 12). Cellular proliferation and viability are measured indirectly according to the change in absorbance (13). Although being sensitive and widely accepted, this method has some limitations (14). The assay is labor intensive and requires a large number of manipulation steps, and this may increase the within-subject variability in results. In addition, the signal intensity is affected by several parameters including the concentration of MTT and the length of the incubation period. A final major drawback of the MTT assay is that it can only be used to make a single time point evaluation. The cytotoxic nature of the assay also limits the end-point to ≤ 4-16h.

As an alternative non-invasive and label-free approach, the impedance-based xCELLigence RTCA detection platform enables dynamic, real-time monitoring of cellular responses, including cell proliferation (15). The technique provides continuous access to information about cell growth with a high temporal and spatial reliability, thereby increasing the amount and quality of acquired data (16). We evaluated the xCELLigence RTCA as a method to monitor myoblast proliferation in real-time. Therefore, we compared the results from the conventional MTT assay with parallel continuous data recorded by the xCELLigence RTCA technology. By doing so, xCELLigence may emerge as a novel means for the study of HFrEF myoblast proliferation.

METHODS

xCELLigence cell proliferation assay

Proliferation of myoblasts was monitored using the xCELLigence RTCA Dual Plate (DP) system (Westburg). For this purpose, HFrEF myoblasts were seeded in duplicate in special modified microtiter 16-well plates (E plates). Each well bottom was covered with interdigitated gold micro-electrodes in order to detect cell-to-electrode impedance responses. These responses reflect the biological status of myoblasts, including cell number. E-plates were placed into the RTCA DP device station, located in a humidified incubator at 37°C and 5% CO_2. The RTCA DP device station, connected to an outside electronic analyzer, continuously transfers the measured impedance data to a computer. Background impedance was determined using 50µl SKGM medium and subsequently, 100µl of myoblast suspension was added. At start, optimal conditions were determined for cell concentration. Therefore, different myoblast cell numbers (625–2x10^4 cells/well) were seeded in the E-Plate. Impedance was monitored with a programmed signal detection schedule in 15-minute intervals for a total of 120 hours. The impedance value of each well was expressed as a cell index (CI) value, calculated as the cell index at a given time point subtracted by the cell index at baseline (SKGM medium alone). Two replicates of each myoblast culture were monitored and averaged for statistical analysis.

MTT assay

Myoblasts were seeded into 96-well plates and grown for 24 hours in a humidified 5% CO_2 atmosphere at 37°C. After 48, 72 and 96 hours, myoblasts were incubated with 0.5 mg/ml MTT for 4 hours at 37°C. The medium was removed and 150 µl of DMSO was added. Optical density (OD) was measured at 590 nm wavelength in a microplate reader (Biorad 550 microplate reader). All cultures were assayed in triplicate and averaged for statistical analysis.

Statistics

Statistical significance of results was determined by the Mann-Whitney *U* test. The high-resolution data of the xCELLigence RTCA system were extrapolated to the matching data points of the MTT assay. The agreement between xCELLigence-generated values and MTT values was calculated according to the Spearman's rank correlation method (Rho, ρ). Intra- and inter-assay coefficient of variance (CV) were calculated for each method separately to

evaluate assay reproducibility. Data was analyzed using the software statistical package for the social sciences (SPSS, IBM SPSS Statistics Inc, Version 20.0). A p-value <0.05 was considered as statistically significant. All data are represented as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

HFrEF patients are characterized by progressive skeletal muscle weakness, wasting and degeneration. Human primary myoblasts isolated from muscle biopsies derived from HFrEF patients provide a pertinent model to investigate pathological mechanisms associated with skeletal muscle wasting. We report an *in vitro* assay to evaluate myoblast proliferation in real-time by means of the xCELLigence RTCA system.

The xCELLigence RTCA proliferation assay delivered a continuous growth curve (**Fig. 6**). The first portion of the growth curve, the cell attaching stage (I), was characterized by a rapid increase in cell impedance. In the second lag phase (II), HFrEF myoblast cells did not yet proliferate, while in the third phase (log phase or exponential phase, III) HFrEF myoblast proliferation was observed. Different concentrations of HFrEF myoblast cells were tested (i.e.; 625-2x10^4 cells/well). As we expected, the CI increased depending on the number of HFrEF myoblasts that attached on the electrodes at the bottom of the well and the time at which the HFrEF myoblasts entered the logarithmic growth phase. Moreover, electrode impedance depends on the number of cells in the well. As such, the xCELLigence system allows for robust assessments of myoblast behavior in real-time. The CI's of the HFrEF myoblast populations with the two highest initial cell numbers (i.e.; 1x10^4 and 2x10^4 cells/well at start) showed a constantly increasing CI over approximately 100 hours. Then, the CI began to decrease, suggesting that these HFrEF myoblasts cultures had reached confluence and that cells started to die due to contact inhibition. Other HFrEF myoblast populations (i.e.; 625-5000 cells/well) continued to proliferate for up to 120h. The slowed growth of these cultures may well be attributed to the lower starting cell numbers, which will have utterly delayed the achievement of saturation density and the resulting contact inhibition. Since the HFrEF myoblast population with 1x10^4 cells/well at start reached its highest level of CI around 96h without entering a confluent phase, we considered this population as the optimal cell density for the study of HFrEF myoblast proliferation. We used this cell population in the subsequent experiments for comparison of xCELLigence RTCA with MTT assay results.

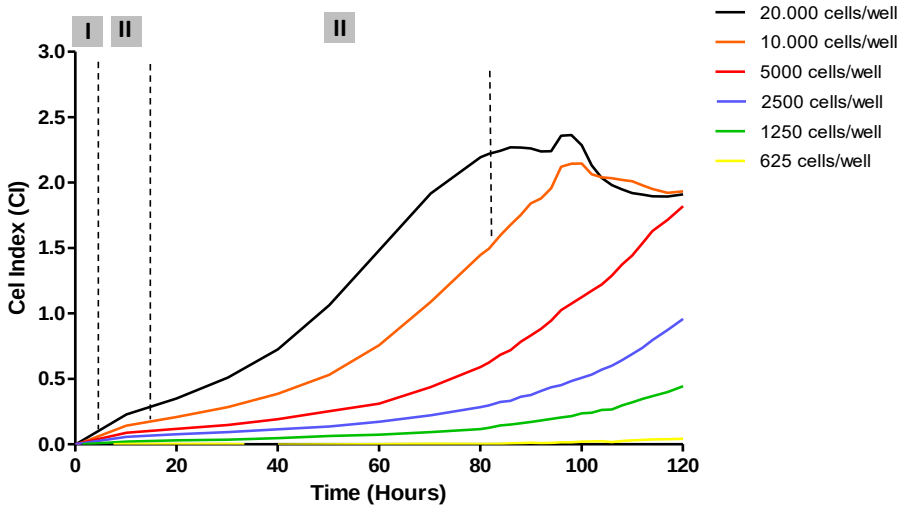


Figure 6. Dynamic monitoring of the impedance-based proliferation of HFrEF myoblasts

HFrEF myoblast cells were seeded at different cell densities (625 - 20 000 cells/well) and growth curves were continuously monitored for up to 120 hours and automatically recorded using the impedance-based xCELLigence RTCA DP System.

To date, measuring myoblast proliferation using the xCELLigence RTCA system has only been described by Will et al. in pig myoblasts (17). This is the first study evaluating the proliferation of myoblast cultures obtained from HFrEF patients simultaneously by means of the xCelligence RTCA technology and the MTT assay. Cell culture conditions and media used for the RTCA system were similar to that applied for the MTT counterpart experiments. A strong linear relationship was noticed, indicating a highly correlative nature between the xCELLigence RTCA system and the MTT assay (ρ=0.921). Moreover, no significant differences between CI (xCelligence) and OD (MTT) were observed when HFrEF myoblast proliferation was followed for up to 96 hours (**Fig. 7**; $p>0.05$). Our results are consistent with other studies showing a high correlation between the MTT results and the CI values of the impedance-based RTCA system (18, 19). Smaller intra- and inter assay variations were indicated on xCELLigence RTCA. However, variance component analysis resulting from both techniques revealed CVs below 15%. Intra- and inter assay CVs were respectively, 4.8% and 7.8% for xCELLigence and 6.6% and 8.9 % for MTT.

In conclusion, these experiments point out the xCELLigence‘s potential as an alternative to the MTT assay for HFrEF myoblast proliferation *in vitro*, supporting the studying of skeletal muscle wasting in HFrEF patients.

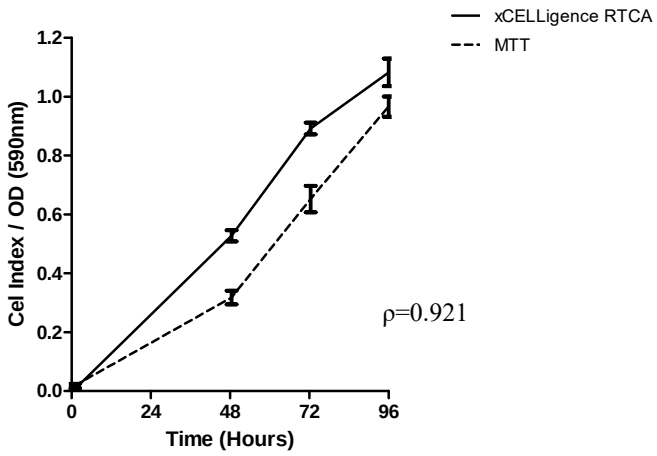


Figure 7. Time-dependent proliferation profiles of HFrEF myoblasts

Growth curves of HFrEF myoblasts (n=8) were determined using the xCELLigence RTCA DP system (CI; full line) or the MTT assay (OD; dotted line). Error bars represent the SEM of five independent experiments.

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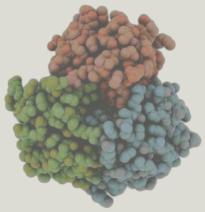

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Chapter 5

Phenotyping of primary heart failure skeletal muscle cell cultures

Adapted from:

Sente T, Van Berendoncks AM, Jonckheere AI, Rodenburg RJ, Lauwers P, Van Hoof V, Wouters A, Lardon F, Hoymans VY, Vrints CJ. Primary skeletal muscle myoblasts from chronic heart failure patients exhibit loss of anti-inflammatory and proliferative activity. BMC Cardiovasc Disord. May 2016.

ABSTRACT

Background: Peripheral skeletal muscle wasting is a common finding with adverse effects in chronic heart failure (HF). Whereas its clinical relevance is beyond doubt, the underlying pathophysiological mechanisms are not yet fully elucidated. We aimed to introduce and characterize the primary culture of skeletal muscle cells from individual HF patients as a supportive model to study this muscle loss. **Methods and results:** Primary myoblast and myotubes cultures were successfully propagated from the *m. vastus lateralis* of 6 HF patients with reduced ejection fraction (HFrEF; LVEF <45%) and 6 age and gender-matched healthy donors. HFrEF cultures were not different from healthy donors in terms of morphology, such as myoblast size, shape and actin microfilament. Differentiation and fusion indexes were identical between groups. Myoblast proliferation in logarithmic growth phase, however, was attenuated in the HFrEF group (p=0.032). In addition, HFrEF myoblasts are characterized by a reduced TNFR2 expression and IL-6 secretion (p=0.017 and p=0.016; respectively). **Conclusion:** Biopsy derived primary skeletal muscle myoblasts of HFrEF patients produce similar morphological and myogenic differentiation responses as myoblasts of healthy donors, though demonstrate loss of anti-inflammatory and proliferative activity.

Keywords

Heart failure; muscle wasting; myoblast cultures; xCELLigence

INTRODUCTION

Skeletal muscle wasting is observed in a variety of chronic diseases including chronic heart failure (HF) (1-4). Muscle wasting is present in approximately 70% of chronic HF patients (5-7). The loss of muscle mass during HF has a significant impact on the patients' quality of life and is associated with high morbidity (6). The mechanisms that underlie HF-related skeletal muscle wasting, however, are currently not clear. A number of hypotheses have been put forth to explain the loss of muscle mass, some of which are physiologic, including prolonged immobilization and malnutrition, or pathologic, such as insulin resistance, impaired myogenesis and inflammation. Until now, mechanistic data generated in this domain have been confined to animal experiments, human muscle biopsy specimens, C_2C_{12} mouse myoblasts and L6 myotubes cell lines. Although animal models and immortalized cell lines are convenient sources to study the basic features of muscle cells, they are devoid of typical human traits. Specimens of skeletal muscle tissue have been used mainly to study changes in histologic and (ultra)structural features. In recent years, however, the primary cell culture model has gained wide acceptance (8-10). Studies indicated that human primary skeletal muscle cells adequately retain phenotypic and genotypic traits of the donor, including morphological, metabolic and biochemical similarities, in a controlled *in vitro* environment and hence are a highly relevant means to study skeletal muscle alterations *in vitro* (11-13). As discussed in **chapter 4**, by generating *in vitro* cultures of primary skeletal muscle myoblasts and myotubes from HF patients, we may increase the near future ability of identifying novel mechanisms contributing to loss of skeletal muscle mass in this patient population. In the present study, we aimed to characterize primary cultures of skeletal muscle from HF origin in relation to healthy donor cells.

MATERIAL AND METHODS

Patient population and controls

The study population consisted of eight patients with systolic HF as a result of dilated cardiomyopathy or ischemic heart disease, recruited from the Heart Failure Clinic of the Antwerp University Hospital (UZA, Edegem, Belgium). All patients had a left ventricular ejection fraction (LVEF) of <45% (HF with reduced EF; HFrEF) and were classified as New York Heart Association (NYHA) functional class II-III. Patients were on a stable dose of

HFrEF medication for at least one month prior to enrollment. Exclusion criteria were recent acute coronary syndrome (≤3 months), valvular disease requiring surgery and acute myocarditis or pericarditis. Patients with acute or chronic infections, allergies, cancer, inflammatory diseases, diabetes mellitus treated with thiazolidinediones or fibrates, renal failure and musculoskeletal abnormalities were excluded to avoid possible metabolic interference. Eight subjects matched for age and gender, no medication intake and without any significant medical history were recruited as controls. The study conforms with the principles outlined in the Declaration of Helsinki and was approved by the local Ethics Committee of the Antwerp University Hospital (committee for medical ethics UZA - UAntwerp). All participants provided written informed consent before enrollment.

Myoblast Cell Culture

Biopsy samples were collected of the *musculus vastus lateralis* using the Bergstrom needle technique, as shown in **chapter 4** (14). Muscle specimens were trimmed of connective tissue and fat, minced into ±1mm^3 fragments and enzymatically dissociated by a series of incubations in 0.05% trypsin/EDTA/collagenase. The supernatant of successive dissociations was centrifuged at 230 x g for 5 min. The resulting cell suspension was purified from fibroblasts by a pre-plating step in collagen-coated 25cm^2 culture flasks containing skeletal muscle growth medium (SKGM; Lonza, Allendale, NJ) (15). After 45 min of incubation, the non-adhering cells were transferred into a new collagen-coated flask. Cells were cultured in a humidified 5% CO_2 atmosphere at 37°C and growth medium was refreshed every two days until cells reached 70% confluence. Cultures were tested for myogenicity by immunostaining using the muscle-specific antibody desmin (1:100, D1033, Sigma-Aldrich, St. Louis, MO, USA). Only cultures with a myogenic purity >90% were used for further analysis. All experiments were analyzed blinded and performed at a similar passage 4.

Assessment of myotubes differentiation and fusion index

In order to induce myogenic differentiation of myoblasts, growth medium was replaced by differentiation medium consisting of serum free Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (1:1; Lonza) supplemented with 2% (v/v) horse serum (Gibco, Life Technologies, Gent, Belgium). At day 6, cells were fixed in 4% paraformaldehyde. Myotubes and nuclei were visualized by immunofluorescence using an antibody against desmin and 4',6-diamidino-2-phenylindole (DAPI), respectively (see ''*in vitro* immunocytochemistry

section'' for details). Myotubes were defined as cells containing at least three nuclei within one continuous cell membrane (16). The differentiation index (DI) was calculated as the percentage of desmin-positive cells in relation to the total number of nuclei. In order to quantitatively assess the extent of myoblast fusion, a myogenic fusion index (MFI) was determined as the average number of myogenic nuclei present in multinucleated myotubes. The fusion index was analyzed in duplicate by counting at least 250 nuclei from 10 randomly selected fields of view. The deformed myotubes index (DMI) was evaluated as indicated by Yip et al. and quantified analogously to the MFI (17).

Proliferation kinetics

Myoblast proliferation and viability were continuously monitored using the xCELLigence Real-Time Cellular Analysis (RTCA) system (Westburg, Leusden, The Netherlands), according to the manufacturer's guidelines. Methodological considerations as described in **chapter 4** were taken into account. Briefly, myoblasts were grown in special modified 16-well plates (E-plates) with microelectrodes on the bottom of each well detecting electrical impedance-based attachment, spreading and proliferation of the myoblast cultures. Background impedance (SKGM medium alone) was measured and cells were seeded at a density of 10.000 cells/well in growth medium. After 30 min at room temperature (RT) to allow cell attachment, plates were locked in the RTCA device and electrical impedance was measured and expressed as a dimensionless parameter termed cell index (CI), a relative change in electrical impedance representing cell status (18). The CI was continuously monitored in 15 min intervals with a programmed signal detection schedule for a total time of 172 h. Two replicates of each culture were run.

***In vitro* immunocytochemistry and immunohistochemistry**

Cultures were grown on collagen-coated glass chamber slides at a density of $5x10^3$ cells and allowed to adhere for 24 h. Next, cells were fixed in 4% paraformaldehyde in PBS-D for 20 min at 4°C and permeabilized in 0.1% Triton X-100 in PBS-D for 5 min. Cells were incubated with primary antibody overnight and with secondary antibody for 1 h. The following primary mouse antibodies were used: monoclonal anti-desmin (1:200; Sigma-Aldrich), monoclonal anti-α-actinin (1:100; Sigma-Aldrich) and monoclonal anti-α-tubulin (1:200; Sigma-Aldrich), and combined with either a donkey anti-mouse IgG secondary antibody, Alexa Fluor 546 conjugated (1:800; Molecular probes, Eugene, OR, USA) or a goat anti-mouse IgG secondary

antibody, Alexa Fluor 546 conjugated (1:800; Vector Laboratories, Burlingame, CA, USA). Actin microfilaments were visualized by applying fluorescein isothiocyanate (FITC)-conjugated phalloidin (50µg/mL in methanol; Sigma-Aldrich) for 20 min in the dark. Immunohistochemical detection of the senescence marker acetyl-p53 in muscle biopsies and cell cultures was performed using the primary antibody anti-p53 (Acetyl-Lys317; 1:200; Abcam). Nuclei were counterstained with DAPI. Cells were visualized using an EVOS fluorescent microscope (Westburg, Leusden, The Netherlands). From each sample, fifteen microphotographs were captured and analyzed manually. The ultrastructural morphology was analyzed using the software program Image J (Version 1.45s, National Institutes of Health, USA). All specimens were evaluated by two blinded investigators who were unaware of clinical data and group assignment.

Flow cytometric analysis

Cell cultures were evaluated for muscle specific marker expression using flow cytometry at several time points during myogenesis. Cells were trypsinized and centrifuged at 230 x g for 5 min. The cell pellet was resuspended in permeabilization buffer for 10 min at a concentration of $1x10^5$ cells/ml and incubated with primary antibodies in the dark for 30 min. The following primary antibodies were used: phycoerythrin conjugated mouse anti-human Pax3 (R&D Systems, Minneapolis, MN, USA), phycoerythrin conjugated rabbit anti-human Pax7 (Bioss Inc., Woburn, MA, USA), phycoerythrin conjugated rabbit anti-human MyoD1 (Bioss Inc.), Alexa fluor 488 conjugated mouse anti-human myogenin (R&D Systems) and rabbit phycoerythrin conjugated anti-human myf6 (MRF4) (Bioss Inc.). Gating was implemented based on negative control staining by using mouse IgG1 Alexa 488 conjugated and mouse IgG2A (R&D Systems) phycoerythrin conjugated isotype antibodies (Bioss Inc.). Cell viability was assessed using the dead cell discriminator dye 7-aminoactinomycin (7-AAD) and Annexin V-FITC (Becton Dickinson, Biosciences, Erembodegem, Belgium). Cells were stained with DRAQ5 (BioStatus Limited; Leicestershire, UK) to exclude cellular debris and non-nucleated cells. Cells were analyzed on a FacsCantoTM II flow cytometer (Becton Dickinson). A minimum of 30.000 events was recorded for each analysis. Data analysis was done with FacsDiva 6.1.2 software. Representative graphs of the muscle specific marker expressions are provided in Additional file 1: **Figure S1**.

Senescence-associated β-Galactosidase activity

Senescence-associated beta-galactosidase (SA-β-gal) activity was assessed with the *β*-Galactosidase Staining Kit (Biovision Research Products, Palo Alto, CA, USA). The protocol was performed according to the manufacturer's instructions. In brief, cells were fixed for 10 min at RT and incubated overnight in freshly prepared acidic *β*-gal staining solution containing 5-bromo-4-chloro-3-indolyl *β*-D-galactopyranoside (X-Gal) at 37°C. Senescence was determined by phase contrast (Olympus Optical Co., Tokyo, Japan) under bright field illumination in 10 randomly selected fields as the ratio of SA-*β*-gal positive (blue) cells to the total number of cells. Analyses were performed in triplicate by two independent observers.

Cytokine production

Concentrations of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-10, interferon-γ (IFN-γ), and IL-1β were determined in cell culture supernatant by a multiplex enzyme-linked immunosorbent assay (ELISA) technique based on electrochemiluminescence (Meso Scale Discovery (MSD) technology, Meso Scale Diagnostics, Rockville, MD, USA) and according to the manufacturer's instructions. Plates were read on a SECTOR® Imager 6000 instrument. Data was analyzed using the Discovery Workbench 3.0 software. All standards and samples were measured in duplicate.

RNA isolation and quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted using the Qiazol reagent technique followed by RNA cleanup (RNeasy Mini Kit, Qiagen). 1µg of isolated total RNA was reverse-transcribed using the iScript^{TM} cDNA Synthesis Kit (Bio-rad Laboratories, Nazareth, Belgium). RT-PCR gene-specific forward and reverse primers (Eurofins MWG Operon; Ebersberg, Germany) were designed: TNFR1-F: 'ACC AGG CCG TGA TCT CTA TG', TNFR1-R:'CAG CTA TGG CCT CTC ACT CC', TNFR2-F:'CTC AGG AGC ATG GGG ATA AA'', TNFR2-R:'AGC CAG CCA GTC TGA CAT CT'. PCR amplification with EVAGreen supermix was performed on a CFX96TM Real-Time PCR Detection system (Bio-rad). Gene expression was normalized using the reference genes TATA box binding protein (TBP) and beta-2-microglobulin (B2M). Relative quantification of gene expression levels was performed by analyzing the RT-PCR data using the delta delta Ct ($2^{-\Delta\Delta Ct}$) calculation. All samples were run in duplicate.

Biochemical analyses

Fasted peripheral venous serum was collected from all HFrEF patients and healthy controls. Creatinine, total cholesterol, triglycerides, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol levels, glucose and high sensitivity C-reactive protein (hsCRP) were assessed immediately on Dimension Vista 1500 instruments (Siemens Healthcare Diagnostics NV/SA, Beersel (Huizingen) Belgium) using reagents from Ortho Clinical Diagnostics. Bioelectrical impedance analysis was used for assessment of body composition (Omron body fat monitor BF 300).

Statistical analysis

Experimental triplicates or duplicates were averaged for statistical analysis. Categorical variables were compared with the Pearson's Chi-square (X_2) test. Mann-Whitney U test was used to compare differences between both groups (HFrEF versus Control). Results are presented as mean ± standard error of the mean (SEM). Differences are considered statistically significant if the p-value is less than 0.05 ($P<0.05$). Statistical analyses were performed using SPSS software (IBM SPSS Statistics Inc, Version 20.0, Chicago, IL, USA).

RESULTS

Patient characteristics

The clinical characteristics of the patients and the healthy donors are provided in **table 1**. All HFrEF patients received standard HF treatment: 75% of the patients received a beta-blocker, 62.5% an angiotensin converting enzyme (ACE)-inhibitor, 87.5% were on diuretics, 50% were treated with an angiotensin II receptor antagonist and 75% were on statin therapy. Age, gender and body mass index (BMI) were similar between groups. HFrEF patients had a pro-inflammatory blood profile (hsCRP, p=0.007) and a dyslipidemic state (triglycerides, p=0.003) compared to the control group.

Morphological analysis of HFrEF myoblasts

Myoblasts were successfully initiated from six control subjects and six HFrEF patients (**Fig 1A, 1B**). The percentage of desmin-positive myoblasts in each culture was found to exceed 90% (91.09 ± 1.89%, HFrEF vs. 92.14 ± 1.76%, controls; p=0.608; **table 2**, **Fig. 1A-D**). The percentage of desmin-positivity remained similar between groups during the subsequent

differentiation step (**Table 2**). No significant differences in myoblast size or shape were observed between both groups. Myoblasts presented as small elongated cells to flat slightly rounded cells. There were also few large multipolar cells. Cultures were stained for phalloïdin in order to evaluate the actin microfilament arrangement (19). Stress fibers were also well-organized in myoblasts of HFrEF patients (**Fig. 1E, 1F**).

Table 1 Clinical characteristics of HFrEF patients and controls

Characteristic	**Controls** **n=8**	**HFrEF** **n=8**	***p-value***
Age (Years)	55 ± 1.8	53 ± 4.5	0.939
Gender (% Male)	62.5 %	62.5%	1.000
Weight (Kg)	83.1 ± 6.4	74.6 ± 6.8	0.367
BMI (Kg/m^2)	27.0 ± 1.3	23.3 ± 1.7	0.088
LVEF (%)	N.A.	31.3 ± 6.0	N.A.
Total cholesterol (mmol/L)	5.02 ± 0.18	4.45 ± 0.51	0.469
HDL (mmol/L)	1.53 ± 0.15	1.25 ± 0.24	0.101
LDL (mmol/L)	3.05 ± 0.29	2.52 ± 0.42	0.363
Triglycerides (mmol/L)	1.00 ± 0.09	1.77 ± 0.17	**0.003**
Glucose (mmol/L)	4.75 ± 0.16	5.51 ± 0.39	0.151
Serum Creatinine (mmol/L)	76.91 ± 3.54	103.43 ± 17.68	0.279
hsCRP (mg/L)	<2.9	22.0 ± 11.3	**0.007**

N, Number of subjects; HFrEF, Heart failure with reduced ejection fraction; BMI, Body mass index; LVEF, Left Ventricular Ejection fraction; HDL, High density lipoprotein; LDL, Low density lipoprotein; hsCRP, high sensitivity C-reactive protein. Data are expressed as mean ± SEM. Significant p-values are highlighted in bold.

Differentiation of HFrEF myoblasts

Myotubes differentiation (**Fig. 2**) started at 48-72h after the switch from growth to differentiation medium. Multinucleated myotubes became apparent on a background of mononucleated myoblasts. The DI was slightly decreased in HFrEF myoblast cultures compared to controls (46.57 ± 3.29% versus 55.49 ± 2.67%), although this observation did not reach significance (p=0.065). Both cultures grew to normal differentiated myotubes marked by a very low percentage of deformed myotubes (DMI of 3.10 ± 0.47%, HFrEF; 1.96 ± 0.36%, control, p=0.394). No significant difference in fusion competence was observed between myotubes from HFrEF patients and control subjects (**Fig. 2G**). Similarly, the size of HFrEF and control myotubes was not statistically different. The maturation and sarcomere

assembly were evaluated by means of α-actinin staining and were not different between groups (**Fig. 2A**). The α-tubulin and actin filament networks were also identical in myotubes of HFrEF patients (**Fig. 2D, 2F**) and controls (**Fig. 2C, 2E**).

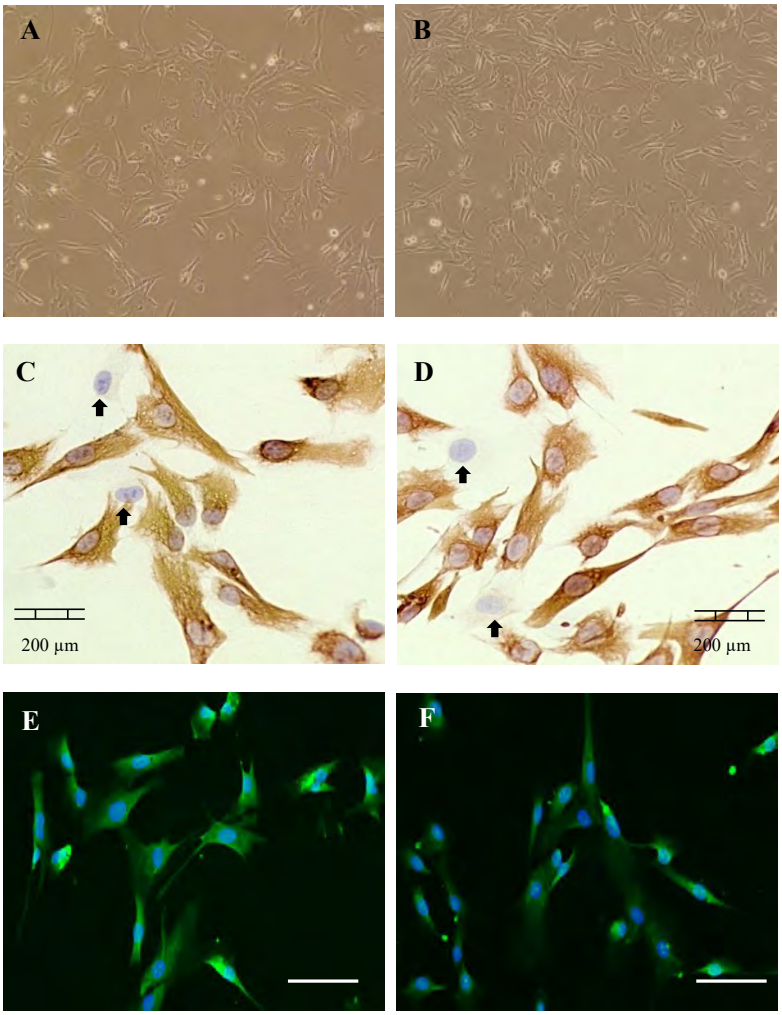

Figure 1. Comparative morphological analysis of HFrEF and control myoblasts

Phase contrast (4X) and immunohistochemical (10X) images of a representative control **(A, C)** and HFrEF **(B, D)** culture. Desmin-positive myoblasts **(red)** are surrounded by few fibroblasts **(blue**, arrows ⬆). Actin microfilament organization using phalloïdin **(green)** and DAPI **(blue)** immunostaining of a representative control **(E)** and HFrEF **(F)** culture, 20X magnification. Scale bar = 200 µm. N=6 HFrEF and 6 control cultures.

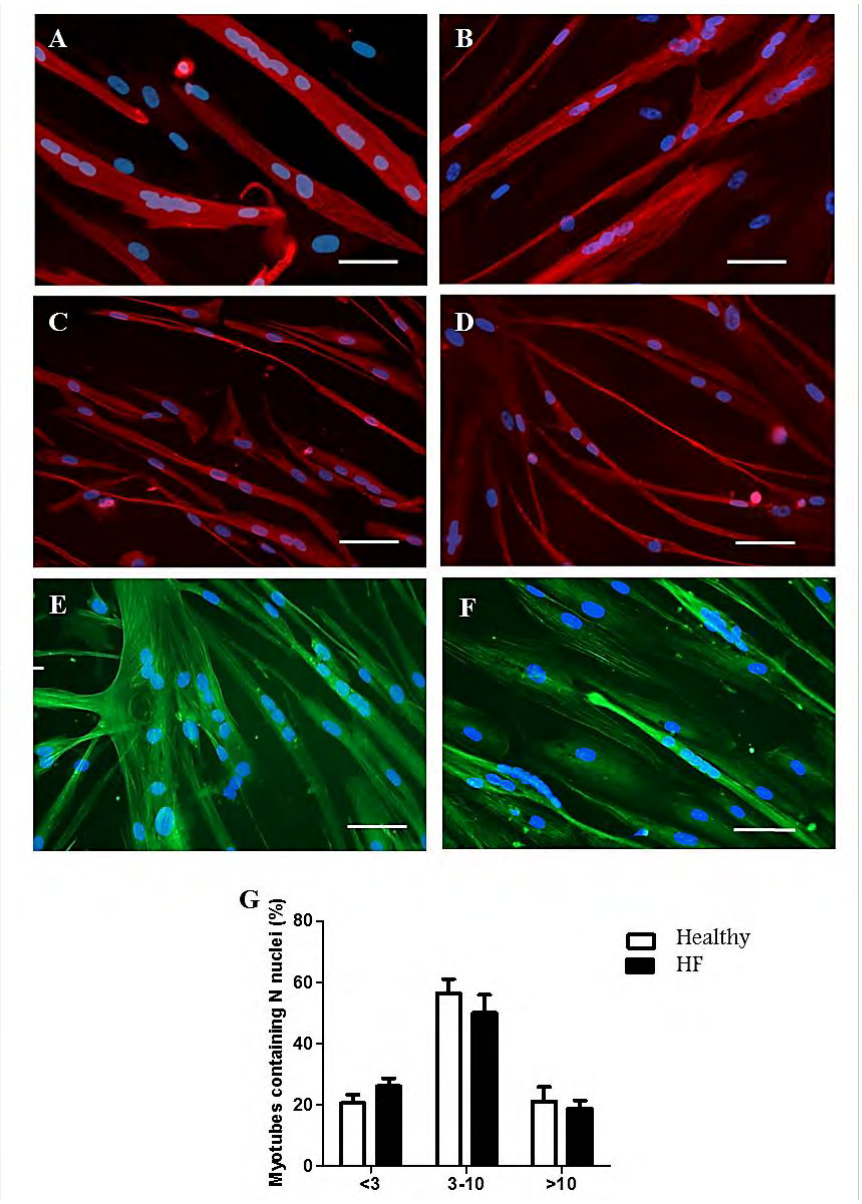

Figure 2. HFrEF and control cultures upon induction of myotubes differentiation

Immunofluorescent images of myogenic cultures from control subjects **(left)** and HFrEF patients **(right)** reacted with antibodies against α-actinin **(A, B)**, α-tubuline **(C,D)** and phalloïdin **(E, F)**. Magnification of 20X. Nuclei were stained with DAPI **(blue)**. Quantification of the number of nuclei (N) present in control and HFrEF myoblast cells **(G)**. Scale bar = 200 µm. Data represent mean ± SEM. (N=6 controls and HFrEF patients).

Quantitative analysis of myoblast viability

The mean numbers of early (Annexin-V^+/7-AAD^-) and late apoptotic cells (Annexine-V^+/7-AAD^+) were not significantly different between skeletal muscle cells from HFrEF patients and control subjects (p=0.352 and p=0.476, respectively; **Table 2**).

Table 2 Myoblast and myotubes characteristics in HFrEF patients versus controls

Characteristic	Controls n=6	HFrEF n=6	*p-value*
Myogenicity (% Desmin positive cells)			
Myoblasts	92.14 ± 1.76	91.09 ± 1.89	0.608
Myotubes	90.80 ± 0.43	92.30 ± 1.19	0.485
Growth Characteristics			
Maximum Cell Index (Time in hours)	114.5 ± 12.05	136.5 ± 5.16	0.214
Cell Index (at 90hours)	1.80 ± 0.35	1.0 ± 0.10	**0.032**
Differentiation Capacity			
Differentiation Index (DI)	55.49 ± 2.67	46.57 ± 3.29	0.065
Deformed Myotubes Index (DMI)	1.96 ± 0.36	3.10 ± 0.74	0.394
Myogenic Fusion Index (MFI)	57.33 ± 2.40	50.41 ± 1.98	0.093
< 3 Nuclei	20.67 ± 2.45	26.17 ± 2.55	0.266
3-10 Nuclei	56.50 ± 4.59	50.17 ± 5.89	0.574
> 10 Nuclei	21.17 ± 4.64	18.83 ± 2.63	0.905
Viability (% alive cells)			
7-AAD^-	97.83 ± 0.51	97.05 ± 0.60	0.476
Annexin-V^-	92.25 ± 1.34	90.59 ± 1.66	0.352

N, Number of subjects; HFrEF, Heart failure with reduced ejection fraction. Data are expressed as mean ± SEM. Significant p-values are highlighted in bold.

Proliferative capacity of HFrEF myoblasts

The increase in CI from 50 h to 100 h was less pronounced in myoblasts cultures from HFrEF patients. In particular, the mean CI taken during the logarithmic growth phase at 90 h was significantly lower in HFrEF cultures (p= 0.032). Myoblasts of HFrEF patients reached their maximum CI at 136.53 ± 5.16 h, whereas myoblasts of control subjects showed a maximum CI at 114.49 ± 12.05 h (p=0.214; **Fig. 3**).

Pax3 and Pax7 expression in HFrEF muscle cells

Satellite cell marker Pax3 showed a low expression (1-2%) in both groups of myotubes cultures, indicating the rare presence of satellite cells (Additional File 1; **Fig S1**). Pax7 expression at day 0 was present in 72.72 ± 11.80% of myoblasts from HFrEF patients and in

78.82 ± 8.13% of myoblasts from controls. The expression level decreased to 51.65 ± 1.86% and 59.74 ± 2.67% at day 6 for respectively HFrEF patients and controls (Additional File 1; **Fig S1**). There was no statistically significant difference in Pax7 expression between groups at either time point.

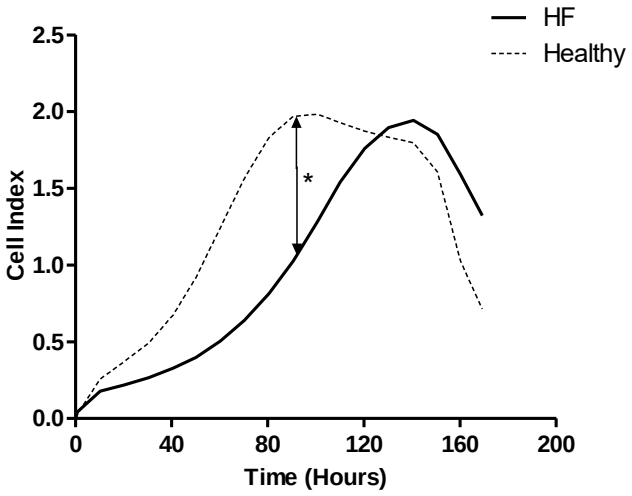

Figure 3. Representative growth kinetics of HFrEF and control myoblasts

Growth curves were generated using the xCELLigence system. (N=8 controls and HFrEF patients) *p<0.05

Myogenic regulatory factors in HFrEF muscle cells

The expression of the transcription factors MyoD and MRF4 remained stable over time during the process of myogenesis in both myotubes cultures (Additional File 1; **Fig S1**). A steady increase in myogenin expression within 48 h after the change from proliferation to differentiation medium was detected in both groups, with levels reaching 3-fold of those found on day 0 (Additional File 1; **Fig S1**). Overall, no significant differences were observed between groups in the percentages of MyoD, Myogenin and MRF4.

Cellular senescence

Muscle biopsies and myoblast cultures from patients and controls displayed no significant differences regarding cellular senescence. First, the expression of acetyl-p53 was similar between groups in both muscle biopsies (1.68 ± 0.95 for HFrEF vs. 0.68 ± 0.25 for controls;

p= 0.307; **Fig. 4A**) and myoblast cultures (1.31 ± 0.42 for HFrEF vs. 1.63 ± 0.41 for controls; p=0.590; **Fig. 4B**). Second, myoblast cultures of HFrEF patients demonstrated an equal amount of SA-β-gal positive cells as controls (p=1.000), which is in accordance with the results for acetyl-p53 (**Fig. 4C**). Myoblast cultures of HFrEF patients consisted of 8.06 ± 1.01% SA-β-gal positive cells, whereas cultures of controls demonstrated 7.86 ± 0.39% SA-β-gal positivity.

mRNA expression levels of TNFR1 and TNFR2

No statistical difference was detected for the gene expression level of the death receptor TNFR1 between myoblast cultures of patients and controls. In contrast, TNFR2, which functions as a survival receptor, was significantly down-regulated in patient-derived myoblasts (p=0.017; **Fig. 4D**).

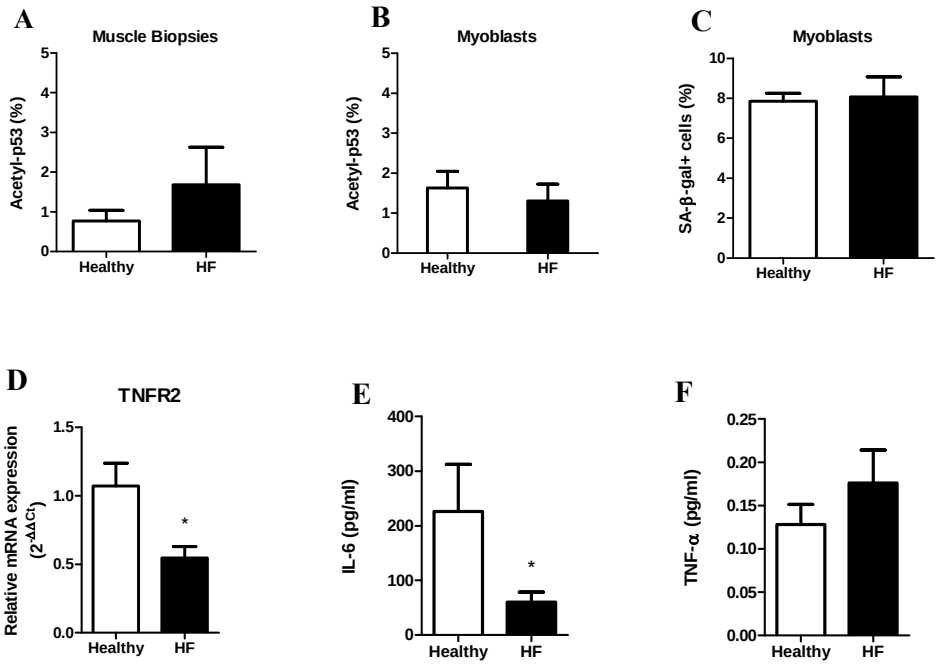

Figure 4. Assessment of senescence and inflammatory characteristics

Representative graphs of mean percentage of acetyl-p53 positive cells in muscle biopsies **(A)** and myoblast cultures **(B)**. Quantification of the number of SA-β-gal positive cells present in myoblast cells from control subjects and HFrEF patients **(C)**. mRNA expression level of TNFR2 in myoblasts from HFrEF patients and control subjects **(D)**. IL-6 **(E)** and TNF-α **(F)** secretion from cultured myoblast cells. Data are mean ± SEM. (N=8 controls and HFrEF patients). *p<0.05

Cytokine response in cultured myoblasts

Myoblast cultures at 72 h revealed a significantly lower IL-6 secretion (p=0.016) by HFrEF myoblasts (59.95 ± 18.82 pg/ml) compared to control myoblasts (226.11 ± 86.07 pg/ml; **Fig. 4E**). TNF-α secretion was somewhat increased in HFrEF myoblast cultures, however, this was not statistically significant (**Fig. 4F**). In addition, no significant difference in IL-10 secretion was observed. Myoblasts did not secrete IL-1β and IFN-γ (mean lower limits of detection of the assays: 0.04 pg/ml and 0.20 pg/ml for IL-1 β and IFN-γ, respectively).

DISCUSSION

Human skeletal muscle cell cultures have been extensively studied in chronic diseases such as type 2 diabetes, obesity and chronic obstructive pulmonary disease (COPD) (12, 13, 20). The present study established for the first time primary cell cultures from the skeletal muscle of patients with HFrEF and analyzed these for phenotypic and proliferative characteristics. The main findings can be summarized as follows:

First, primary myoblasts and myotubes from HFrEF patients demonstrate a morphology, myogenic differentiation capacity, viability and senescence that is comparable to muscle cells of healthy donors. Second, myoblasts of HFrEF patients exhibit an altered proliferative and inflammatory activity, supported by the diminished expression of the survival receptor TNFR2 and lower IL-6 secretion.

In the present study we demonstrate that satellite cell-derived myoblasts from HFrEF patients are able to differentiate *in vitro* into multinucleated myotubes. In addition, we show that skeletal myoblasts and myotubes from patients with HFrEF have a similar size, shape and myogenic differentiation ability as muscle cell cultures from healthy donors. These findings are in line with previous studies in patients with COPD and type-2 diabetes (20, 21). In these patients, the myotubes' myogenic fusion index and commitment to terminal differentiation were not different from myotubes of healthy donors. Also, the expression patterns of the myogenic regulatory factors MyoD, Myogenin and MRF4 were similar to healthy donor cultures.

HFrEF myoblasts further demonstrated a delay in proliferation kinetic in comparison to myoblasts of age- and gender-matched healthy donors. Therefore, it could be possible that the satellite cells of the HFrEF patients had already undergone multiple cell divisions *in vivo* to replenish damaged muscle fibers, resulting in slower culture rates of cell division. A state of cellular senescence, however, was not yet detected in the HFrEF myoblasts. Instead, we observed a reduced expression of the TNF-α receptor TNFR2, but not of TNFR1. Of note, Torre-Amione et al. already reported a diminished expression of myocardial TNFR1 and TNFR2 in patients with advanced HF in 1996 (22). Recently, patients with HF were found to have elevated levels of circulating soluble TNFR2, which the authors attributed to increased tissue shedding (23). Furthermore, we observed that the secretion of IL-6 was significantly reduced in comparison to the IL-6 release by the healthy donor myoblasts. TNFR1 and TNFR2 are the two major transducers of TNF-α signals. Ligation of TNF-α with TNFR1 leads to caspase activation and induces apoptotic cell death (24). In contrast, TNFR2 signaling activates nuclear factor-kappa β (NF-κβ) and signal transducer and activator of transcription 3 (STAT3), and thereby promotes cell proliferation, cytokine production and cell survival (25). IL-6 is a pleiotropic cytokine that is, among other cells, also produced by growing myofibers and associated satellite cells. It stimulates the robust activation and proliferation of satellite and myoblasts cells in both autocrine and paracrine manners via STAT3 signaling, and therefore acts as a novel mediator in controlling muscle regeneration (26-28). In this regard, Serrano et al showed that if IL-6 is genetically deleted, satellite cell proliferation and migration become attenuated, leading to a reduction in myofiber size (29). Therefore, based on our findings and the results from previous studies, it seems that persistent exposure of skeletal muscle cells to high systemic and local levels of TNF-α in HFrEF induces a refractory cell state, provoking a decrease in muscle cell secretion of IL-6 and a reduction in TNFR2 expression, thereby attenuating cell proliferation, and this plausibly without affecting the endogenous synthesis of IL-6 (30, 31). In this regard, *in vitro* studies by Hamilton et al. demonstrated that a single 10-hour incubation step of colon cancer cells with TNF-α leads to endogenous IL-6 through NF-κβ activation and promotes TNFR2 expression via the autocrine effects of IL-6 (32). Yet, repeated exposure of cells to TNF-α was shown to weaken the secretion of IL-6 (33). Hence, the lower secretion of IL-6 may well contribute to the decrease in proliferative competitiveness of the HFrEF myoblasts.

Another potential mechanism underlying reduced myoblast proliferation in HFrEF is the lack of physical activity in these patients (34-37). It has been demonstrated that both physical

training and a single bout of exercise positively influence satellite cell function, myoblast proliferation, and cytokine expression and secretion (38-40). Resistance training, for instance, increases satellite cell content and activation status, and stimulates satellite cell proliferation by IL-6 induced activation of STAT3 signaling (41, 42). Recently, Begue et al. also indicated that resistance exercise training promotes satellite cell proliferation by IL-6 induced activation of STAT3 signaling (43). Furthermore, metabolic disturbances such as hyperglycaemia, insulin resistance, mitochondrial dysfunction and a decreased activation of 5' adenosine monophosphate-activated protein kinase (AMPK) and p38 mitogen-activated protein kinase (MAPK) were shown to impair the proliferation of porcine myoblasts and C_2C_{12} mouse myoblasts (44-49). In addition, growth differentiation factor myostatin was shown to negatively regulate the self-renewal of satellite cells and to inhibit C_2C_{12} muscle cell activation, proliferation, myogenic differentiation and protein synthesis (50-52). As such, distinct mechanisms might be involved in the altered inflammatory and proliferative actions of HFrEF myoblasts.

STUDY LIMITATIONS

Cells from both HFrEF patients and control subjects were cultured under the same *in vitro* environmental conditions which did not reflect the prevailing inflammatory milieu to which skeletal muscles of HFrEF patients are exposed to *in vivo*. Circulating TNF-α was not measured, however, previous studies have indicated increased serum TNF-α levels in HFrEF (1, 22, 23). HFrEF patients were on optimal medical treatment and thus, were administered a number of pharmacological agents including ACE-inhibitors, beta-blockers, diuretics and statins. In this regard, and although well-tolerated by the majority of patients, evidence has indicated that statins may affect (either positively or negatively) skeletal muscle function (53-56). In our study, we observed no differences in myoblast number, morphology, or differentiation capacity among HFrEF patients on statin therapy (75% of patients) and those not taking the drug (25%). In addition, it has been shown that chronic kidney disease (CKD), diabetes mellitus and COPD, common co-morbidities in patients with HFrEF, may affect muscle cell parameters including proliferation and differentiation capacities (20, 57, 58). Therefore, HFrEF patients with major comorbidities were excluded from the study. Finally, results are to be confirmed in a wider range of patients with HFrEF.

CONCLUSION

In this study, we show that myoblasts derived from HFrEF patients have altered proliferative and reduced anti-inflammatory activity if compared to healthy donor cells. We believe that the *in vitro* cultivation of biopsy-derived primary skeletal muscle myoblasts and myotubes is a promising tool for future research on muscle wasting in HFrEF.

ACKNOWLEDGMENTS

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Figure S1

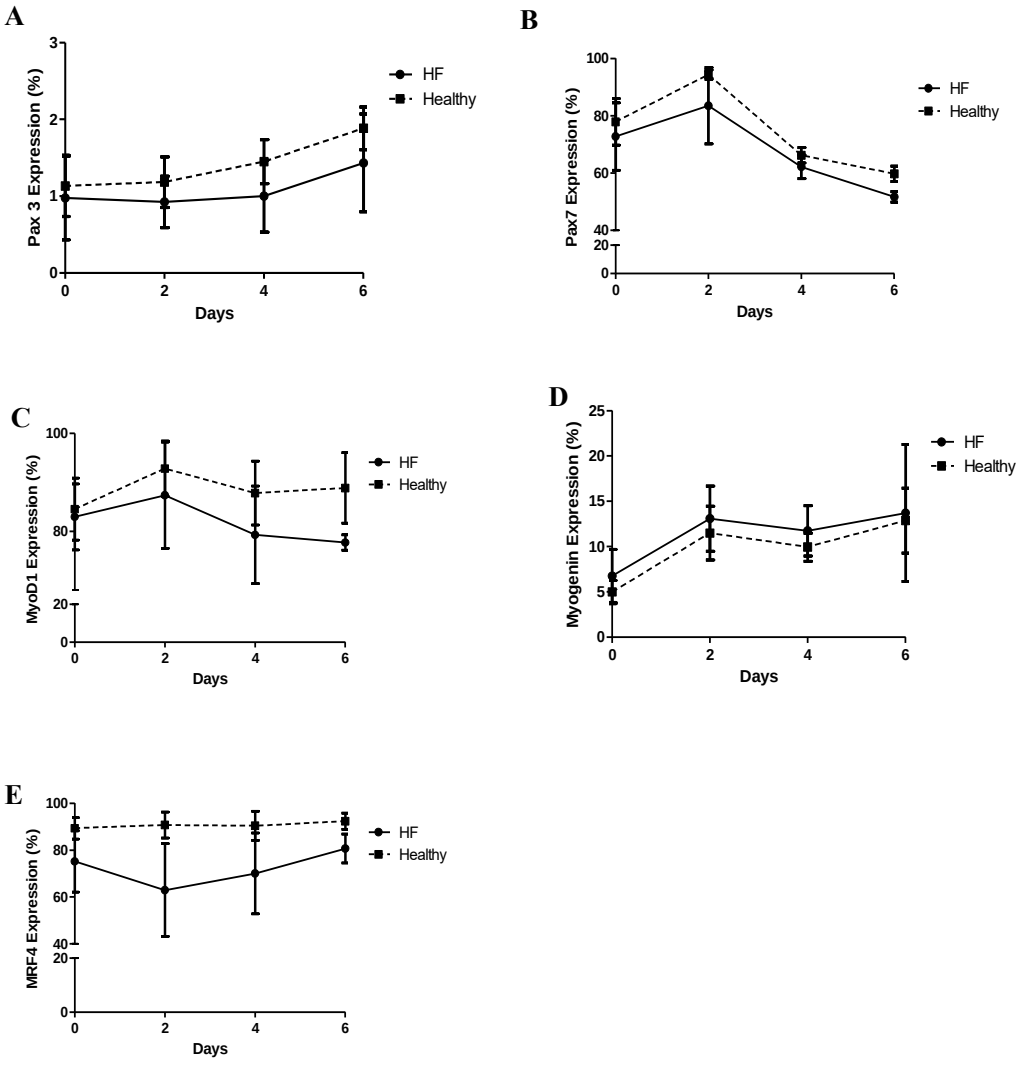

Figure S1. Differentiation of HFrEF myotubes

Representative graphs showing the expression pattern of Pax3 **(A)**, Pax7 **(B)** and the myogenic regulatory factors MyoD **(C)**, Myogenin **(D)** and MRF4 **(E)** in muscle cells cultivated from HFrEF patients and controls following six days in differentiation medium. Satellite cell marker Pax3 **(A)** showed a low expression in both myoblasts groups indicating the relatively absence of satellite cells. A slightly increased Pax7 expression was observed within 48h after having changed the proliferation medium to differentiation medium **(B)**. As the myoblasts matured, the level of this transcription factor gradually decreased whereby the reduction in Pax7 expression was more pronounced in HFrEF, although not statistically significant myoblasts. Expression of MyoD and MRF4 remained stable over time during myogenesis **(C, E)**. A steady increase in myogenin expression within 48 h after the change from proliferation to differentiation medium was detected in both groups with levels reaching 3-fold of those found on day 0 **(D)**. Data are mean ± SEM. (N=6 controls and HFrEF patients).

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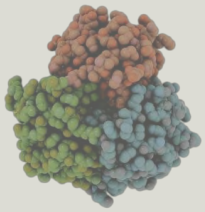

6

Chapter 6

Underlying mechanism of adiponectin resistance in heart failure: roles of AdipoR1 and inflammation

Adapted from:

Sente T, Van Berendoncks AM, Fransen E, Vrints CJ, Hoymans VY. Tumor necrosis factor-α impairs adiponectin signalling, mitochondrial biogenesis and myogenesis in primary human myotubes cultures. Am J Physiol Heart Circ Physiol. Feb 2016

ABSTRACT

Background: Skeletal muscle metabolic changes are common in patients with chronic heart failure (HF). Previously, we demonstrated a functional skeletal muscle adiponectin resistance in HF patients with reduced left ventricular ejection fraction (HFrEF). We aimed to examine the impact of adiponectin receptor 1 (AdipoR1) deficiency and TNF-α treatment on adiponectin signalling, proliferative capacity, myogenic differentiation and mitochondrial biogenesis in primary human skeletal muscle cells. **Methods**: Primary cultures of myoblasts and myotubes were initiated from the *m. vastus lateralis* of 10 HFrEF patients (LVEF; 31.30 ± 2.89%) and 10 age-and gender matched healthy controls. Healthy control cultures were transfected with siAdipoR1 and/or exposed to TNF-α (10ng/ml; 72h). **Results**: Primary cultures from HFrEF patients preserved the features of adiponectin resistance *in vivo*. AdipoR1 mRNA was negatively correlated with time to reach maximal cell index (r=-0.7319, p=0.003). SiRNA-mediated AdipoR1silencing reduced pAMPK (p<0.01), AMPK activation (p=0.046) and myoblast proliferation rate (xCELLigence RTCA; p<0.0001). Moreover, TNF-α decreased the mRNA expression of genes involved in glucose (APPL1, p=0.0002; AMPK, p=0.021), lipid (PPARα, p=0.025; ACADM, p=0.003) and mitochondrial (FOXO3, p=0.018) metabolism, impaired myogenesis (MyoD1, p=0.053; Myogenin, p=0.048) and polarized cytokine secretion towards a growth-promoting phenotype (Il-10, IL-1β, IFN-γ, p<0.05 for all; Meso Scale Discovery technology). **Conclusion**: Major features of adiponectin resistance are retained in primary cultures from the skeletal muscle of HFrEF patients. In addition, our results suggest that an increased inflammatory constitution contributes to adiponectin resistance and confers alterations in skeletal muscle differentiation, growth and function.

Keywords

Chronic heart failure; primary muscle cell cultures; adiponectin resistance; siAdipoR1; inflammation

INTRODUCTION

Profound energetic impairment of peripheral skeletal muscles is a presenting feature in patients with chronic heart failure (HF). Alterations of skeletal muscle metabolism in these patients is characterized by a complex network of anabolic and catabolic factors and signalling pathways, including a role of disrupted adiponectin signalling (1-3). Adiponectin is an abundant adipocyte-derived plasma protein with anti-diabetic, anti-inflammatory and anti-atherogenic actions able to beneficially affect vascular function and energy homeostasis (4). Adiponectin mainly acts through interaction with the G-protein-coupled skeletal muscle receptor, adiponectin receptor 1 (AdipoR1), thereby enhancing glucose uptake and transport, and stimulating muscle fatty acid β-oxidation (FAO) (4, 5). These actions of adiponectin require the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) and increased activity of peroxisome proliferator-activated receptor α (PPARα) (4-6).

In patients with existing ischemic heart disease, high serum levels of adiponectin are paradoxically linked with an increased risk for heart failure and mortality (7, 8). In HF with reduced left ventricular ejection fraction (HFrEF), elevated serum adiponectin also relates with metabolic impairment, advanced disease state and symptomatic status (3, 7, 9). Ample evidence further points to a state of adiponectin resistance in the skeletal muscle of HFrEF patients (10, 11). These patients exhibit an increased skeletal muscle mRNA and protein content of adiponectin in comparison to healthy subjects, whereas the underlying signalling cascade is deactivated (10). The latter was illustrated by a reduction in the mRNA levels of AdipoR1, AMPK as well as in phosphorylated AMPK. Further, a down-regulation of PPARα was observed together with a decrease of the target genes involved in glucose (hexokinase 2; HK2) and lipid (acyl-CoA dehydrogenase C-4 to C-12 straight chain; ACADM) metabolism.

Recent findings suggest that the presence of skeletal muscle adiponectin resistance in HFrEF is, at least partly, due to down-regulation of AdipoR1 (12-14). Phosphorylation of AdipoR1 observed in cardiomyocytes isolated from failing mice hearts resulted in receptor desensitization and subsequent down-regulation, as well as in impaired adiponectin signalling and cardio-metabolism (15). Along with downregulation of AdipoR1, also long-term inflammation has been implicated in the development of adiponectin resistance. Increased presence of inflammatory mediators in HFrEF (e.g.; tumor necrosis factor alpha, TNF-α; interleukin (IL)-6) contributes to immune activation and, in that, can have detrimental effects on adiponectin signalling (2, 16-19). In this regard, Bruun et al. showed a direct inhibitory

effect of endogenous cytokines, and in particular of TNF-α, on human adipocyte mRNA of adiponectin (20). Vice versa, adiponectin might act as a protective mechanism by weakening inflammation, since adiponectin has been shown to be capable of suppressing TNF-α secretion from macrophages (21). Upregulation of circulating adiponectin in HFrEF might therefore occur to counteract the already increased concentrations of pro-inflammatory cytokines (22). Increased circulating adiponectin is further strongly correlated with reduced skeletal muscle mass and muscle strength in HFrEF (3, 23). A study by Goto et al. also demonstrated a positive association between muscle mass and the expression of AdipoR1 in atrophied skeletal muscles of mice and in C_2C_{12} cells (24). Finally, adiponectin has been shown to influence muscle cell proliferation and regeneration (25).

The adiponectin resistant state in the skeletal muscle of HFrEF patients and its precise relation with AdipoR1 and inflammation, however, are not completely understood. The aims of the present study were (1) to examine adiponectin resistance in cultured primary skeletal muscle cells from HFrEF patients and (2) to study the significance of AdipoR1 deficiency and TNF-α exposure on adiponectin signalling, proliferation, myogenic differentiation and mitochondrial biogenesis in primary cultures from healthy controls.

MATERIAL AND METHODS

Patients and controls

The study population consisted of ten patients with systolic heart failure as a result of dilated cardiomyopathy or ischemic heart disease (LVEF<35%; NYHA II-III), recruited from the Heart Failure Clinic of the Antwerp University Hospital UZA, (Edegem, Belgium). HFrEF patients were on a stable medical regimen for at least one month before enrollment. Exclusion criteria were recent acute coronary syndrome (≤3 months), valvular disease requiring surgery, malignant ventricular arrhythmia and acute myocarditis or pericarditis. Infections (acute/chronic), allergies, cancer, inflammatory diseases, diabetes, renal failure and musculoskeletal abnormalities were excluded to avoid possible metabolic interference. Referent controls were ten age- and gender matched healthy subjects with no medication intake and no evidence of cardiovascular disease. Cardiovascular disease was excluded by performing a complete medical history, a comprehensive physical examination, an electrocardiogram (ECG) and an echocardiogram. Informed consent was obtained from all participants. The study was approved by the local Ethics Committee of the Antwerp

University Hospital and is in accordance with the Declaration of Helsinki.

Cardiopulmonary exercise testing

HFrEF patients and control subjects underwent a cardiopulmonary exercise test (CPET) on a treadmill (Medical Jaeger, Würzburg, Germany) or cycle ergometer (Ergoline, Schiller AG, Baar, Switzerland) in non-fasting conditions. A ramp protocol started with 20W, whereas workload was increased with incremental steps of 10 or 20W/min. until exhaustion. Respiratory gas exchange data were determined continuously and permitted determination of ventilation (VE), oxygen uptake (VO_2) and carbon dioxide production (VCO_2) (Cardiovit CS-200 Ergo-Spiro, Schiller AG, Baar, Switzerland). Peak oxygen consumption (VO_2 peak) was expressed as the highest obtained VO_2. Twelve-lead ECG and heart rate were recorded continuously and blood pressure was measured every 2 min. All study subjects underwent echocardiographic examinations. Muscle biopsies were collected within one week.

Biochemical Analysis

Fasted blood samples were collected from all patients and controls. Levels of creatinine, total cholesterol, triglycerides, low-density (LDL) and high-density lipoprotein (HDL) cholesterol, glucose and high sensitivity C-reactive protein (hsCRP) were assessed on Dimension Vista 1500 instruments using reagents from Ortho Clinical Diagnostics (Siemens Healthcare Diagnostics NV/SA, Beersel/Huizingen, Belgium). Bioelectrical impedance analysis was performed for the assessment of body composition (Omron body fat monitor BF 300). Circulating total adiponectin was measured using an enzyme-linked immunosorbent assay (ELISA, R&D Systems, Abingdon, UK). Serum TNF-α concentrations were measured using a high-sensitivity human TNF-α-specific ELISA (R&D Systems).

Primary muscle cell cultures and TNF-α treatment

Muscle biopsies of the *m. vastus lateralis* were obtained using the Bergstrom needle technique, as described in **chapter 4** (26-28). Satellite cells were isolated and cultured into collagen-coated flasks containing SKGM at 37°C in a humidified 5% CO_2 atmosphere. Differentiation was initiated by DMEM/F12 medium containing 2% horse serum (Gibco; Invitrogen, Grand Island, NY, USA). In each experiment, a part of the myotubes cultures was incubated for 72h with 10ng/ml recombinant human TNF-α (Sigma-Aldrich, St. Louis, MO, USA) with or without siAdipoR1.

Lipid-mediated AdipoR1 silencing

Myoblasts cultures derived from control subjects were grown in collagen-coated 6-well plates until cell density reached approximately 70% confluence. For lipofection, a 2µM solution of siRNA (ON-TARGETplus siRNA human AdipoR1, Dharmacon, Tournai, Belgium) in 1X siRNA buffer was prepared. siRNA and DharmaFECT2 transfection reagent were each diluted in antibiotic- and serum-free differentiation medium with or without TNF-α (10ng/ml), incubated for 5 min. at RT and then combined. The siRNA-DharmaFECT2 mixture was allowed to equilibrate at RT for 20 min. and added drop wise to each well (final concentration 25nM). Control cultures were prepared in a similar manner but without the addition of siRNA. ON-TARGETplus Non-Targeting siRNA pool and ON-TARGETplus GAPDH Control Pool (Dharmacon) were used as a negative and positive control. Western blot and RT-PCR were performed to assure silencing efficiency at 72h after lipid-mediated transfection.

RNA isolation and quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted using the Qiazol reagent technique followed by RNA cleanup using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). 1µg of total RNA was reverse-transcribed using the iScriptTM cDNA Synthesis Kit (Bio-rad Laboratories, Nazareth, Belgium). RT-PCR gene-specific forward and reverse primers (Eurofins MWG Operon, Ebersberg, Germany) were designed for the genes listed in **table 1**. PCR amplification was performed on a CFX96TM Real-Time PCR Detection system (Bio-rad Laboratories). The specificity and the purity of the amplified product were checked by electrophoresis on agarose minigels and melting point dissociation curves. Gene expression levels were normalized to TATA box binding protein (TBP) and beta-2-microglobulin (B2M). Relative quantification of gene expression levels was performed by using the $2^{-\Delta\Delta Ct}$ calculation. All samples were run in duplicate.

Cytokine production

Cell culture supernatant was collected and concentrated by centrifugation at 4000 x g for 15 min. with Amicon Ultra-4 centrifugal filter device (Millipore). Levels of TNF-α, IL-6, IL-10, interferon-γ (IFN-γ) and IL-1β were determined before and after transfection by means of multiplex Meso Scale Discovery technology (Meso Scale Diagnostics, Rockville, MD, USA). Plates were read on a SECTOR® Imager 6000 instrument and data was analyzed using the Discovery Workbench 3.0 software (Meso Scale Diagnostics).

Table 1. Oligonucleotide primers for RT-PCR amplification

Gene	GenBank accession number	Oligonucleotide sequence	T_A	Length of Amplicon (bp)
AdipoQ	NM_004797.2	5'-GCTGGGAGCTGTTCTACTGC 5'-CGATGTCTCCCTTAGGACCA	62	233
AdipoR1	NM_015999	5'-CGGCTCATCTACCTCTCCAT 5'-CACAAAGCCCTCAGCGATAG	58	174
AMPK	NM_206907	5'-ACTGTACCAGGTCATCAGTACACC 5'-CCACCATATGCCTGTGACAA	60	218
HK2	NM_000189	5'- GACCACATTGTCCAGTGCAT 5'- TTTGTCCACTTGAGGAGGATG	62	137
PPAR-alpha	NM_005036.4	5'-CCTCTCAGGAAAGGCCAGTA 5'-CAGTGAAAGATGCGGACCTC	60	141
ACADM	NM_000016	5'-GCTACCAAGTATGCCCTGGA 5'-CCCAAGCTGCTCTCTGGTAA	60	133
APPL1	NM_012096	5'-AGTGCAGCAACACACCTGAC 5'-TGGGTAATGGGGAACATCAT	60	188
PGC-1 alpha	NM_013261	5'-AGCTGCTGAAGAGGCAAGAG 5'-TTCCCCTAAACCAAGCACAC	55	156
FOXO3	NM_001455	5'-GGGGAACTTCACTGGTGCTA 5'-GAGAGCAGATTTGGCAAAGG	53	143
MyoD1	NM_002478	5'-TGCCACAACGGACGACTTC 5'-CGGGTCCAGGTCTTCGAA	60	233
Myogenin	NM_002479	5'-GCCTCCTGCAGTCCAGAGT 5'-AGTGCAGGTTGTGGGCATCT	60	187
TBP	NM_003194	5'-GAGAGCCACGAACCACGC 5'-ACATCACAGCTCCCCACCAT	62	178
B2M	NM_0040482	5'-ACCCCCACTGAAAAAGATGA 5'-ACTTTCAAACCTCCATGATG	55	114

AdipoQ, adiponectin; AdipoR1, adiponectin receptor 1; AMPK, adenosine monophosphate activated protein kinase alpha1; HK2, hexokinase 2; PPAR-alpha, peroxisome proliferator-activated receptor alpha; ACADM, Acyl-Coenzyme A dehydrogenase, APPL1, Adaptor protein phosphotyrosine interaction PH domain and leucine zipper containing 1; PGC-1alpha, Peroxisome proliferator-activated receptor gamma coactivator 1 alpha; FOXO3, Forkhead box O3; MyoD1, Myogenic differentiation 1; TBP, TATA box binding protein; B2M, Beta-2-microglobulin; TA, Annealing temperature.

Western blotting

Myotubes cultures were lysed and homogenized in RIPA buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 1 mM NaF, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, and 1 mM phenylmethylsulphonyl fluoride, Sigma-Aldrich) plus protease and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany) and centrifuged at 230 x g for 10 min. to obtain lysates. Protein concentration was determined using a BCA Protein Assay Kit (Thermo Scientific, Barrington, IL, USA). Lysates were mixed in Laemmli sample buffer (Bio-Rad Laboratories) and β-mercapto-ethanol (Sigma-aldrich)

and heat-denaturated for 5 min. Equal volumes of protein were loaded on 4-12% SDS–PAGE gels (Invitrogen). After gel electrophoresis, proteins were transferred to a reduced-fluorescence PVDF membrane (Immobilon-FL, Millipore, Bedford, MA, USA) and blocked in Odyssey blocking buffer mixed 1:1 with TBS (Li-Cor, Lincoln, NE) for 1h. Primary antibodies were incubated overnight at 4°C in Odyssey blocking buffer mixed 1:1 with TBS-T. Fluorescent secondary antibodies were incubated for 1h in Odyssey blocking buffer plus TBS-T and 0.01% SDS (Dako, Glostrup, Denmark) at RT. AdipoR1 (1:1000), total AMPK (1:1000), Thr^{183} phosphorylated AMPK alpha1 (1:1000) and Thr^{172} phosphorylated AMPK alpha2 (1:1000) antibodies were purchased from Abcam (Cambridge, MA, UK). Fluorescence intensity data was normalized to β-actin expression (Sigma–Aldrich) and visualized using a Lumi-Imager (Roche Diagnostics).

Myoblast proliferation by means of real time cell analysis

Myoblast proliferation was examined using the xCELLigence Real-Time Cellular Analysis (RTCA) system (Westburg, Leusden, The Netherlands), as summarized in **chapter 4**. Briefly, myoblasts were grown in modified 16-well plates (E-plates, Westburg) with microelectrodes on the bottom of each well for impedance-based detection. 100µl of cell-free growth medium was added to the wells and placed at RT for 30 min. to measure background impedance. Then, 50 µl of myoblasts suspension was seeded into each well ($1x10^4$ cells/well). After 30 min. at RT to allow cell attachment, plates were transferred in the RTCA device and impedance was monitored and expressed as cell index (CI) (29). After approximately 24h, cell culture medium was changed and cells were transfected with siAdipoR1 alone or combined with TNF-α (10 ng/ml). The CI was monitored every 15 min. for a total time of 180h at 37°C in a 5% CO_2 atmosphere and data was analyzed using the RTCA software. Two replicates of each culture were run.

Senescence-associated β-Galactosidase activity

Senescence-associated beta-galactosidase (SA-β-gal) activity was assessed with the *β*-Galactosidase Staining Kit (Biovision Research Products, Palo Alto, CA, USA) at 72h. Myotubes were fixed and incubated overnight in freshly prepared acidic *β*-gal staining solution containing 5-bromo-4-chloro-3-indolyl *β*-D-galactopyranoside (X-Gal) at 37°C. Senescence was determined by phase contrast under bright field illumination in ten randomly selected fields (Olympus Optical Co., Tokyo, Japan).

Histological examination

Cells were grown on collagen-coated glass chamber slides and fixed in acetone for 10 min. at 4°C and permeabilized in 0.1% Triton X-100 for 5 min. Actin microfilaments were visualized by applying FITC-conjugated phalloidin (50 µg/mL in methanol; Sigma-Aldrich) for 20 min. in the dark. Immunohistochemical detection of adiponectin was performed using the primary mouse anti-human adiponectin antibody (1:100; Abcam). The human pre-adipocyte cell strain Simpson-Golabi-Behmel syndrome was used as a positive control. Nuclei were counterstained with DAPI. Fifteen fields of each culture were randomly chosen for semi-quantitative analysis of staining intensity using the software program Image J (Version 1.45s, National Institutes of Health, USA).

MTT assay

Viability was determined via methylthiazole tetrazolium bromide (MTT) assay. Therefore, myotubes were seeded into 96-well plates and grown in a humidified 5% CO_2 atmosphere at 37°C. After 72 hours, cultures were incubated with 0.5 mg/ml MTT for 4 hours at 37°C. The medium was removed and 150 µl of DMSO was added. Optical density (OD) was measured at 590 nm wavelength in a microplate reader (Biorad 550 microplate reader, Nazareth, Belgium). All cultures were assayed in triplicate and averaged for statistical analysis.

Statistical analysis

Statistical analyses were conducted using JMP Pro 11 (Sas Institute Inc., Cary, NC, USA). Baseline differences between the HFrEF patients and the matched controls were tested with the Mann-Whitney U test. Association with categorical variables were compared with the Pearson's Chi-square (X^2) test. Comparison of the different conditions was performed by one-way analysis of variance (ANOVA) followed by a post hoc analysis with Dunnett's correction for multiple testing. The agreement between myoblast proliferation and mRNA expressions was calculated according to the Spearman's rank correlation method. Statistical significance was designated at $p<0.05$. All data are represented as mean ± standard error of the mean (SEM).

RESULTS

Clinical characteristics of the study population

Clinical characteristics are listed in **table 2**. Patients and controls were comparable with respect to age, gender and BMI. In comparison to the healthy subjects, HFrEF patients demonstrated impaired exercise capacity (VO_2 peak, p=0.016; maximal workload, p=0.001 and work efficiency, p=0.007), a dyslipidemic state (reduced total cholesterol, p=0.042 and HDL, p=0.032) and a pro-inflammatory profile (increased hs-CRP and hs-TNF-α, p=0.001 and p=0.019, respectively). Total serum adiponectin was significantly higher in the HFrEF group (p=0.038).

Table 2 Clinical characteristics of HFrEF patients and healthy control subjects

Characteristic	Controls N=10	HFrEF N=10	*P-value*
Age (years)	53.90 ± 1.43	57.40 ± 3.94	0.085
Gender (% male)	70%	70%	1.000
Weight (kg)	81.11 ± 3.51	70.47 ± 3.07	0.063
BMI (kg/m^2)	27.05 ± 1.49	23.00 ± 0.92	0.063
LVEF (%)	N.A.	31.30 ±2.89	N.A.
Ischemic etiology (%)	N.A	60%	N.A.
Total cholesterol (mmol/L)	5.56 ± 0.36	4.16 ± 0.37	**0.042**
HDL (mmol/L)	1.70 ± 0.12	1.28 ± 0.19	**0.032**
LDL (mmol/L)	3.07 ± 0.34	2.53 ± 0.31	0.305
Triglycerides (mmol/L)	1.34 ± 0.25	1.48 ± 0.18	0.246
Glucose (mmol/L)	4.67 ± 0.11	5.44 ± 0.27	**0.036**
Serum Creatinine (mmol/L)	0.84 ± 0.04	0.95 ± 0.05	0.118
hsCRP (mg/L)	0.14 ± 0.10	2.71 ± 1.15	**0.001**
VO_2 Peak (ml/kg/min)	36.44 ± 2.67	18.30 ± 1.65	**0.016**
Maximal workload (watt)	213.00 ± 15.50	105.00 ± 11.07	**0.001**
Work efficiency (Watt ml^{-1} kg^{-1} min^{-1})	6.14 ± 0.24	4.38 ± 0.15	**0.007**
Serum adiponectin (mg/l)	10.97 ± 1.41	16.72 ± 2.10	**0.038**
Serum TNF-α (pg/ml)	0.82 ± 0.13	1.49 ± 0.25	**0.019**

N, Number of subjects; HFrEF, Heart failure with reduced ejection fraction; BMI, Body mass index; LVEF, Left Ventricular Ejection fraction; HDL, High density lipoprotein; LDL, Low density lipoprotein; hsCRP, high sensitivity C-reactive protein. Data are expressed as mean ± SEM. Significant p-values are highlighted in bold.

Features of adiponectin resistance in primary HFrEF myotubes

We observed that 39.5% of HFrEF myotubes cultures expressed adiponectin mRNA compared to 16.6% of healthy donor cultures. Overall, adiponectin mRNA expression was higher in HFrEF cultures with a mean Ct value of 38.51 ± 1.14 versus 40.12 ± 1.24 in control cultures. Immunohistochemistry documented the presence of adiponectin protein expression in primary muscle cultures. Cultures of HFrEF patients stained abundantly for adiponectin protein, whereas fewer staining was detected in cultures of control subjects (p=0.058; **Fig. 1A, 1B**). Adiponectin secretion was not significantly different between HFrEF and control cultures (p=0.467, **Fig. 1C**). AMPK phosphorylation was approximately three times lower in HFrEF myotubes compared to controls with no significant difference in total protein expression of AMPK indicating a reduced activation of AMPK (pAMPK/AMPK, p=0.046 **Fig. 1D**). In addition, the mRNA expression level of AdipoR1 was lower in the HFrEF group than in healthy controls (p=0.051). Similarly, mRNA expression levels of AMPK (p=0.022) and HK2 (p=0.059) were decreased in myotubes from HFrEF patients (**Fig. 1E**). No major differences in gene expression level between groups were detected for PPARα (p=0.228) and its target gene in lipid metabolism ACADM (p=0.101). Likewise, no difference in mRNA expression was observed for APPL1 (p=0.573; **Fig. 1E**).

Silencing of AdipoR1 expression by siRNA

The efficiency of AdipoR1 silencing (siAdipoR1) in healthy myotubes was evaluated at the target mRNA and protein level. A difference of approximately four Ct values for AdipoR1 mRNA was observed between the non-transfected and siAdipoR1 transfected cultures ($p<0.0001$), representing a transfection efficiency of 83.56 ± 4.21% (**Fig. 2C**), comparable to the results obtained for myotubes transfected with the ON-TARGETplus GAPDH Control Pool (positive control). AdipoR1 protein expression was also significantly reduced in siAdipoR1 transfected cultures (**Fig. 2A, 2B**, $p<0.0001$).

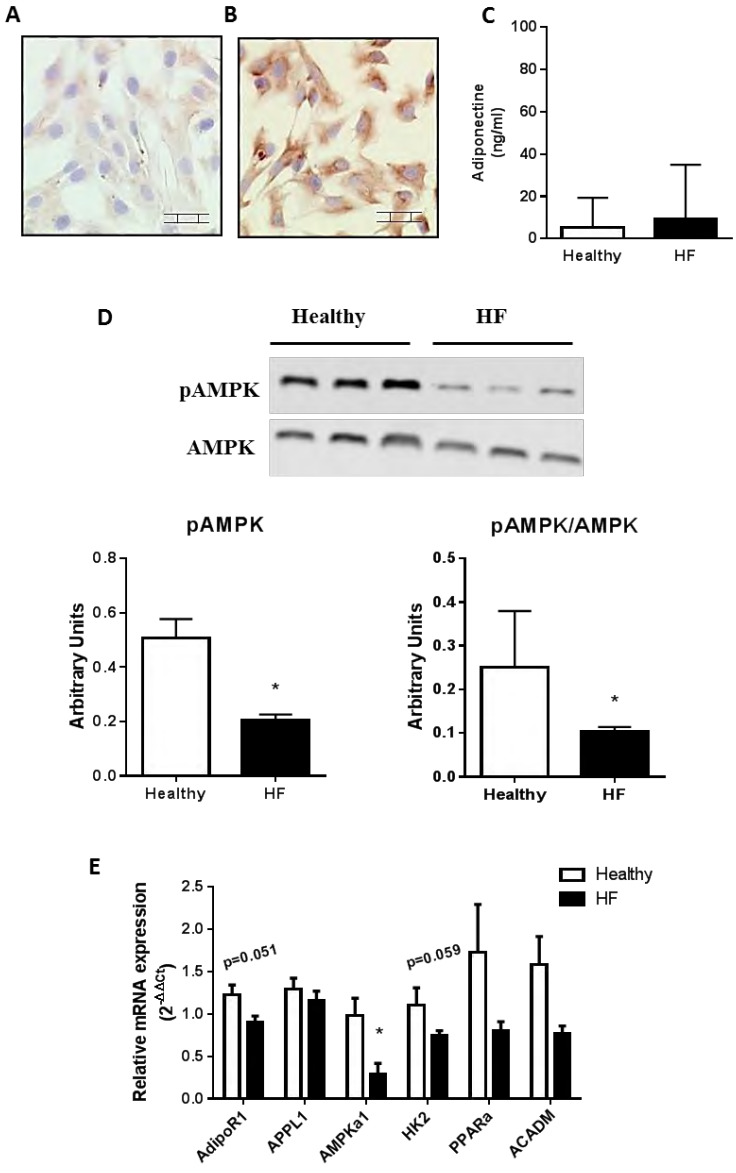

Figure 1. Adiponectin and genes involved in lipid- and glucose metabolism in primary muscle cell cultures of HFrEF patients and control subjects

Adiponectin protein expression in myoblasts from control subjects **(A)** and HFrEF patients **(B)**; 20x magnification; scale bar= 200µm. Adiponectin levels in culture supernatant measured by ELISA **(C)**. Representative western blots and mean protein expression values of phosphorylated AMPK and total AMPK **(D)**. mRNA content of metabolic gene expressions quantified by RT-PCR **(E)**. Data are represented as mean ± SEM. (N=8) *p<0.05 vs. baseline.

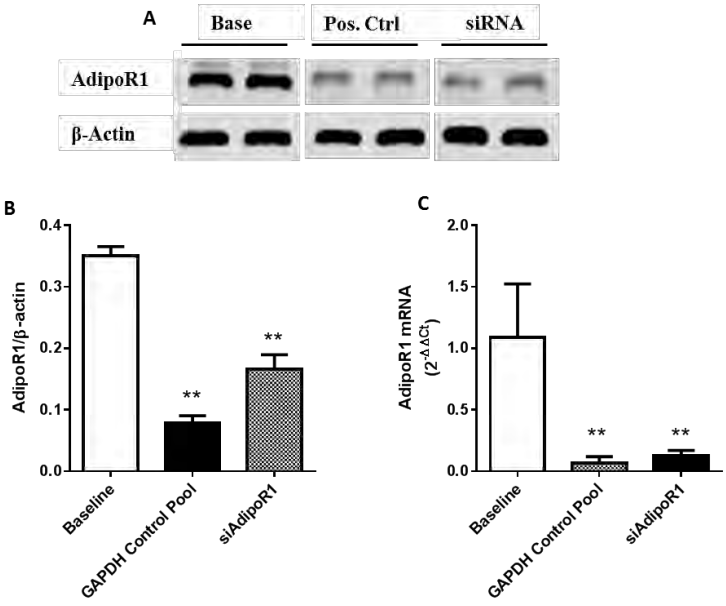

Figure 2. Silencing of AdipoR1 by siRNA

Silencing efficiency of AdipoR1 protein and mRNA content in primary myotubes cultures transfected with anti-AdipoR1 siRNA was monitored by western blot **(A, B)** and RT-PCR **(C)**. ON-TARGETplus GAPDH Control Pool was used as a positive control. N=5. **p<0.01 vs. baseline.

TNF-α exposure down-regulates the adiponectin signaling pathway

Silencing of AdipoR1 and/or treatment with TNF-α did not alter the expression of adiponectin mRNA (siAdipoR1 transfected myotubes 1.43 fold up-regulation vs baseline; myotubes incubated with TNF-α 1.25 fold up-regulation vs baseline; TNF-α activated, siAdipoR1 transfected cells 2.11 fold up-regulation vs baseline). Likewise, silencing of AdipoR1 and/or treatment with TNF-α did not influence the expression of adiponectin protein (**Fig. 3A, 3B**). Moreover, inhibition of AdipoR1 did not affect the mRNA expression levels of AMPK, APPL1, HK2, PPARα and ACADM (all p>0.05, **Fig. 3C**). In contrast, incubation with TNF-α resulted in reduced mRNA expressions of AdipoR1, APPL1, AMPK, PPARα, and ACADM (p<0.0001; p=0.0002; p=0.021; p=0.025; p=0.003 respectively, TNF-α activated vs baseline cells; p<0.0001; p=0.0006; p=0.019; p=0.035; p=0.023; p=0.037 respectively, for TNF-α activated, siAdipoR1 transfected vs baseline cells; **Fig. 3C**). The phosphorylation of AMPK was significantly reduced in siAdipoR1 transfected as well as in TNF-α activated cultures (p<0.010 for all vs baseline), with no significant difference in total AMPK protein expression, suggesting a decreased activation of AMPK (**Fig. 3C**). In addition, exposure of myotubes to TNF-a led to a significant increase in adiponectin secretion (p=0.008).

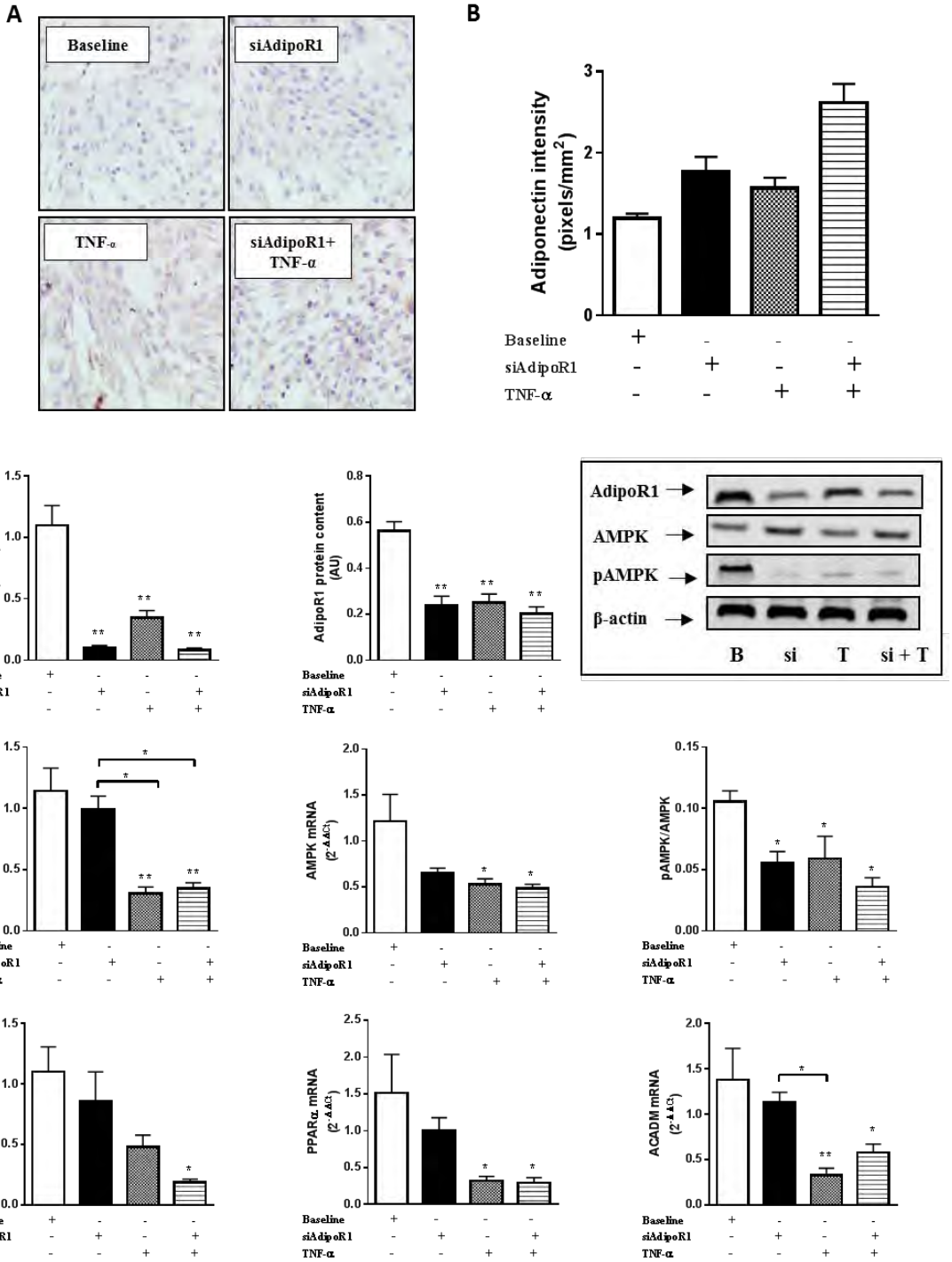
A
Baseline
siAdipoR1
TNF-α
siAdipoR1+ TNF-α
B
Adiponectin intensity (pixels/mm2)
Baseline
siAdipoR1
TNF-α
C
AdipoR1 mRNA (2-ΔΔCt)
AdipoR1 protein content (AU)
AdipoR1
AMPK
pAMPK
β-actin
B
si
T
si + T
APPL1 mRNA (2-ΔΔCt)
AMPK mRNA (2-ΔΔCt)
pAMPK/AMPK
HK2 mRNA (2-ΔΔCt)
PPARα mRNA (2-ΔΔCt)
ACADM mRNA (2-ΔΔCt)

Figure 3. Impact of AdipoR1 silencing on adiponectin signalling in the presence or absence of TNF-α

Immunohistochemical staining of adiponectin within primary myotubes cultures **(A)**. A semi-quantitative analysis was used for the analysis of adiponectin intensity **(B)**. mRNA expression of AdipoR1, APPL1, AMPK, HK2, PPARα and ACADM in myotubes from control subjects cultured for 72 hours with siAdipoR1, TNF-α or both using quantitative RT-PCR. Western blot was done to determine AdipoR1 protein content and phosphorylation of AMPK Representative western blots (a single control subject) and statistical graphs (mean values of all controls) are shown **(C)**. Data are represented as mean ± SEM. p-values refer to the pairwise comparison with regard to the baseline. *p<0.05; **p<0.01. N=8.

TNF-α exposure disrupts mitochondrial function and decreases myogenesis

SiAdipoR1 transfection did not induce statistical differences in mitochondrial biogenesis-related genes when compared to baseline myotubes, whereas exposure of myotubes to TNF-α significantly reduced the mRNA expression of FOXO3 (p=0.018 vs baseline) but not of PGC-1α (**Fig. 4A, 4B**). TNF-α further attenuated the mRNA expression of the differentiation markers myogenic differentiation 1 (MyoD1) and myogenin in both the absence (p=0.053 and p=0.048, respectively) and presence of siAdipoR1 (p=0.047; p=0.031 respectively) compared to baseline (**Fig. 4C, 4D**).

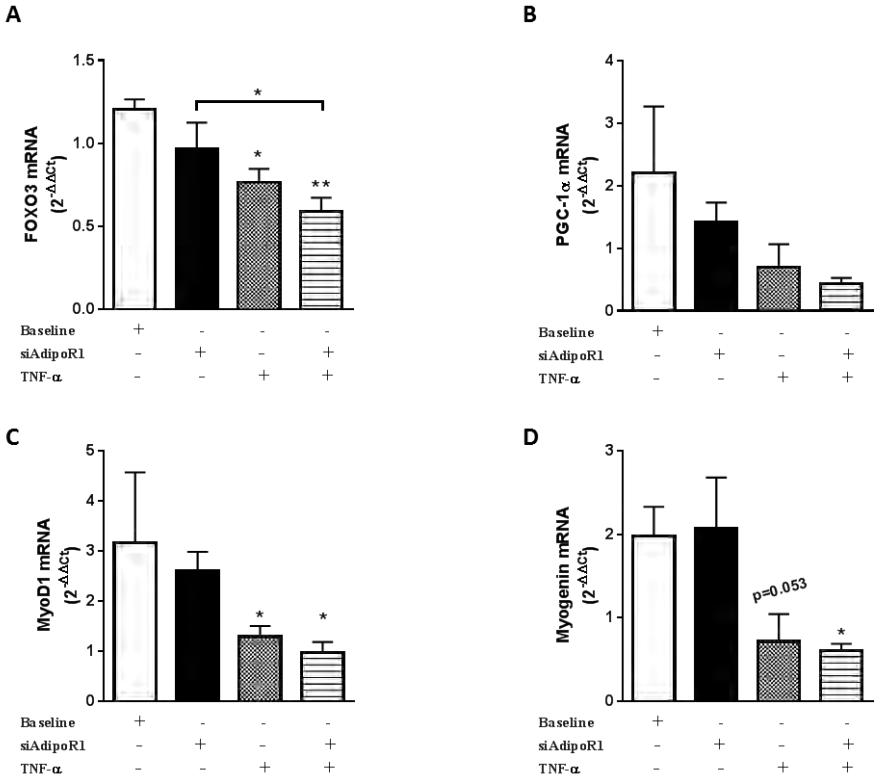

Figure 4. Impact of AdipoR1 silencing and TNF-α exposure on mitochondrial biogenesis and myogenesis in primary myotubes cultures

mRNA expression levels of genes involved in mitochondrial metabolism (FOXO3) **(A)**, biogenesis (PGC-1α) **(B)** and myogenesis (MyoD1, Myogenin; **C, D)** were determined in control myotubes cultures treated with siAdipoR1, TNF-α or both for 72h. Data are represented as mean ± SEM. *p<0.05; **p<0.01 vs. respective baseline or for pairwise comparisons as indicated by brackets. N=8.

Silencing of AdipoR1 decreases myoblast proliferation capacity

Results on myoblast proliferation were obtained by recording the cellular impedance, represented by the CI over time, using the xCELLigence RTCA system. SiAdipoR1 myoblasts reached their maximum CI at 116.55h ± 1.12, siAdipoR1 cells also treated with TNF-α had a maximum CI at 97.45h ± 0.54, whereas baseline cultures showed a maximum CI at 84.27 ± 1.42 (**Fig. 5A, 5B**). The time to reach the maximum CI was significantly delayed in siAdipoR1 cultures and in siAdipoR1cultures treated with TNF-α ($p<0.0001$ vs baseline). TNF-α treatment, however, partially restored myoblast proliferation over time (siAdipoR1 vs. siAdipoR1+TNF-α $p<0.0001$). Overall, the time needed to reach the maximum CI was negatively correlated with the mRNA expression of AdipoR1 (maximal CI over time, $r=-0.732$, $p=0.003$; **Fig. 5C**).

Phenotypical alterations

SiAdipoR1 myotubes demonstrated no differences in cellular senescence, morphology, viability and cytokine secretion compared with baseline cultures (**Fig. 6**). However, cellular senescence, as assessed by the percentage of SA-β-gal positive cells, was increased in siAdipoR1 myotubes that were treated with TNF-α ($p=0.010$ vs. baseline, **Fig. 6A**). TNF-α treatment further induced substantial morphological changes in myotubes including a disorganization of the actin microfilaments (**Fig. 6B**). Cell viability decreased after treatment with TNF-α ($p=0.029$). Furthermore, TNF-α treatment of siAdipoR1 myotubes was accompanied by an increased secretion of IL-10, IL-1β and IFN-γ ($p<0.0001$; $p=0.002$; $p=0.023$; respectively vs baseline; **Fig. 6D**).

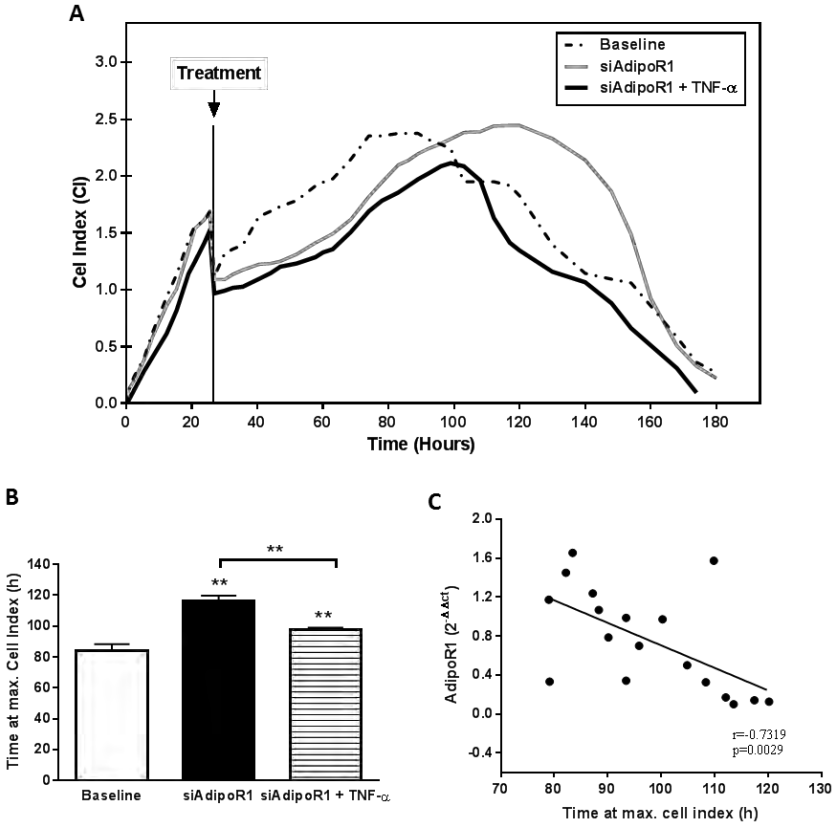

Figure 5. Representative growth kinetics of myoblasts transfected with siAdipoR1 and TNF-α

Growth curves were generated using the xCELLigence system **(A)**. Time (h) needed to reach maximal cell index (CI) **(B)**. Scatter plot of the correlation between mRNA expression levels of AdipoR1 and time at maximal CI **(C)**. Data are represented as mean ± SEM. N=8. *p<0.05; **p<0.01 vs. respective baseline or for pairwise comparisons as indicated by brackets.

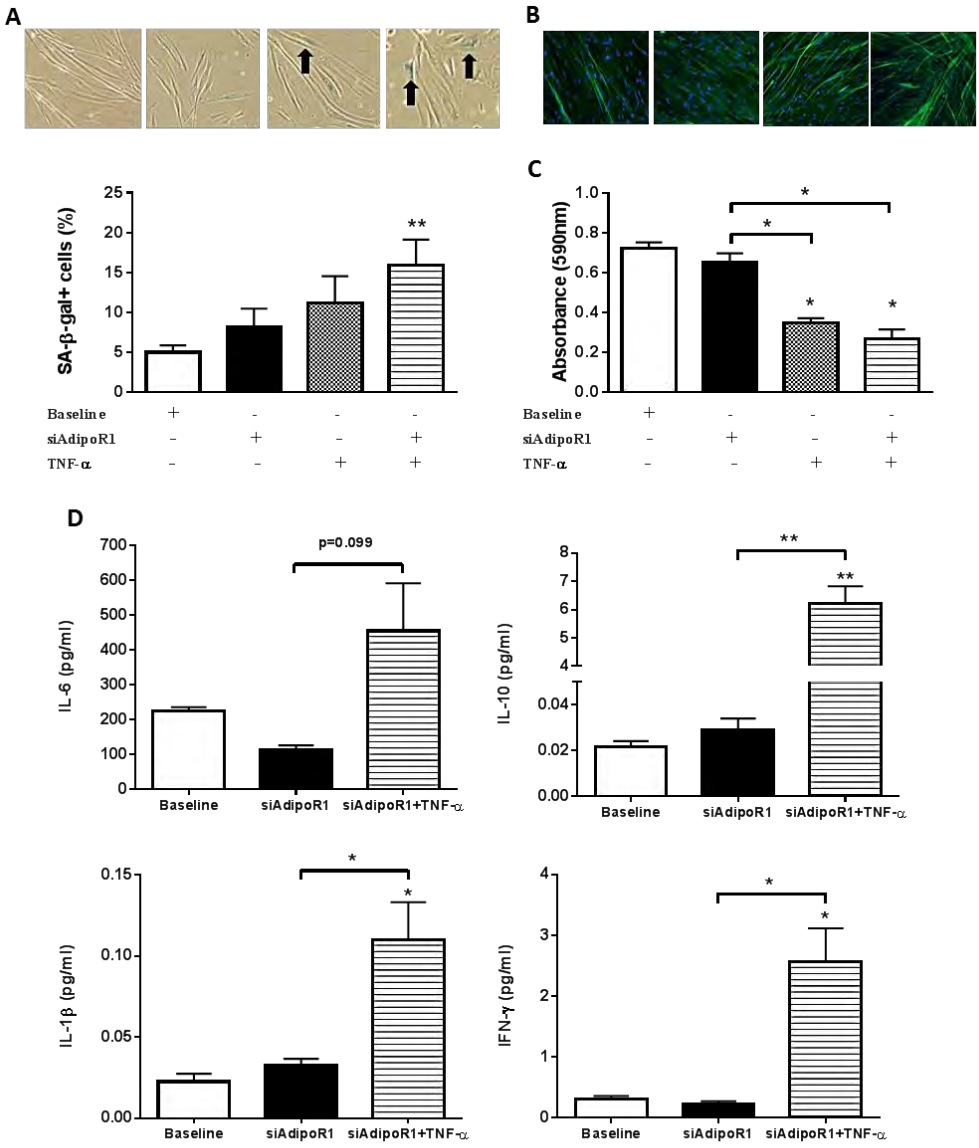

Figure 6. Effect of AdipoR1 silencing and TNF-α on myotubes morphology, senescence, viability and cytokine secretion

Representative images from myotubes stained with the senescent marker SA-β-gal **(A)**. Arrows indicate myotubes exhibiting a strong perinuclear blue cytoplasm staining (⬆). Actin microfilaments where visualized by phalloïdin staining **(B)**, 20X magnification. Effect of siAdipoR1, TNF-α or both on myotubes viability assessed by MTT absorption **(C)**. Supernatant levels of IL-6, IL-10, IL-1β and IFN-γ were quantified by Meso Scale Discovery (MSD) **(D)**. Data are represented as mean ± SEM. N=8. *p<0.05; **p<0.01 vs. respective baseline or for pairwise comparisons as indicated by brackets.

DISCUSSION

In the present study we aimed to gain insight into the roles of AdipoR1 and TNF-α in the process of skeletal muscle adiponectin resistance in HFrEF. The main findings of our study can be summarized as follows:

(1) Primary cultures from the skeletal muscle of HFrEF patients preserve the principal characteristics of adiponectin resistance *in vivo*, including a reduced expression of AdipoR1 and several downstream genes with impaired cellular energy metabolism signalling as a prominent feature.
(2) Silencing of AdipoR1 in healthy donor cultures attenuates myoblasts proliferation and AMPK activation.
(3) TNF-α exposure of healthy donor myoblasts impairs adiponectin signalling (i.e.; reduced AdipoR1, AMPK, PPARα and ACADM), decreases myogenic differentiation capacity and polarizes cytokine secretion toward a growth-promoting phenotype. The siAdipoR1-induced reduction in myoblast proliferation was partially restored by TNF-α.

Features of impaired skeletal muscle adiponectin signalling in HFrEF are preserved *in vitro*

The concept of a deregulated adiponectin system in HFrEF was first introduced by Kintscher and Skurk (30, 31). These authors reasoned that adiponectin in HFrEF, present at a high level in the blood stream of these patients, could probably no longer exert its cardiovascular protective actions and that there might be a local adiponectin system in the human heart. At that time, a decreased mRNA and protein expression of AdipoR1 was observed in the left ventricle of infarcted mice hearts, supporting the notion of an adiponectin resistance at the receptor level induced by the failing myocardium (30). A few years later, Van Berendoncks et al. described the existence of a functional adiponectin resistance in skeletal muscle of patients with mild to moderate HFrEF (10). In the present study, we demonstrate that features representing defects in adiponectin signalling in skeletal muscle from HFrEF patients are adequately preserved in primary cell cultures. Our findings are consistent with the results reported by McAinch et al. showing that myotubes initiated from severely obese and diabetic subjects retain the *in vivo* changes in cellular regulation *in vitro*, reflecting the metabolic physiology of the muscle donor (32). Thus, although adiponectin is primarily secreted by white adipose tissue, recent evidences demonstrated that adiponectin is also produced by non-

adipose tissues and cell types such as murine and human cardiomyocytes, osteoblasts, hepatocytes, C_2C_{12} myoblasts and skeletal muscle fibers (10, 33-38). In our study, there was no difference in the amount of adiponectin secreted by primary cultures of HFrEF patients and controls. This finding is in line with a recent study by Kreth et al., who showed that the amount of secreted adiponectin by cardiomyocytes of HFrEF patients equals that of cardiomyocytes isolated from healthy myocardium (39). Further, we found that TNF-α treatment of primary human healthy myotubes increased the level of adiponectin in the culture supernatant. A previous report by Delaigle et al. already demonstrated that, in response to a cytokine combination of IFN-$_Y$ with TNF-α, the mRNA and protein expression levels of adiponectin become upregulated *in vivo* and *in vitro* in human and rodent myotubes (34).

TNF-α as a major component in the pathogenesis of impaired adiponectin signalling

We confirm here that inhibition of AdipoR1 leads to a significant decrease in AMPK activation. AMPK is a major key player in energy homeostasis and therefore, a reduced activation capacity of AMPK signalling disturbs glucose metabolism. In this regard, Yamauchi and Iwabu et al. have shown that skeletal muscle AdipoR1 null-mice are glucose intolerant and insulin resistant as a result from a lack of adiponectin-dependent activation of AMPK (12, 13). Furthermore, Koentges and colleagues recently studied the role of AdipoR1 in the regulation of myocardial energetics and revealed that the AMPK/PPARα/PGC-1α signalling axis is impaired in AdipoR1$^{(-/-)}$ hearts of diabetic mice (14). Diabetes is known to be associated with elevated levels of TNF-α (40). In our study, the expression levels of several downstream adiponectin-related genes, including AMPK, PPARα and ACADM were reduced if siAdipoR1 myoblasts also received co-treatment with TNF-α. Exposure of healthy human myoblasts to TNF-α, however, sufficed to induce a significant loss of AdipoR1 expression and to promote a reduction in the expressions of APPL1, AMPK, PPARα and ACADM. In previous studies, TNF-α addition to primary cultures of rat neonatal cardiomyocytes or cultured L6 myotubes induced a decrease in the mRNA and protein expression levels of AdipoR1 and AMPK, whereas mice with cardiac over-expression of TNF-α demonstrated a reduced expression of PPARα, PGC-1α and a diminished FAO capacity (41-43). In this regard, Steinberg et al. also showed that TNF-α signalling through TNFR1 suppresses AMPK activity and that TNFR1 null-mice thus display a lower activation and phosphorylation of AMPK (43). The precise mechanism by which TNF-α decreases the expression of AdipoR1 and AMPK in skeletal muscle, however, is not clear but could possibly involve the phosphatidylinositol 3-kinase (PI3K)/Akt and FOXO1 pathways (44-47).

Furthermore, in a study by Bordenave et al., exercise training of patients with type 2 diabetes improved muscle mitochondrial oxygen consumption and lipid oxidation and this appeared to be independent of the circulating concentrations of insulin and adiponectin (48). These data suggest that skeletal muscle adiponectin resistance in HFrEF may result from metabolic, mitochondrial and inflammatory defects which *de facto* precede alterations in circulating adiponectin.

TNF-α exposure induces pro-inflammatory and structural alterations in primary myotubes cultures

Declines in skeletal muscle oxidative capacity and mitochondrial biogenesis as well as an impaired myogenesis are characteristic symptoms of HFrEF patients (49). In this regard, Martinez et al. observed a reduced myogenin protein expression in the skeletal muscle of rats with myocardial infarction-induced heart failure (50). Down-regulation of the myogenic regulatory factors MyoD1 and myogenin has been accompanied by impairment of myoblast differentiation and induction of fibre-type shift in the glycolytic direction, resulting in a decreased oxidative capacity and mitochondrial content of muscle cells (51-53). In our study, TNF-α attenuated the expression of MyoD1 and myogenin in myotubes whether the receptor AdipoR1 had been silenced or not. We obtained similar results for FOXO3. It has been shown that FOXO3 regulates mitochondrial metabolism and reduces oxidative stress, but can also induce muscle wasting via an E3 ubiquitin ligase atrogin-1/muscle atrophy F-box (MAFbx)/muscle ring finger-1 (MuRF-1) dependent pathway (54). The literature on TNF-α and FOXO3, however, is conflicting (54, 55). Yet, Li et al. showed that treatment of human aortic endothelial cells with AICAR, an AMPK activator, leads to increased FOXO3 phosphorylation and nuclear translocation. This increase could be prevented by AMPK siRNA, indicating that AMPK is able to induce the activation of FOXO3 (55, 56). The inflammatory-induced decreases in MyoD1, myogenin and FOXO3 could therefore be important factors underlying altered muscle energy metabolism and impaired mitochondrial biogenesis in HFrEF patients.

AdipoR1 mRNA expression and proliferation rate of primary myoblasts

In our study, the proliferation rate of primary myoblasts cultures was delayed in case of siRNA silencing of AdipoR1. Overall, the degree of AdipoR1 mRNA expression was negatively correlated with the time needed to reach the maximum CI. In previous research,

metabolic disturbances such as hyperglycaemia, mitochondrial dysfunction and decreased activation of AMPK were shown to impair the growth of porcine primary vascular smooth muscle cells and porcine myoblasts (25, 57, 58). Indeed, optimal energy homeostasis is of crucial importance to promote cell survival and cell growth. In our study, there was however, no correlation between AMPK activation and the proliferation rate of siAdipoR1 myoblasts cultures. Podbregar and co-workers showed that prior exposure of cultured human skeletal muscle cells to TNF-α leads to increased IL-6 secretion (59). IL-6, but also IL-1β and IFN-γ, can promote myoblast proliferation and myotubes formation (60-62). In this regard, the altered cytokine production profile induced by treatment of siAdipoR1 myoblasts with TNF-α may have contributed to the recovery in cell proliferative capacity. The higher cellular senescence and apoptotic scores in response to TNF-α, however, might have hampered the normalization of myoblast proliferation to control levels.

CONCLUSION

In conclusion, features of adiponectin resistance are preserved in primary cultures from the skeletal muscle of HFrEF patients. Silencing of AdipoR1 attenuates myoblasts proliferation and activation of AMPK. Exposure of primary myotubes to TNF-α leads to impairment of adiponectin signalling, myogenesis and mitochondrial biogenesis, suggesting that an increased inflammatory constitution contributes to adiponectin resistance and skeletal muscle dysfunction in HFrEF.

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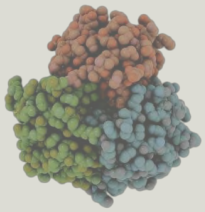

Chapter 7

Heart failure myoblast proliferation and relation with skeletal muscle features

Adapted from:

Sente T, Van Berendoncks AM, Beckers P, Possemiers N, Wouters A, Huyge I, Fransen E, Vrints CJ, Hoymans VY. Association between primary myoblasts proliferation and parameters of functional capacity. Cardiovasc Pathol. Sep. 2016

ABSTRACT

Objective: Patients with chronic heart failure and reduced ejection fraction (HFrEF) are characterized by exercise intolerance and peripheral muscle abnormalities. In **chapter 5**, we demonstrated that primary myoblasts from chronic HFrEF patients have impaired proliferative activity. The present study was designed to investigate whether myoblast proliferation is associated with the degree of impairment in skeletal muscle mass, strength and exercise capacity. **Methods and results:** Primary myoblasts cultures were initiated from the skeletal muscle (*m. vastus lateralis*) of seven chronic HFrEF patients (mean LVEF 24.71 ± 3.49%) and eleven healthy subjects. All subjects underwent clinical assessments including anthropometric and muscle strength measurements and cardio-pulmonary exercise testing. Compared to controls, patients showed a reduction in exercise capacity ($\dot{V}O_2$ peak, maximal workload, work efficiency), leg lean mass and muscle strength (upper and lower limbs, quadriceps; all $p<0.05$). The myogenic differentiation potential of the myoblasts was similar between groups. The *in vitro* myoblast proliferation of myoblast, as evaluated by xCELLigence technology, was postively associated with leg lean mass (partial R-square=0.472, $p<0.01$), VO_2 peak (partial R-square=0.438, $p=0.012$) and work efficiency (partial R-square=0.239, $p=0.026$), when accounted for disease status. **Conclusion:** The proliferative ability of primary human myoblasts *in vitro* is associated with leg lean mass and parameters of exercise capacity.

Keywords: Heart failure, muscle atrophy, exercise capacity, myoblast proliferation, xCELLigence

INTRODUCTION

Patients with chronic heart failure and reduced ejection fraction (HFrEF) have a wide range of structural and functional disorders including hemodynamic, neurohormonal and metabolic changes (1, 2), which severely compromise quality of life. One of the major disabling symptoms in chronic HFrEF is exercise intolerance. Patients with HFrEF encounter great difficulties in doing submaximal performances during prolonged time, and thus, with fulfilling activities of daily life. Skeletal muscle weakness, along with impaired aerobic capacity, is an important determinant of exercise intolerance in HF. Weakness of the skeletal muscles is largely dependent on the quantity of skeletal muscle mass and the quality of contractile proteins (3, 4). Loss of skeletal muscle mass already occurs early in HFrEF and has been linked to reduced maximal isometric muscle strength and low peak oxygen uptake (VO_2 peak) (5).

Satellite cells act as myogenic precursors and are required for skeletal muscle regeneration (6). In response to exercise or muscle trauma, satellite cells become activated, proliferate as myoblasts, and then differentiate and either fuse into existing damaged muscle fibers or form *de novo* myofibers (6, 7). Satellite cell dysfunction is therefore considered a major underlying factor contributing to muscle wasting (8). In **chapter 5**, we demonstrated that satellite cell-derived myoblasts isolated from the *m. vastus lateralis* of chronic HFrEF patients are characterized by a time delay in proliferative activity (9). The aim of the present study was to examine whether primary myoblast proliferation is inextricably linked with the degree of impairment in skeletal muscle mass, contractile strength and parameters of exercise capacity, including VO_2 peak, maximal work load and work efficiency.

MATERIAL AND METHODS

Patients and controls

Seven chronic HFrEF patients with stable and optimally treated systolic dysfunction (LVEF <35%; NYHA II-III) due to ischemic heart disease were recruited from the Heart Failure Clinic of the Antwerp University Hospital (UZA). Patients with recent acute coronary syndrome or revascularization (≤3 months), valvular disease requiring surgery, myocarditis or pericarditis, cancer, inflammatory or infectious (acute/chronic) diseases, diabetes mellitus, renal failure, or allergic conditions were excluded. A group of eleven sedentary healthy

subjects without cardiovascular disease were enrolled as controls. The study was approved by the local Ethics Committee (UZA - University of Antwerp) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Clinical and biochemical analyses

All patients and controls underwent a clinical evaluation, echocardiography and a symptom-limited cardiopulmonary exercise test (CPET) on a bicycle (Ergoline, Schiller AG, Baar, Switzerland) in non-fasting conditions (10). Body composition was determined using a Lunar Prodigy Advance Full Size Dual-energy x-ray absorptiometry (DEXA) scanner (GE Healthcare, Diegem, Belgium). The 1-repeated maximum (1-RM) of different skeletal muscle groups was assessed as an indicator of muscle strength using a multifunctional fitness machine (Unica, Technogym, Gambetola, Italy). Fasted blood samples were collected in serum tubes, centrifuged at 3000 rpm for 15 min. and frozen at -80°C. Creatinine and high sensitivity C-reactive protein (hsCRP) were assessed (Dimension Vista 1500 instruments, Siemens Healthcare Diagnostics NV/SA, Beersel/Huizingen, Belgium).

Primary skeletal muscle cell cultures

Muscle biopsies *(m. vastus lateralis)* were taken using the Bergström needle technique as previously described in **chapter 4** (9, 11-13). Biopsy samples were minced and enzymatically dissociated by a series of incubations in 0.05% trypsin/EDTA/collagenase. Satellite cells were collected, suspended in skeletal muscle growth medium (SKGM) and cultivated in culture flasks. Growth medium was refreshed every two days until cells reached 70% confluence. Cultures were expanded for three passages. Desmin staining (1:100, Sigma-aldrich, St. Louis, MO, USA) was used to verify myogenic purity.

FACS analysis

Cell viability and expression of the myogenic markers paired box 7 (Pax7) and myogenic differentiation 1 (MyoD1) were evaluated by flow cytometry as reported in **chapter 5** (9). Cultures were seeded in six-well culture plates (2.5×10^4 cells/well) and grown for 48 hours. The following phycoerythrin conjugated primary antibodies were used: rabbit anti-human Pax7 and rabbit anti-human MyoD1 (Bioss Inc., Woburn, MA, USA). Viability was assessed using Annexin-V and 7-AAD according to the manufacturer's instructions (Becton Dickinson, Biosciences, Erembodegem, Belgium). Events were recorded and analyzed using a FacsCantoTM II flow cytometer (Becton Dickinson) and FacsDiva 6.1.2 software.

xCELLigence cell proliferation assay

Proliferation was monitored using the xCELLigence Real-Time Cell Analyzer RTCA (Westburg, Leusden, The Netherlands). In **chapter 4**, we evaluated this assay for cell proliferation of myoblast cultures with results compared by direct cell counting and a conventional colorimetric assay (e.g.; MTT assay). Myoblasts were seeded in special modified microtiter 16-well plates (E plates). Each well bottom was covered with interdigitated gold micro-electrodes in order to detect cell-to-electrode impedance responses. E-plates were placed into the RTCA device station, located in a humidified incubator at 37°C and 5% CO_2 and connected to an outside electronic analyzer. Background impedance (SKGM) was determined and subsequently, myoblasts suspension was added. At start, optimal conditions were determined for cell concentration. For this purpose, different myoblast cell numbers (625–2x10^4 cells/well) were seeded in the E-Plate. Impedance was monitored with a programmed signal detection schedule in 15-minute intervals for a total of 180 hours. The impedance value of each well was expressed as a cell index (CI) value, calculated as the CI at a given time point subtracted by the CI at baseline (SKGM medium). Two replicates of each myoblast culture were monitored and averaged for statistical analysis.

Statistical analysis

Differences between groups were compared using the Mann–Whitney U test. Associations between proliferation rate and different outcomes, accounting for disease status (i.e.; HFrEF or healthy control), were analyzed by multiple linear regression. Briefly, we fitted a model including proliferation, group and their interaction as independent variables and each of the outcomes as a dependent variable. In this model, we tested the significance of the interaction term to analyze whether the association between the outcome and the proliferation rate was different between both groups. In case of a significant interaction, separate models were fitted for both groups. In case the interaction was not significant, the model was refitted with only main effects for proliferation rate and group. In this latter case, the association between proliferation rate and outcome is not significantly different between both groups. All analyses were performed using the software package statistical package for the social sciences (SPSS, IBM SPSS Statistics Inc, Version 20.0, Chicago, IL, USA). Significance was accepted at the 5% level ($p < 0.05$). Data are represented as mean ± standard error of the mean (SEM).

RESULTS

Clinical characteristics

Baseline clinical characteristics of HFrEF patients and healthy controls are shown in **table 1**. Patients and controls were matched for gender, weight and body mass index (BMI). Patients, however, were older (52.45±0.87; controls and 57.57±5.24; HFrEF; p=0.026), had higher serum hsCRP (p<0.001) and demonstrated significant reductions in $\dot{V}O_2$ peak (p=0.003), maximal workload (p<0.001) and work efficiency (p=0.009).

Table 1 Clinical characteristics

Characteristic	Controls N=11	HFrEF N=7	*p-value*
Age (years)	52.45 ± 0.87	57.57 ± 5.24	**0.026**
Gender, % male (n)	27.3 (3)	28.6 (2)	0.623
Weight (kg)	85.42 ± 3.45	78.99 ± 7.31	0.211
BMI (kg/m^2)	27.56 ± 1.11	25.31 ± 1.80	0.328
LVEF (%)	N.A.	24.71 ± 3.49	N.A.
NYHA class (II/III) (n/n)	N.A.	3/4	N.A.
VO_2 peak (ml/kg/min)	35.89 ± 2.01	18.12 ± 1.47	**0.003**
Maximal workload (Watt)	222.73 ± 11.13	90.0 ± 11.95	**<0.001**
Work efficiency (Watt/VO_2)	6.33 ± 0.18	4.94 ± 0.36	**0.009**
Creatinine (mmol/L)	0.82 ± 0.05	1.18 ± 0.69	0.390
hsCRP (mg/L)	0.26 ± 0.11	1.33 ± 0.16	**<0.001**

HFrEF, Heart failure with reduced ejection fraction; BMI, Body mass index; LVEF, Left Ventricular Ejection fraction; NYHA, New York Heart Association; hsCRP, high sensitivity C-reactive protein. Data are expressed as mean ± SEM. Significant p-values are highlighted in bold.

Skeletal muscle features

Leg lean mass (p=0.044), muscle strength in both upper and lower limbs (p<0.001) and quadriceps muscle strength (p=0.002) were significantly reduced in HFrEF patients. Culture viability of myoblasts from HFrEF patients was not different from control myoblasts (p=0.131 for Annexin-V+/7-AAD-; p=0.439 for Annexine-V+/7-AAD+; **table 2**). No differences were detected in the proportion of desmin positive cells between HF and control cultures (p=0.099). In addition, no differences were found between myoblasts of patients and healthy controls in the expression level of Pax7 and MyoD1 (p=0.947 and p=0.912, respectively), pointing toward a similar myogenic potential of the myoblast populations.

Dynamic monitoring of HFrEF myoblast cells

As shown in **chapter 4**, the xCELLigence RTCA proliferation assay produced a continuous growth curve (**Fig. 1**). Three distinct phases could be observed, (I) the cell attaching stage, characterized by a rapid increase in cellular impedance, (II) a static lag phase in which myoblasts likely fully spread but do not actively proliferate and III) a log or exponential phase representing myoblast proliferation. The CI's of the conditions with the two highest seeding cell numbers (i.e.; $1x10^4$ and $2x10^4$ cells/well at start) showed a continuous increase in CI over the first 100 hours. Then, the CI's began to decrease, suggesting that the myoblasts had reached confluence and started to die due to contact inhibition. In the other conditions, (i.e.; 625-5000 cells seeded/well) HFrEF myoblasts continued to proliferate for up to 120 hours. Since the myoblast population with $1x10^4$ cells/well at start reached its highest level of CI around 96h without entering the confluent phase, we considered this population as the optimal cell density for the study of chronic HFrEF myoblast proliferation.

Table 2 Skeletal muscle characteristics

Characteristic	Controls N=11	HFrEF N=7	*p-value*
Myogenicity			
Desmin (%)	92.88 ± 0.93	86.03 ± 4.96	0.099
Pax7 (%)	85.44 ± 2.47	85.49 ± 3.95	0.947
MyoD1 (%)	47.50 ± 3.02	47.88 ± 3.95	0.912
Viability			
$Annexin^+/7\text{-}AAD^-$	7.70 ± 0.65	6.00 ± 0.56	0.131
$Annexin^+/7\text{-}AAD^+$	2.50 ± 0.35	2.15 ± 0.29	0.439
Growth Characteristics			
Maximum cell index	1.46 ± 0.02	1.33 ± 0.04	**0.008**
Time to reach max. cell index (h)	99.20 ± 0.48	140.66 ± 0.83	**<0.001**
Proliferation rate (CI/h; 24-96h)	0.014 ± 0.0007	0.009 ± 0.0010	**0.001**
Muscle Mass			
Total lean mass (kg)	55.67 ± 3.36	51.75 ± 3.83	0.328
Leg lean mass (kg)	19.64 ± 1.37	15.11 ± 1.48	**0.044**
Muscle Strength			
Upper limbs (kg)	57.90 ± 1.91	34.56 ± 3.54	**<0.001**
Lower limbs (kg)	47.43 ± 1.82	29.34 ± 2.81	**<0.001**
Quadriceps (kg)	31.87 ± 1.47	22.71 ± 1.34	**0.002**

HFrEF, Heart failure with reduced ejection fraction. Pax7, paired box 7; MyoD1, Myogenic differentiation 1. Data are expressed as mean ± SEM. Significant p-values are highlighted in bold.

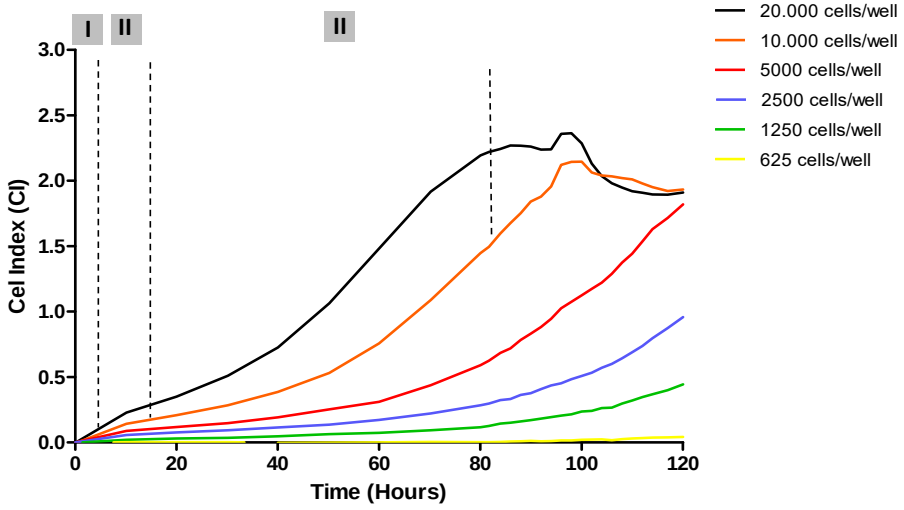

Figure 1. Dynamic monitoring of the impedance-based proliferation of myoblasts

Myoblast cells were seeded at different cell densities (625 - 20 000 cells/well) and growth curves were continuously monitored for up to 120 hours and recorded using the impedance-based xCELLigence RTCA System.

Impaired proliferation of myoblasts in HFrEF

Figure 2 demonstrates the proliferation curve of HFrEF and control myoblasts derived from xCELLigence plate readings. During the logarithmic growth phase (48 to 96h), the proliferation of myoblasts from healthy controls was more pronounced. In particular, the time to reach the maximal CI ($p<0.001$; **Fig. 2A, 2B**) was significantly different between cultures from patients and controls. The CI from control myoblasts peaked already at 99.20 ± 2.48h, whereas HFrEF myoblasts reached their maximum CI at 140.66 ± 5.83h. Furthermore, the mean proliferation rate (i.e.; cell index over time) of control myoblasts was significantly higher compared to HFrEF myoblasts ($p=0.001$; **Fig. 2A, 2C**).

Myoblast proliferation is associated with leg lean mass

The effect of myoblast proliferation on leg lean mass, total lean mass and muscle strength, including quadriceps, upper and lower limbs strength, was not significantly different between HFrEF patients and healthy controls. However, myoblast proliferation is strongly associated with leg lean mass (partial R-square=0.472, $p<0.001$; **Fig. 3A**) when accounted for disease status, but not with total lean mass, quadriceps muscle strength, or muscle strength of the upper and lower limbs (partial R-square=0.007 to 0.188, all $p>0.05$).

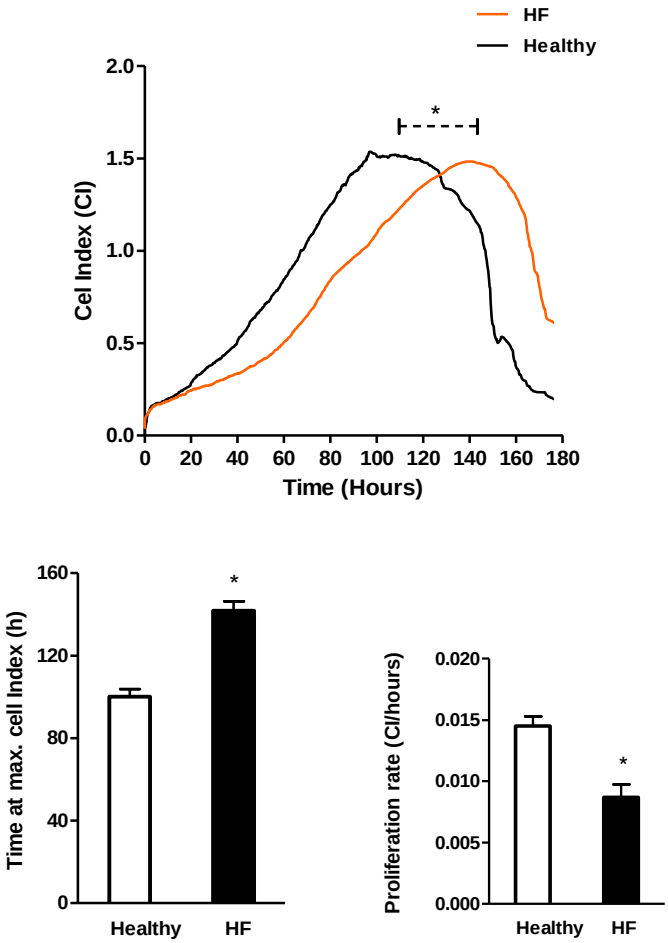

Figure 2. Proliferation kinetics of HFrEF and control myoblasts

Representative growth kinetics of HFrEF and control myoblasts **(A)**. Time to reach maximal cell index **(B)**. Mean rate of myoblast proliferation calculated as cell index over time (hours in culture) during a time period of 24 - 96 hours **(C)**. Results represent mean ± SEM. *p<0.05.

Myoblast proliferation is associated with parameters of exercise capacity

For none of the parameters of exercise capacity, any significant interaction was observed between myoblast proliferation and disease status. Myoblast proliferation was, however, associated with VO_2 peak (r=0.438, p=0.012) and work efficiency (partial R-square=0.239, p=0.026; **Fig. 3B, 3C**) when accounted for disease status. No association was present between proliferation and maximal workload (partial R-square=0.017, p>0.05).

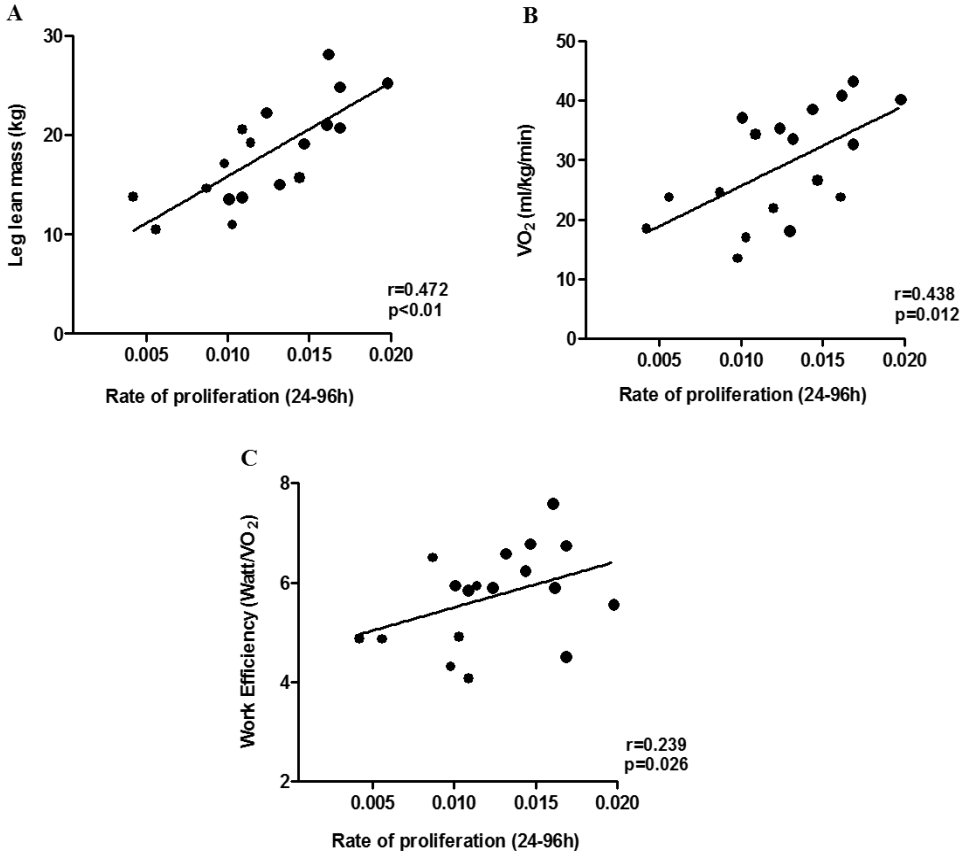

Figure 3. Associations between myoblast proliferation and *in vivo* muscle parameters

Scatter plots of the association between proliferation rate and leg lean mass **(A)**, VO_2 peak **(B)** and work efficiency **(C)**.

DISCUSSION

A major consequence of chronic HFrEF is the reduction in skeletal muscle mass resulting in progressive muscle weakness, degeneration and loss of muscle strength (5, 14). Using the xCELLigence RTCA technology, we recently reported (**chapter 5**) the presence of impaired proliferative activity of satellite cell-derived myoblasts in patients with HFrEF (9). We now extend our previous findings by demonstrating that the proliferative ability of primary myoblasts *in vitro* is associated with several *in vivo* parameters of functional capacity, including leg lean mass, VO_2 peak and work efficiency.

Muscle mass maintenance is mediated by mechanisms controlling the activation, proliferation, differentiation and self-renewal of satellite cells and myoblasts (15). Poor activation and proliferation cause a decline in the myoblasts population resulting into the incomplete replacing of skeletal muscle mass (16). In this regard, Zhang et al. observed less satellite cell activation and suppressed myotubes formation in isolated satellite cells from mice with chronic kidney disease(17). These mice also demonstrated abnormal muscle regenerative capacity, resulting in increased loss of muscle mass. Clinical studies further demonstrated that loss of skeletal muscle mass in non-cachectic patients with chronic HFrEF is associated with a reduction in VO_2 peak (18, 19). VO_2 peak is closely associated with mitochondrial function in HFrEF patients (20). Mitochondrial activity plays a central role in myoblast proliferation (21). In this respect, it has been shown that mitochondrial biogenesis is impaired in HFrEF (22). The positive impact of exercise, both physical training and a single bout of exercise, on satellite cell function is well recognized (23, 24). Resistance training, for instance, is able to increase satellite cell content, its activation status and myonuclear number, but also stimulates the proliferative and differentiation capacities of myoblasts (25-27). Besides a reduction in exercise capacity and advanced the advanced disease status, HFrEF patients with muscle wasting were also reported to have lower muscle strength (5). In this study, however, we did not find any association between primary myoblast proliferation and strengths of the quadriceps, or upper and lower limbs.

A potential limitation of this study is that age was significantly different between patients and controls. Baj et al., however, showed that donor age, with the study participants being between 41-91 years, has no effect on growth kinetics of primary skeletal muscle cells (28).

CONCLUSION

In conclusion, when compared to healthy controls, cultivated satellite cell-derived myoblasts from patients with chronic HFrEF demonstrate loss of proliferative activity. This ability of primary myoblasts to proliferate *in vitro* is closely associated with clinical parameters like leg lean mass and exercise capacity.

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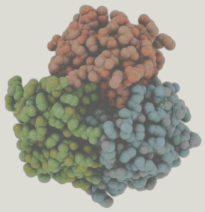

Chapter 8

Adiponectin, inflammation and skeletal muscle dysfunction in heart failure

Sente T, Beckers P, Van Berendoncks AM, Possemiers N, Lauwers P, Van Hoof V, Huyge I, Lemmens K, Vrints CJ, Hoymans VY. STAT3/TNFR2 signalling in skeletal muscle of chronic heart failure patients with reduced ejection fraction; relation with adiponectin, inflammation, exercise intolerance and skeletal muscle dysfunction. In preparation. 2016

ABSTRACT

Background: STAT3/TNFR2 signalling, possibly mediated via IL-6 and TNF-α, is emerging as a critical mediator of proliferation. In previous work, we observed that primary skeletal myoblasts from chronic HFrEF patients exhibit loss of proliferative and anti-inflammatory activities. **Purpose:** This study evaluates STAT3/TNFR2 signalling in skeletal muscle of chronic HFrEF patients and the relationship with adiponectin, exercise capacity and skeletal muscle mass and function. **Methods:** Biopsies from the *m. vastus lateralis* were obtained from 20 HFrEF patients (LVEF<35%, NYHA II-III) and 30 age- and gender- matched healthy subjects. Muscle tissue was examined for mRNA expression profiles of adiponectin, TNF-α, TNFR1/2, IL-6 and STAT3. Fasting blood samples were analyzed for TNF-α, sTNFR2, IL-6 and adiponectin using ELISA. All participants underwent cardio-pulmonary exercise testing. Quadriceps strength (1-RM) and cross-sectional area (CT scanning) were determined. **Results:** mRNA expression of STAT3 (p=0.043) and TNFR2 (p=0.028) were reduced in skeletal muscle tissue of HFrEF patients, whereas TNF-α mRNA was increased (p=0.043); IL-6 mRNA remained unchanged (p=0.067). pSTAT3 (p=0.032) and activation of STAT3 (pSTAT3/STAT3; p=0.008) were also reduced in HFrEF patients. Circulating TNF-α (p= 0.028), sTNFR2 (p=0.009) and IL-6 (p= 0.019) levels were increased in HFrEF patients. Both TNF-α and IL-6 showed a (near) significant negative correlation with the mRNA expressions of STAT3 (r=-0.498, p=0.048; r=-0.522, p=0.056) and TNFR2 (ρ=-0.463, p=0.064; ρ=-0.629; p=0.012). Circulating (p=0.049) and muscle adiponectin (p=0.044) concentrations were increased in HFrEF patients. Adiponectin in circulation correlated negatively with TNFR2 mRNA (ρ=-0.562, p=0.042), and positively with TNF-α mRNA (ρ=0.735, p=0.038) but not with the mRNA expressions of STAT3 and IL-6. In addition, circulating adiponectin showed a negative correlation with exercise capacity, quadriceps strength and quality, whereas the mRNA expression of adiponectin in skeletal muscle did not. STAT3/TNFR2 signalling in the skeletal muscle of HFrEF patients was not related with exercise capacity and neither with quadriceps strength, CSA or quality. **Conclusion:** These data indicate that STAT3/TNFR2 signalling in the skeletal muscle of HFrEF patients is attenuated. Both systemic inflammation and increased blood adiponectin are associated with impaired STAT3/TNFR2 signalling in HFrEF; no relationship however was found between STAT3/TNFR2 signalling and parameters of exercise capacity or skeletal muscle mass, strength and quality.

Keywords: Heart failure, skeletal muscle, adiponectin, TNFR2, STAT3

INTRODUCTION

Chronic heart failure (HF) with reduced left ventricular ejection fraction (HFrEF) is a clinical syndrome associated with diverse metabolic disturbances, including abnormalities of skeletal muscle energy metabolism. Skeletal muscle dysfunction is highly prevalent in patients with HFrEF (1-5). With increasing severity of disease, these patients lose muscle bulk, especially in their lower legs and arms (1). Yet, besides quantity, muscle quality is also affected in HFrEF (6). This skeletal muscle weakness in HFrEF negatively impacts exercise capacity (7-9). Poor physical capacity, including reduced quadriceps maximal isometric strength, peak oxygen uptake (peak VO_2) and VE/VCO_2 slope, is widely recognized as an independent predictor of worse prognosis in patients with HFrEF (10). Skeletal muscle abnormalities hence curtail the ability of HFrEF patients to perform normal daily life activities and comprise their active life expectancy (11, 12). The exact pathophysiological mechanisms underlying skeletal muscle dysfunction in HFrEF are however still unclear.

Systemic inflammation possibly contributes to HFrEF-related skeletal muscle changes. Toth et al. found that elevated serum levels of tumour necrosis factor-alpha (TNF-α) and interleukin (IL)-6 in HFrEF are associated with peripheral muscle alterations (most notably muscle loss, reduced muscle strength and contractile dysfunction) and decreased peak VO_2 (13). In addition, it has been shown that biopsy samples from the quadriceps muscle of HFrEF patients demonstrate changes suggestive of inflammation, such as an up-regulated expression of TNF-α (14). In previous work, we observed that satellite cell-derived myoblasts from HFrEF patients have a time delay in their proliferative activity, show a diminished expression of TNF-α receptor 2 (TNFR2; p75) and a reduced IL-6 secretion. Recent *in vitro* evidence from colon cancer cells has further demonstrated that signal transducer and activator of transcription 3 (STAT3), typically activated by circulating IL-6 or TNF-α, induces TNFR2 expression (15). STAT3 is involved in satellite cell proliferation and muscle repair and, as such, helps to counteract muscle loss (16-18). Hence, we speculate that systemic and local immune activation in HFrEF impairs muscle STAT3/TNFR2 signalling, thereby attenuating myoblast proliferation and promoting loss of muscle mass. A profound decreased expression and phosphorylation (i.e.; activation) of STAT3 has already been observed in the myocardium of patients with dilated cardiomyopathy (19). At present, however, there are no data available on STAT3/TNFR2 signalling in the skeletal muscle of HFrEF patients.

Recent studies in murine cardiac fibroblasts and cardiomyocytes have also established adiponectin as an activator of STAT3 signalling (20, 21). In this regard, it was shown that circulating adiponectin positively influences muscle cell proliferation and regeneration, and that STAT3, similar to adiponectin, exerts cardioprotective actions (22-24). These findings thus seem to support a role for adiponectin in STAT3/TNFR2 signalling. In non-cachectic HFrEF patients, however, high plasma adiponectin has been associated with muscle loss and exercise intolerance (25, 26). Plasma adiponectin levels in HFrEF were also closely correlated with circulating TNF-α, which is indicative of systemic immune activation (27, 28). Finally, in HFrEF, not only circulatory adiponectin is increased, but so is the expression of adiponectin in skeletal muscle (29-33).

Therefore, in the present study we aimed 1) to examine whether STAT3/TNFR2 signalling in the skeletal muscle of HFrEF patients is attenuated, and 2) to study its relationship with inflammation (systemic and local), adiponectin concentrations, parameters of exercise capacity and quadriceps mass, strength and quality.

MATERIAL AND METHODS

Subjects

Twenty patients with HFrEF and systolic dysfunction due to ischemic or dilated cardiomyopathy were recruited between November 2010 and January 2015 from the Heart Failure Clinic of the Antwerp University Hospital (UZA). Patients were included if left ventricular ejection fraction (LVEF) was <35% (assessed by echocardiography), if they were in NYHA functional class II-III and if they had been stable on medical treatment and symptoms for a minimum of three months prior to inclusion. Patients with recent acute coronary syndrome or revascularization (≤3 months), valvular disease requiring surgery, malignant ventricular arrhythmia and cerebrovascular disease were excluded. Other exclusion criteria were acute myocarditis or pericarditis, musculoskeletal abnormalities, infections (acute and chronic), allergies, cancer, inflammatory diseases, diabetes mellitus and renal failure. Thirty age-and gender matched sedentary subjects with a normal electrocardiogram (ECG), left ventricular function and lipid profile, no medication intake and with no symptoms or history of cardiovascular disease served as healthy controls. All study subjects underwent a clinical evaluation including echocardiographic examinations by a cardiologist. Daily

physical activity was measured using a questionnaire-based assessment. The study was approved by the Ethics Committee of the Universal Hospital Antwerp (committee for medical ethics UZA - UAntwerp) and complies with the principles outlined in the Declaration of Helsinki. All participants gave written informed consent before enrolment.

Skeletal muscle biopsy

Skeletal muscle biopsies from the *m. vastus lateralis* of the left leg were collected using the Bergström needle technique under aseptic conditions and local anaesthesia, as previously described in **chapter 4** (32, 34, 35). Any visible non-muscle tissue, such as macroscopic fat residues and fibrous tissue, were removed. Biopsies were either fixed with 4% buffered formaldehyde or immediately snap frozen in liquid nitrogen and stored at -80°C. The procedure was separated at least one week from exercise testing.

Cardiopulmonary exercise testing

A symptom-limited cardiopulmonary exercise test (CPET) was performed on a treadmill (Medical Jaeger, Würzburg, Germany) or bicycle ergometer (Cardiovit CS-200 Ergo-spiro, Schiller AG, Baar, Switzerland) in non-fasting conditions (36). A ramp protocol started with an equivalent of 20 or 40W, whereas workload was increased with incremental steps equivalent of 10 or 20W/min. Respiratory gas exchange data were determined continuously and permitted determination of ventilation (VE), oxygen uptake (VO_2) and carbon dioxide production (VCO_2). Peak oxygen consumption (VO_2 peak) was expressed as the highest obtained VO_2 during exercise and was also expressed as percentage of the predicted value (%VO_2 peak predicted). Maximal work-economy was defined as maximal workload at VO_2 peak (Wattmax/VO_2 peak). Twelve-lead ECG and heart rate were recorded continuously and blood pressure was measured baseline and every 2 min. On-line analysis of VE/VO_2 and VE/VCO_2 curves permitted to encourage patients to exercise up to exhaustion.

Biochemical analyses

Fasted venous blood samples were collected from all patients and controls. Levels of creatinine, total cholesterol, triglycerides, low-density (LDL) and high-density lipoprotein (HDL) cholesterol, glucose and high sensitivity C-reactive protein (hsCRP) were immediately assessed on Dimension Vista 1500 instruments using reagents from Ortho Clinical Diagnostics (Siemens Healthcare Diagnostics NV/SA, Beersel/Huizingen, Belgium). Plasma was separated by centrifugation and aliquots were stored at -80°C until further analysis.

Glomerular filtration rate (GFR) was calculated using the chronic kidney disease epidemiology collaboration (CKD-EPI) formula. Circulating total (low, middle, high molecular weight) adiponectin concentrations were measured using a commercially available enzyme-linked immunosorbent assay (ELISA, R&D Systems, Abingdon, UK). Concentrations of TNF-α, soluble TNF receptor 2 (sTNFR2) and IL-6 were determined using (high-sensitive) ELISA kits (R&D Systems). For TNF-α, the mean sensitivity was 0.106 pg/ml, for sTNFR2 it was 0.6 pg/ml and for IL-6 0.039 pg/ml. Internal controls (low, medium, high) were implemented to value assay performance (Quantikine Immunoassay Controls, R&D Systems). N-terminal pro-brain natriuretic peptide (NT-proBNP) was determined with a sandwich immunoassay on an Elecsys 2010 (Roche diagnostics, GmbH, Mannheim, Germany).

Anthropometric variables

Body composition was determined using bioelectrical impedance analysis (Omron body fat monitor BF 300). Lean body mass was calculated by total weight minus fat mass. Waist circumference was recorded in expiratory position at the level of the umbilicus. Hip circumference was measured at the widest point at the level of the greater trochanters. Waist-to-hip ratio (WHR) was calculated. The anatomic cross-sectional area (CSA) of the quadriceps muscle was assessed by ultrafast computerized tomography (CT) scanning (Brilliance 64, Philips Medical Systems, Best, The Netherlands) in 17 HFrEF patients and 10 healthy controls. Scans were performed in the supine position and 6 mm transaxial slices were scanned at the mid-femur level.

Peripheral muscle strength

The 1-Repeated Maximum (1-RM) was assessed as an indicator of muscle strength using a multifunctional fitness machine (Unica, Technogym, Gambetola, Italy). In addition, muscle strength was measured using a linear isokinetic dynamometer (Aristokin, Lode BV, Groningen, The Netherlands). A row-movement was applied to evaluate the ability to perform complex movements of both arms and legs. Linear isokinetic speed was set at 100 cm/s and minimal force at 5 Newton (N). Torque measurements were gravity corrected and start–stop angles were set at 90° and 30°. Subjects performed several submaximal practice efforts. Maximal muscle strength was taken as the mean performance of three maximal efforts, with a rest period of at least 1 min in between. HFrEF patients or controls with low back pain were

not tested on the linear isokinetic dynamometer. Muscle quality refers to myofibril contractile function and is expressed as strength over CSA.

RNA isolation and quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from muscle biopsies (5-15mg) using the Qiazol reagent technique followed by RNA cleanup using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands), as illustrated in **chapter 4** (35). RNA concentrations were determined by ultraviolet absorbance at 260 nm (A_{260}) using a Nanodrop (ND)-1000 spectrophotometer (NanoDrop Technologies Inc., Delaware, United States). Purity was estimated by A_{260}/A_{280} ratio determination. RNA integrity (RIN) was assessed by microcapillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent technologies, Germany). The quality of the RNA sample was rated according to the RNA integrity number (RIN) and the ratio of 28S/18S ribosomal RNA. Oligo-dT first-strand cDNA was synthesized from 1µg of total RNA using the iScriptTM cDNA Synthesis Kit (Bio-rad Laboratories, Nazareth, Belgium). RT-PCR was performed for mRNA relative quantification on a CFX96TM Real-Time PCR Detection system (Bio-rad Laboratories) as previously described (32). Gene-specific forward and reverse primers (Eurofins MWG Operon, Ebersberg, Germany) were as follows: TNF-α-F: TCCTTCAGACACCCTCAACC, TNF-α-R: AGGCCCCAGTTTGAATTCTT, TNFR1-F: ACCAGGCCGTGATCTCTATG, TNFR1-R: CAGCTATGGCCTCTCACTCC, TNFR2-F: CTCAGGAGCATGGGGATAAA, TNFR2-R: AGCCAGCCAGTCTGACATCT, IL-6-F: TACCCCCAGGAGAAGATTCC, IL-6-R: TTTTCTGCCAGTGCCTCTTT, STAT3-F: TGTGCGTATGGGAACACCTA, STAT3-R: GGCGAACCCTGTTCATCTTA, AdipoQ-F: GCTGGGAGCTGTTCTACTGC, AdipoQ-R: CGATGTCTCCCTTAGGACCA. Gene expression levels were normalized to TATA box binding protein (TBP) and beta-2-microglobulin (B2M) (35, 37, 38). Values for the cycle threshold (Ct) were analyzed using the CFX96^{TM} software (Version 1.6; Bio rad Laboratories, CA, USA). Relative quantification of gene expression levels was performed by using the $2^{-\Delta\Delta Ct}$ calculation. All samples were run in duplicate.

Western blotting

Western blotting was performed as described in **chapter 6** (38). Briefly, protein extracts of the *m. vastus lateralis* from both HFrEF patients and control subjects were loaded on 4-12% SDS–PAGE gels and separated for 45 min. at 165V (Invitrogen, Grand Island, NY, USA). After gel electrophoresis, proteins were transferred to a reduced-fluorescence PVDF

membrane (Immobilon-FL, Millipore, Bedford, MA, USA) for 1h at 100V. Thereafter, the blots were blocked in Odyssey blocking buffer mixed 1:1 with TBS (Li-Cor, Lincoln, NE) for 1h at room temperature (RT), followed by incubation with a primary antibody, total STAT3 (1:1000) and Y^{705} phosphorylated STAT3 (1:1000; Abcam, Cambridge, MA) at 4°C overnight. After washing, membranes were incubated with fluorescent secondary antibodies for 1h in Odyssey blocking buffer plus TBS-T and 0.01% SDS (Dako, Glostrup, Denmark) at RT. Fluorescence intensity data was normalized to β-actin expression (Sigma–Aldrich) and visualized using a Lumi-Imager (Roche Diagnostics).

Statistical analysis

Experimental triplicates or duplicates were averaged for statistical analysis. Evaluation of normality was performed using the Kolmogorov–Smirnov test. Differences between groups (HFrEF versus controls) were compared using the independent Student t-test or Mann-Withney U test for continuous variables, as appropriate. Categorical variables were compared with the Pearson's Chi-square (X_2) test or Fisher's exact test. Correlations are expressed as univariate analyses and calculated using the Pearson's correlation coefficient for normally distributed variables and the nonparametric equivalent Spearman's rank correlation coefficient for other variables. Significance was accepted at the 5% level ($p < 0.05$). All data are presented as mean ± standard error of the mean (SEM) or median with interquartile range (IQR), depending on the distribution of the data. Statistical analyses were performed using the software package statistical package for the social sciences (SPSS, IBM SPSS Statistics Inc, Version 23.0, Chicago, IL, USA).

RESULTS

Clinical characteristics

Twenty HFrEF patients (85% men) with a mean age of 55 years were included in the study. HF was of ischemic etiology in 65% of patients, the remaining patients suffered from idiopathic cardiomyopathy. Mean LVEF was 27% (±1.89). Thirteen patients (65%) were in NYHA functional class II and 7 (35%) were classified as NYHA class III. The NT-proBNP level was increased (1015.0 [412.3; 2074.0] pg/ml) when compared to the reference limits observed in healthy subjects (39). All patients received standard HF treatment. Clinical characteristics are listed in **table 1**. HFrEF patients and healthy subjects were matched with

respect to age and gender. HFrEF patients demonstrated a dyslipidemic state (reduced total cholesterol, p=0.003; HDL, p=0.005 and LDL, p=0.031 and increased triglycerides, p=0.012, despite statin treatment) and an increased waist to hip ratio (0.90 [0.84; 0.94] versus 1.00 [0.94; 1.07]; p=0.002). In addition, HFrEF patients exhibited a pro-inflammatory profile (increased circulating hsCRP, p=0.000; TNF-α, p=0.028; sTNFR2, p=0.009 and IL-6, p=0.019) and mild renal impairment (increased creatinine, p=0.025 and decreased eGFR, p=0.009). Total serum adiponectin levels were higher in the HFrEF group (p=0.049). After stratification according to disease state (ischemic or non-ischemic HF), no significant differences in clinical characteristics were found between both groups (p>0.05 for all).

Exercise capacity and quadriceps strength and mass

Parameters of exercise capacity, quadriceps features and linear isokinetic measurements are shown in **table 2**. Exercise capacity was impaired in HFrEF patients (decreased VO_2 peak, p<0.001; maximal workload, p<0.001 and work efficiency, p=0.013) compared to healthy subjects. In addition, slope (p=0.007) and VE/VCO_2 slope (p=0.001) were increased in the HFrEF group. Quadriceps strength (p=0.035) and CSA (p=0.007) were lower in HFrEF patients compared to healthy subjects. Quality of quadriceps muscle (strength/CSA) was less in the HFrEF group with a trend towards statistical significance (p=0.085). Linear isokinetic parameters were all significantly decreased in HFrEF patients (p<0.05), except for time to maximal force (time to Fmax, p=0.531).

STAT3/TNFR2 signalling in skeletal muscle of HFrEF patients

No significant difference in local IL-6 mRNA expression was observed between HFrEF patients and controls (p=0.067, **Fig. 1A**). Both, STAT3 (p=0.042; **Fig. 1B**) and TNFR2 (p=0.029; **Fig. 1C**) mRNA expression levels were reduced in HFrEF patients compared to healthy controls. A significantly increased TNFR1/TNFR2 mRNA ratio (p=0.005; **Fig. 1D**) was documented in the skeletal muscle of HFrEF patients. In addition, HFrEF patients had elevated local muscle mRNA TNF-α levels (p=0.043; **Fig. 1E**). No significant difference in local TNFR1 mRNA expression was observed between both groups (p=0.676, **Fig. 1F**).

The phosphorylation state of STAT3 (**Fig. 2A, B**) and the ratio between phosphorylated- and total STAT3 (pSTAT3/STAT3; **Fig. 2A, C**) were significantly reduced in patients with HFrEF compared to control subjects (p=0.032, p=0.008; respectively), indicating a decreased STAT3 activation.

Table 1 Clinical characteristics

Characteristic	Controls n= 30	HFrEF n= 20	*p-value*
Demographics			
Age (Years)	56.49 ± 1.08	55.37 ± 2.41	0.675
Gender (male/female)	26/4	17/3	0.871
BMI (kg/m^2)	24.9 (22.7; 28.5)	24.3 (21.9; 28.8)	0.507
Heart failure measurements			
Etiology (% ischemic)	N.A.	65%	N.A.
LVEF (%)	≥60%	27.0 ± 1.89	N.A.
NYHA Class (II/III) (n/n)	N.A.	13/7	N.A.
NT-proBNP (pg/ml)†	N.A.	1015.0 (412.3; 2074.0)	N.A.
Medication (N)			
Beta-blocker	0	18	N.A.
ACE-I and/or ATII	0	17	N.A.
Diuretics	0	16	N.A.
Statins	0	13	N.A.
Lipid status (mmol/L)			
Total Cholesterol	209.80 ± 7.35	170.82 ± 10.12	**0.003**
HDL	60.57 ± 2.52	46.82 ± 3.78	**0.005**
LDL	126.23 ± 6.85	101.12 ± 8.89	**0.031**
Non-HDL-C	3.60 ± 0.17	3.84 ± 0.26	0.442
Triglycerides	84.0 (72.7; 125.0)	115.0 (99.5; 173.5)	**0.012**
Anthropometric data			
Waist circumference (cm)	88.33 ± 1.97	96.10 ± 5.42	0.140
Hip circumference (cm)	97.0 (95.0; 102.0)	96.0 (86.5; 101.8)	0.327
Waist to hip ratio	0.90 (0.84; 0.94)	1.00 (0.94; 1.07)	**0.002**
Total lean body mass (kg)	59.9 (57.0; 65.3)	51.9 (46.6; 62.6)	0.156
Total fat mass (kg)	18.8 (15.7; 27.5)	23.3 (16.6; 30.2)	0.499
Laboratory data			
Glucose	85.87 ± 2.04	96.38 ± 3.92	**0.012**
hsCRP (mg/l)	0.00 (0.00; 0.03)	0.61 (0.00; 1.78)	**0.000**
Creatinine (mmol/L)	0.89 ± 0.02	1.18 ± 0.11	**0.025**
eGFR (ml/min/1.73 m^2)	90.66 ± 1.81	72.73 ± 5.72	**0.009**
Adiponectin (mg/l)††	8.41 ± 1.44	17.80 ± 4.09	**0.049**
TNF-α (pg/ml)‡	0.67 ± 0.34	1.81 ± 0.20	**0.028**
sTNR2 (pg/ml)‡‡	2696.5 ± 172.4	3458.7 ± 203.4	**0.009**
IL-6 (pg/ml)‡	1.12 ± 0.11	3.98 ± 0.37	**0.019**

N, Number of subjects; HFrEF, Heart failure with reduced ejection fraction; BMI, Body mass index; LVEF, Left Ventricular Ejection fraction; NYHA, New York Heart Association; NT-proBNP, N-terminal pro brain natriuretic peptide; ACE-I, Angiotensin-converting enzyme inhibitors; ATII, angiotensin II receptor antagonists; HDL, High density lipoprotein; LDL, Low density lipoprotein; Non-HDL-C, non-HDL cholesterol; hsCRP, high sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate; TNF-α, Tumor necrosis factor-alpha; sTNFR2, soluble TNF receptor 2; IL-6, Interleukin-6.

Values are expressed as mean ± SEM or median (interquartile range).

† Data available for 12 subjects.

†† Data available for resp. 20 and 18 subjects

‡ Data available for resp. 15 and 17 subjects

‡‡ Data available for resp. 20 subjects

Table 2 Exercise capacity, quadriceps features and linear isokinteic measurements

Characteristic	Controls n=30	HFrEF n=20	*p-value*
Maximal exercise capacity			
VO_2 peak (ml/kg/min)	39.89 ± 1.92	18.71 ± 1.52	**<0.001**
% VO_2 peak predicted	28.59 ± 0.57	29.23 ± 1.19	0.632
Maximal workload (watt)	240.33 ± 9.20	103.53 ± 8.87	**<0.001**
% Watt predicted	159.0 (135.7; 190.7)	147.8 (121.6; 187.6)	0.566
Work efficiency (watt ml^{-1} kg^{-1} min^{-1})	6.28 ± 0.27	5.21 ± 0.28	**0.013**
Slope	21.5 (19.9; 25.3)	31.8 (23.0; 39.4)	**0.007**
VE/VCO_2 slope	22.15 ± 0.59	30.71 ± 1.89	**0.001**
Quadriceps parameters			
Circumference (cm)	51.68 ± 0.71	48.92 ± 3.21	0.435
Strength (kg)	61.43 ± 2.15	39.58 ± 7.70	**0.035**
Cross-sectional area (CSA; cm^2)†	82.5 (80.5; 88.2)	43.5 (42.3; 44.2)	**0.007**
Quality (strength/mass)	0.79 ± 0.05	0.58 ± 0.14	0.085
Linear isokinetic measurements			
Mean power (W)	576.52 ± 30.61	259.80 ± 51.37	**<0.001**
Maximal power (W)	957.04 ± 43.22	518.40 ± 108.56	**<0.001**
Work (J)	596 (557; 694)	277 (167; 430)	**<0.001**
Maximal force (N)	957.04 ± 43.22	518.40 ± 108.56	**<0.001**
Time to Fmax. (s)	0.36 ± 0.12	0.41 ± 0.07	0.531
Max. explosive force (N/s)	18900 (12000; 47900)	7400 (3250; 25150)	**0.019**
Mean explosive force (N/s)	3291.43 ± 223.63	1674.60 ± 518.86	**0.006**
Force at 0.25s (N)	806.57 ± 48.23	411.00 ± 143.09	**0.003**
Power at 0.25s (W)	124.09 ± 10.06	70.00 ± 33.22	**0.049**

N, Number of subjects; HFrEF, Heart failure with reduced ejection fraction; VO_2 peak, peak oxygen consumption; %VO_2 peak predicted, percentage of predicted peak oxygen consumption; %Watt predicted, percentage of predicted maximal workload; VE/VCO_2 slope, minute ventilation carbon dioxide production relationship.

Values are expressed as mean ± SEM or median (interquartile range).

† Data available for resp. 10 and 14 subjects.

Local immune activation and skeletal muscle STAT3/TNFR2 signalling in HFrEF

A significant strong positive relationship was shown between TNF-α mRNA and IL-6 mRNA (r=0.778, p=0.003) in HFrEF patients. No correlations were found between TNF-α mRNA, STAT3 mRNA and TNFR2 mRNA in the HFrEF group (p>0.05 for all; **table 3**).

A negative correlation, almost reaching significance, was apparent between the mRNA expression levels of IL-6 and STAT3 (r=-0.498, p=0.052) in HFrEF. No correlation was observed between IL-6 mRNA and TNFR2 mRNA in the HFrEF group (p>0.05; **table 3**).

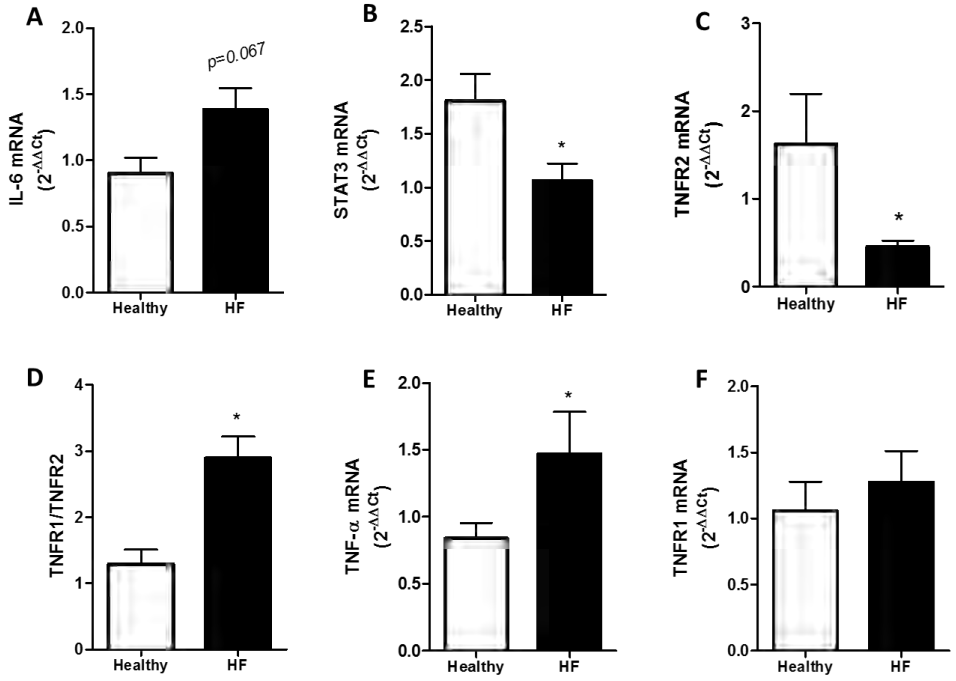

Figure 1. Skeletal muscle inflammation and STAT3/TNFR2 signalling

mRNA transcript levels of IL-6 **(A)**, STAT3 **(B)**, TNFR2 **(C)**, ratio TNFR1/TNFR2 **(D)**, TNF-α **(E)** and TNFR1 **(F)** in skeletal muscles of HFrEF patients versus control subjects. Data are mean ± SEM. *p<0.05

Systemic immune activation and skeletal muscle STAT3/TNFR2 signalling in HFrEF

Serum TNF-α correlated negatively with STAT3 mRNA (r=-0.497, p=0.048, **table 3**), and showed a trend toward being negatively related to the mRNA level of TNFR2 (ρ=-0.463, p=0.064). Serum IL-6 correlated negatively with the mRNA expression levels of STAT3 and TNFR2 (r=-0.522, p=0.056; ρ=-0.629, p=0.012, respectively, **table 3**).

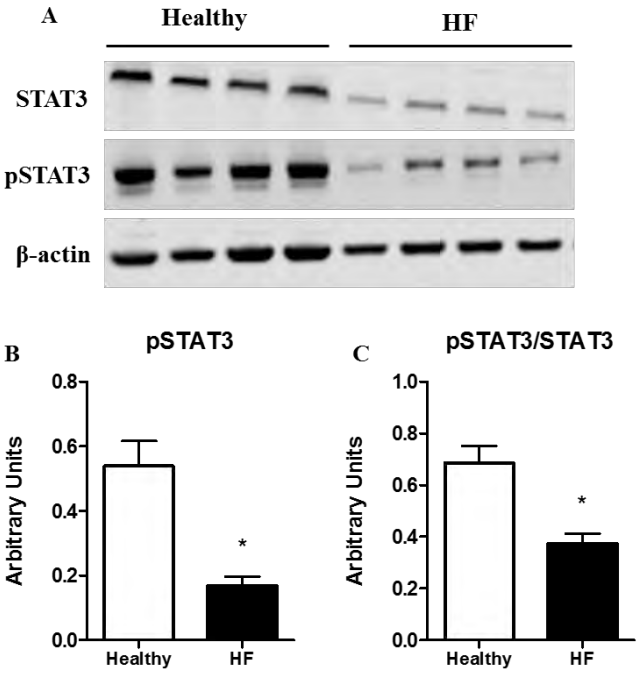

Figure 2. STAT3 phosphorylation within human skeletal muscle.

Western blots of STAT3 **(A)**, pSTAT3 (phospho Y705) **(B)** and β-actin **(C)** in skeletal muscle biopsy samples of patients with HFrEF and control subjects. Top, representative western blots. Bottom shows mean values expressed as mean ± SEM. *p<0.05. N=6.

Table 3 Correlations between parameters of inflammation and muscle STAT3/TNFR2 signalling in HFrEF

	Circulation†				**Skeletal muscle**††			
	TNF-a		**IL-6**		**TNF-a**		**IL-6**	
	r/ρ	*p*	*r/ρ*	*p*	*r/ρ*	*p*	*r/ρ*	*p*
Skeletal muscle								
TNF-α	-0.016	0.964	0.304	0.508	1.000	N.A.	0.778	**0.003**
Il-6	0.071	0.810	0.382	0.526	0.778	**0.003**	1.000	N.A.
STAT3	-0.498	**0.048**	-0.522	**0.056**	0.239	0.471	-0.498	**0.052**
TNFR2	-0.463	0.064	-0.629	**0.012**	-0.389	0.072	0.190	0.651

† *Data available for 17 HFrEF patients*
†† *Data available for 20 HFrEF patients*
Abbreviations see in Table 2

Adiponectin and skeletal muscle STAT3/TNFR2 signalling in HFrEF

Besides increased serum adiponectin, the local mRNA expression of adiponectin within the skeletal muscle was upregulated in HFrEF patients (p=0.044, **Fig. 3A**). As illustrated in **table 4**, serum adiponectin showed a strong positive correlation with skeletal muscle TNF-α mRNA (ρ=0.735, p=0.038), and correlated negatively with TNFR2 mRNA expression (ρ=-0.562, p=0.042, **Fig. 3B**) in HFrEF. No relation was found between circulating adiponectin and the mRNA expressions of IL-6 and STAT3 in the skeletal muscle of HFrEF patients (both p>0.05). In addition, no correlation was observed between adiponectin mRNA expression and the mRNA levels of TNF-α, IL-6 and STAT3 (p>0.05, for all, **table 4**). A moderate negative relation, although not significant, was observed between adiponectin mRNA and TNFR2 mRNA (ρ=-0.449, p=0.062; **Fig. 3C**) in HFrEF patients.

Furthermore, serum adiponectin levels were associated with NT-proBNP levels (ρ=0.480, p<0.001) whereas no correlation was apparent between adiponectin mRNA expression in skeletal muscle and NT-proBNP levels (ρ=0.145, p=0.697) in HFrEF patients. As shown in **table 4**, serum adiponectin correlated positively with circulating levels of hsCRP and TNF-α (ρ=0.597 and ρ=0.565; p<0.05, respectively) whereas no correlations were observed between adiponectin mRNA and serum inflammatory parameters (p>0.05 for all, **table 4**).

Table 4 Univariate correlations between adiponectin, inflammatory parameters and muscle STAT3/TNFR2 signalling in HFrEF

	Serum adiponectin†		**Muscle adiponectin**††	
	ρ	*p*	*ρ*	*p*
Circulation				
vs hsCRP	0.597	**0.053**	0.045	0.821
vs TNF-α	0.565	**0.023**	0.239	0.196
vs *sTNFR2*	0.443	0.072	0.361	0.295
vs *IL-6*	0.196	0.408	0.105	0.222
Skeletal muscle				
vs TNF-α	0.735	**0.038**	0.287	0.144
vs IL-6	0.300	0.124	0.387	0.153
vs STAT3	0.129	0.571	0.256	0.477
vs *TNFR2*	-0.562	**0.042**	-0.449	0.062

† *Data available for 18 HFrEF patients*
†† *Data available for 20 HFrEF patients*
Abbreviations see in Table 2

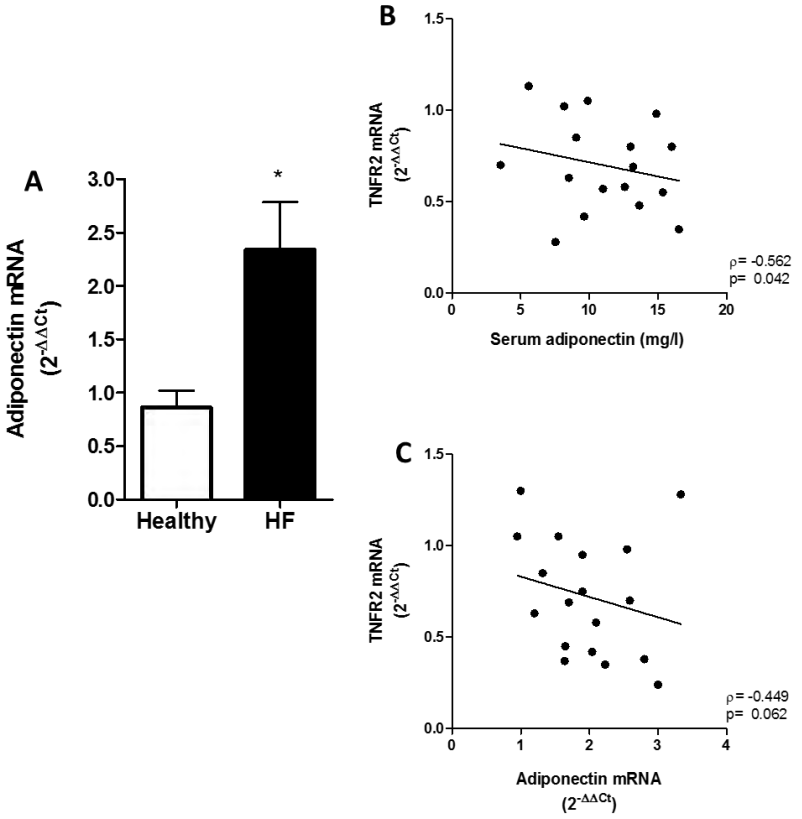

Figure 3. Adiponectin and correlations with muscle TNFR2 mRNA content of adiponectin **(A)** in skeletal muscle of HFrEF patients and control subjects. Scatter plots of the correlation between TNFR2 mRNA and both serum adiponectin **(B)** and muscle adiponectin **(C)** in HFrEF patients. Data are represented as mean ± SEM. *p<0.05

Exercise capacity, quadriceps parameters and skeletal muscle STAT3/TNFR2 signalling in HFrEF

The mRNA expression levels of TNF-α, IL-6, STAT3 and TNFR2 in the skeletal muscle of HFrEF patients did not relate with parameters of exercise capacity (e.g. VO_2 peak, maximal workload, work efficiency; r=-0.544 to r=0.469, p>0.05 for all). In addition, no associations were apparent between mRNA expression levels of TNF-α, IL-6, STAT3, TNFR2 and quadriceps parameters, including strength, CSA and quality (r=-0.324 to 0.312, p>0.05, for all) in HFrEF patients. However, a positive correlation was observed between TNFR2 mRNA and quadriceps circumference (ρ=0.497, p=0.048) in HFrEF. Furthermore, TNF-α mRNA was negatively correlated with quadriceps strength (r=-0.658, p=0.029).

Exercise capacity, quadriceps parameters and circulating adiponectin in HFrEF

Serum adiponectin correlated negatively with exercise capacity (VO_2 peak, maximal workload and work efficiency) and quadriceps strength and quality ($p<0.05$ for all, **table 5**). No associations were documented between muscle adiponectin mRNA expression and exercise capacity or quadriceps parameters ($p>0.05$ for all).

Table 5 Correlations between adiponectin, exercise capacity and quadriceps features in HFrEF

	Circulation†		**Skeletal muscle**††	
	ρ	*p*	*ρ*	*p*
VO_2 peak	-0.534	**0.044**	-0.167	0.270
Maximal workload	-0.361	**0.048**	-0.202	0.392
Work efficiency	-0.687	**0.028**	-0.265	0.273
Quadriceps circumference	-0.754	0.084	-0.422	0.096
Quadriceps strength	-0.853	**0.031**	-0.039	0.875
Quadriceps CSA‡	-0.714	0.071	-0.394	0.260
Quadriceps quality	-0.750	**0.052**	-0.500	0.207

† *Data available for 18 HFrEF patients*
†† *Data available for 20 HFrEF patients*
‡ *Data available for 14 HFrEF patients*
Abbreviations see in Table 2

DISCUSSION

In the present study, we examined whether STAT3/TNFR2 signalling in the skeletal muscle of HFrEF patients is attenuated and related to parameters of systemic and local inflammation, adiponectin concentrations, physical functioning and quadriceps features including strength, mass and quality.

Several novel and interesting findings emerge from this study. In patients with chronic HFrEF:

1. The reduction in gene expressions of skeletal muscle STAT3 and TNFR2 is associated with increased systemic inflammation, but not with parameters of exercise capacity or skeletal muscle strength, mass and quality;

2. Higher circulating adiponectin is associated with reduced gene expression of skeletal muscle TNFR2, decreased exercise capacity and lower quadriceps muscle strength and quality.

The present study is the first to show a down-regulation and decreased activation of STAT3 in skeletal muscle of patients with chronic HFrEF. STAT3 is a known regulator of skeletal muscle regeneration and is involved in satellite cell survival, proliferation and differentiation (16, 40, 41). Further, we observed a decreased expression of TNFR2 in the skeletal muscle of chronic HFrEF patients. In mice subjected to hind limb ischemia, absence of TNFR2 signalling resulted in increased and long-term inflammation, reduced angiogenesis and impaired skeletal muscle regeneration (e.g.; activation, survival and proliferation of satellite cells) during post-ischemic recovery (42). In previous *in vitro* work, we found that primary cultured myoblasts from HFrEF patients have a reduced proliferative activity and a diminished expression of skeletal muscle TNFR2 when compared to myoblasts of healthy donors (**chapter 5**) (35). Therefore, diminished expression levels of STAT3 and TNFR2 may possibly add to reduced satellite cell and myoblast proliferation and loss of skeletal muscle tissue. In the present study, however, we did not find a relationship between impaired STAT3/TNFR2 expression and skeletal muscle mass, strength and quality.

Several mechanisms may account for the impaired skeletal muscle STAT3/TNFR2 signalling in chronic HFrEF including systemic immune activation. Studies have indicated that the pro-inflammatory cytokines IL-6 and TNF-α promote TNFR2 through induction of nuclear factor kappa β (NF-κβ) and activation of STAT3 via the Janus family of tyrosine kinases (JAKs) (15, 43, 44). Moreover, evidence suggests that the promoter region of TNFR2 contains two STAT-binding sequences (45). In the present study, however, increased serum levels of TNF-α and IL-6 in HFrEF were negatively associated with skeletal muscle STAT3/TNFR2 signalling. Furthermore, decreased skeletal muscle TNFR2 expression may be due to the proteolytic cleavage of the extracellular component of the receptor from the muscle cell membrane (receptor shedding), since we detected a significant increased concentration of sTNFR2 in chronic HFrEF patients. Hence, decreasing the expression of TNFR2, is a possible compensatory mechanism to lower systemic inflammation in HFrEF (46). Another potential mechanism underlying reduced myoblast proliferation in HFrEF is the lack of physical activity in these patients (7, 47-49). It has been shown that STAT3 is activated in the skeletal muscle in response to physical activity (17, 50). In addition, moderate aerobic training induces TNFR2 in the rat tibialis anterior muscle (51). We observed, however, no association between muscle STAT3/TNFR2 signalling and exercise capacity in chronic HFrEF patients.

Furthermore, our data demonstrates that increased serum adiponectin, rather than muscle adiponectin, is negatively associated with skeletal muscle TNFR2 expression in chronic

HFrEF, suggesting that serum adiponectin may participate in the impaired skeletal muscle anti-inflammatory and regenerative capacity in chronic HFrEF. The relevance of the association between adiponectin and impaired skeletal muscle regeneration is further demonstrated by the association of increased serum adiponectin with reduced physical function (e.g.; VO_2 peak and maximal workload), skeletal muscle mass and muscle strength in both cachectic and non-cachectic HFrEF patients (25, 26, 52, 53). Our results confirm a strong negative association between serum adiponectin and submaximal exercise performance, quadriceps strength and quality.

CONCLUSION

The present study demonstrates impaired expression of STAT3/TNFR2 genes in the skeletal muscle of HFrEF patients. STAT3/TNFR2 dysregulation in the skeletal muscle of HFrEF patients seems to be associated with both systemic immune activation and increased blood adiponectin concentrations. More knowledge of muscle STAT3/TNFR2 signalling and its association with inflammation and adiponectin, will eventually contribute to novel therapeutic strategies combating muscle wasting in HFrEF.

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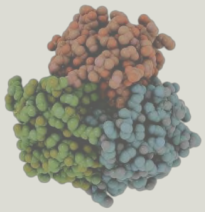

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Chapter 9

Discussion and future perspectives

Partly adapted from:

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Sente T, Van Berendoncks AM, Hoymans VY, Vrints CJ. Adiponectin resistance in skeletal muscle: Pathophysiological implications in chronic heart failure. J Cachexia Sarcopenia Muscle. Oct. 2015.

ADIPONECTIN: TOO MUCH OF A GOOD THING IN HEART FAILURE

Adiponectin was discovered two decades ago as an adipose tissue peptide with a prominent role in improving insulin sensitivity (1). Further details on its physiological and pathophysiological actions have been substantiated in recent years, and clarified in **chapters 1 and 2** (2-4). Adiponectin was initially viewed as a biological marker of overall good cardiovascular health. First major epidemiological studies had shown that high adiponectin levels are associated with reduced heart-attack risk and mortality in healthy middle-aged cohorts (5). However, subsequent studies added a twist to the cardio-metabolic benefits and favorable clinical associations of adiponectin. At the time this thesis started, it was already clear that adiponectin had direct opposite relationships with adverse outcomes in different populations. The Cardiovascular Health Study was the first and to date the largest population-based longitudinal study examining adiponectin levels in older adults (aged 65–100 years) (6). This study reported that both, high and low levels of adiponectin are associated with a significant increased rate of cardiovascular disease (CVD) and mortality (e.g.; Goldilocks principle), whereas in people suffering from chronic heart failure (HF) and reduced ejection fraction (HFrEF) or atrial fibrillation (AF), only high adiponectin levels are related with increased death (7-9). To date, it has been documented that the association of adiponectin with all-cause and cardiovascular mortality is less favorable with increasing cardiovascular risk, shifting from a U-shape relationship in the absence of CVD, HFrEF or AF, to a flat association with CVD, followed by direct linear association with prevalent HFrEF, AF or older age (7, 10, 11). These contrasting findings are now well-known as the "*adiponectin paradox*," as described in detail in **chapter 1** (4). The underlying mechanisms for this paradox remain a major puzzle in the field.

An undeserved bad reputation in heart failure?

It is not yet clear whether increased adiponectin concentrations are beneficial, rather than detrimental to HFrEF patients (12). For a long time, concentrations of adiponectin were believed to relate with HFrEF incidence (13-16). However, a recent study by Frankel et al. refute this assumption, as they observed that neither high nor low concentrations of adiponectin are associated with incident HF, suggesting that adiponectin itself has no major role in the development of HFrEF (17). Instead, the increase in circulating adiponectin concentration might be induced to attenuate the overall cardiovascular risk and/or prevent HFrEF progression. In this regard, it has been demonstrated that adiponectin deficiency in

mice accelerates the transition from left ventricular hypertrophy (LVH) to HFrEF (18). Nevertheless, as we reported, adiponectin may have a two-faced character in HFrEF **(Fig. 1)** (4). In the early stage of HFrEF, the increase in adiponectin production may be part of a compensatory mechanism, but for patients in the final stages of HFrEF, increased adiponectin levels may worsen cardiac dysfunction, leading to an increased risk of mortality (19).

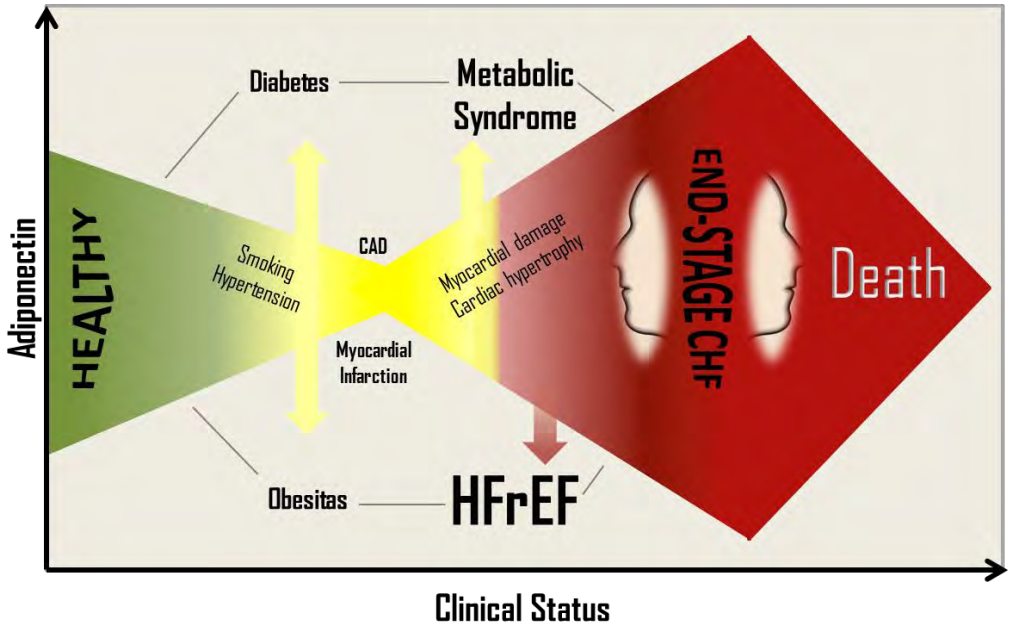

Figure 1. Two faces of adiponectin in HFrEF

Adiponectin is a beneficial adipokine that is present at high levels in lean, healthy individuals. Low levels of adiponectin have been associated with conditions such as obesitas, hypertension, myocardial infarction, CAD and cardiac hypertrophy. In HF, however, increases in circulating adiponectin parallels worsening of prognosis for patients. Based on clinical and experimental evidence, we speculate that adiponectin has two faces in HF. Elevated adiponectin likely serves as a protective and compensatory mechanism for the energy metabolic alterations that occur during the early stages of HF. In contrast, in patients with end-stage HF, high levels of adiponectin will further deteriorate cardiac function, increasing the risk of death. Green, yellow and red colors indicate normal, low and high levels of adiponectin, respectively. Arrows point to disease condition. *Abbreviations: HFrEF: Heart failure with reduced ejection fraction; CAD: coronary artery disease.*

Adiponectin has turned up to be a specific protein of interest in HF, especially in chronic HFrEF. Hence, at the beginning of this thesis, we summarized and discussed the role of adiponectin in HFrEF (**Chapters 1 and 2**). Recent progress in the field and data obtained during this thesis now allow us to elaborate on these original premises (**Fig. 2**).

THE DUAL ROLE OF ADIPONECTIN IN HEART FAILURE

As mentioned in **chapter 1**, adiponectin offers a wide range of myocardial and vascular protective effects and exerts several anti-inflammatory actions (4, 20-24). However, recent experimental data has overturned the dogma that the actions of adiponectin are exclusively cardioprotective and anti-inflammatory in HF (25).

An apparently cardioprotective hormone?

For a long time, adiponectin was believed to be a true cardioprotective hormone in patients with diverse metabolic and cardiovascular disorders. On one hand, diminished plasma levels of total adiponectin were evident in the presence of obesity, diabetes (type 1 and 2), hypertension, insulin resistance, myocardial infarction and coronary artery disease (CAD), and were associated with adverse outcomes (5, 13, 26-32). On the other hand, elevated adiponectin levels have been shown to improve nitric oxide (NO) production, apoptosis, insulin sensitivity and left ventricular (LV) systolic dysfunction (33). Moreover, in a nested case-control study among 18 225 male participants (40 to 75 years) of the Health Professionals Follow-up Study, free of diagnosed cardiovascular disease, men with high total circulating adiponectin concentrations were are at a lower risk of myocardial infarction compared to men with medium-to low adiponectin levels (5). This relationship was independent of inflammation and glycemic status but possibly related to differences in blood lipids. Also, in the Uppsala Longitudinal Study of Adult Men (ULSAM study, 832 healthy men, 70 years of age) a high concentration of total adiponectin was independently associated with a lower risk for CHD (32). A reduction in adiponectin level was further related with the presence and severity of IHD caused by systemic atherosclerosis (34). Hence, several studies have documented a significant inverse relationship between total adiponectin and major risk factors of HF (5, 15, 28, 31) (**Fig. 2**). Furthermore, adiponectin deficiency in early mouse HF models (i.e.; adiponectin KO mice subjected to TAC) caused greater LV remodelling, LV hypertrophy, systolic and diastolic dysfunction and mitochondrial oxidative capacity (18, 35-37). Shibata et al. further demonstrated that adenovirus-mediated over-expression of adiponectin can attenuate LV dysfunction in mice following permanent LAD-ligation induced myocardial infarction, and thus that adiponectin is protective against the development of HFrEF (38).

However, when it comes to chronic HFrEF, several studies have questioned the cardioprotective properties of adiponectin. In **chapter 8**, we demonstrated that higher concentrations of total adiponectin in chronic HFrEF patients are associated with increased N-terminal pro-brain natriuretic peptide (NT-proBNP) levels, a marker of poor prognosis in HFrEF. In addition, as mentioned above, a U-shaped relation between adiponectin and mortality was recently reported (7, 10, 11). However, on the assumption that adiponectin is a true cardio-protective hormone, adiponectin levels might rise in an attempt to mitigate the robust activation of neuro-hormonal and pro-inflammatory responses and metabolic impairment in HFrEF. It is possible that the beneficial actions of adiponectin are insufficient to counteract these adverse processes, even at high levels, which could offer an explanation for its association with a worsening prognosis in established HF. Furthermore, in patients with acute non-ischemic and non-valvular HF, a higher ratio of high-molecular weight (HMW) to total adiponectin level was associated with a better prognosis in multivariate survival analysis. The total and HMW adiponectin levels at the time of hospital admission or discharge had no significant impact on the prognosis (39). Yet, in a paper by Dieplinger et al. a higher total adiponectin level in patients with acute HF was associated with worse prognosis. These conflicting results between studies may relate to differences in the underlying etiology of acute HF (e.g.; dilated cardiomyopathy versus hypertension) or the adiponectin isoform being measured (total versus HMW adiponectin). Still, in the Cardiovascular Health study, a strong correlation was found between total and HMW adiponectin. In addition, a non-linear relationship between HMW and total adiponectin with incident diabetes type 2 (a major risk factor for HF) was documented (7, 40). Currently, available clinical data from HFpEF patients is still limited (19). Hence, the role of adiponectin in HFrEF is to be interpreted with caution, and the underlying conditions and diseases have to be taken into account. At present, it seems that elevated levels of adiponectin exert a predominant cardioprotective effect and act as favourable prognostic indicator in early HF, whereas in chronic disease (at least for HFrEF), high circulating adiponectin concentrations are associated with adverse LV remodelling and accelerated progression toward end-stage HF (15, 41, 42) (**Fig. 2**). Therefore, it looks like adiponectin balances on a tight rope between protective and harmful in HFrEF, while its actions in HFpEF are currently far from clear.

A hidden pro-inflammatory nature?

Most adipokines are pro-inflammatory. Adiponectin, however, exerts anti-inflammatory effects in various cells types and tissues including cardiomyocytes, adipocytes, endothelial cells, macrophages, and adipose tissue (43-46). The production and secretion of adiponectin may beneficially influence the inflammatory reaction, thereby antagonizing various pro-inflammatory activities (47, 48) (**Fig. 2**). Adiponectin inhibits the production of C-reactive protein (CRP) and interleukin (IL)-6 and attenuates tumor necrosis factor alpha (TNF-α) secretion through its ability to modulate 5' AMP-activated protein kinase (AMPK) signalling and suppress nuclear factor-κB (NF-κB) activation (49). In addition, adiponectin reduces the expression of vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1, E-selectin and interleukin (IL)-8 in human endothelial cells, and therefore monocyte attachment (43, 50). Recent findings indicate that adiponectin also combats cellular inflammation by affecting sphingolipid metabolism (51, 52). Furthermore, Tsatsanis et al. provided evidence that adiponectin mediates its anti-inflammatory effects by the induction of macrophage tolerance: exposure of macrophages to high levels of globular adiponectin rendered the cells tolerant toward pro-inflammatory stimuli and adiponectin itself, whereas a decrease in globular adiponectin level and subsequent re-exposure to high doses made macrophages more sensitive to pro-inflammatory stimuli, including its own (53). Adiponectin was also shown to facilitate the uptake of apoptotic cells by macrophages, and by that the inflammatory reaction and immune dysfunction induced by apoptotic bodies could be weakened (54, 55). Hence, several *in vitro* studies support a role for adiponectin in attenuating inflammation (43, 49-55).

In recent years, however, evidence emerged challenging this perspective of adiponectin as an exclusively anti-inflammatory cytokine (**Fig. 2**). In contrast with a reduction in serum adiponectin in metabolic diseases, chronic inflammatory and immune-mediated pathologies such as diabetes type 1, rheumatoid arthritis and HFrEF, account for apparently paradoxically high levels of circulating adiponectin (56). A growing number of clinical studies have illustrated that adiponectin rather exerts pro-inflammatory than anti-inflammatory activities in chronic inflammatory/immune diseases, suggesting that the anti-inflammatory actions of adiponectin in metabolic disorders cannot be applied to chronic inflammatory conditions, in which the levels of adiponectin are elevated (57-59). In chronic HFrEF, elevation of circulating adiponectin occurs concomitantly with an increase in systemic inflammation (e.g.;

rise in CRP, TNF-α and IL-6), as described in **chapter 8**. In view of this, and because of adiponectin's pro-inflammatory actions described above, it seems reasonable to assume that high circulating levels of adiponectin add to the chronic systemic inflammatory condition present in HFrEF. Hence, fluctuating plasma concentrations of adiponectin in HFrEF (with initially reduced levels of adiponectin in CAD or after myocardial infarction, followed by increased levels when HF is established) results in macrophage activation and thereon production of high levels of pro-inflammatory cytokines including TNF-α and IL-6, a hypothesis which has also been put forward by Tsatsanis et al. in the context of obesity (53). In this regard, it was also shown that adiponectin induces pro-inflammatory programs in different cell types, such as the release of various pro-inflammatory cytokines including TNF-α and IL-6 (60, 61).

On the other hand, a compensatory rise in adiponectin levels would be most expected after factoring adiponectin's favourable association with inflammatory factors. In this context, elevated circulating adiponectin may act as a compensatory mechanism for the increase in systemic inflammation, whereby adiponectin levels rise in an attempt to counterbalance the high levels of pro-inflammatory cytokines in chronic HFrEF. However, the question remains open, whether and/or when adiponectin serves as a pro-or anti-inflammatory cytokine in HF (**Fig. 2**).

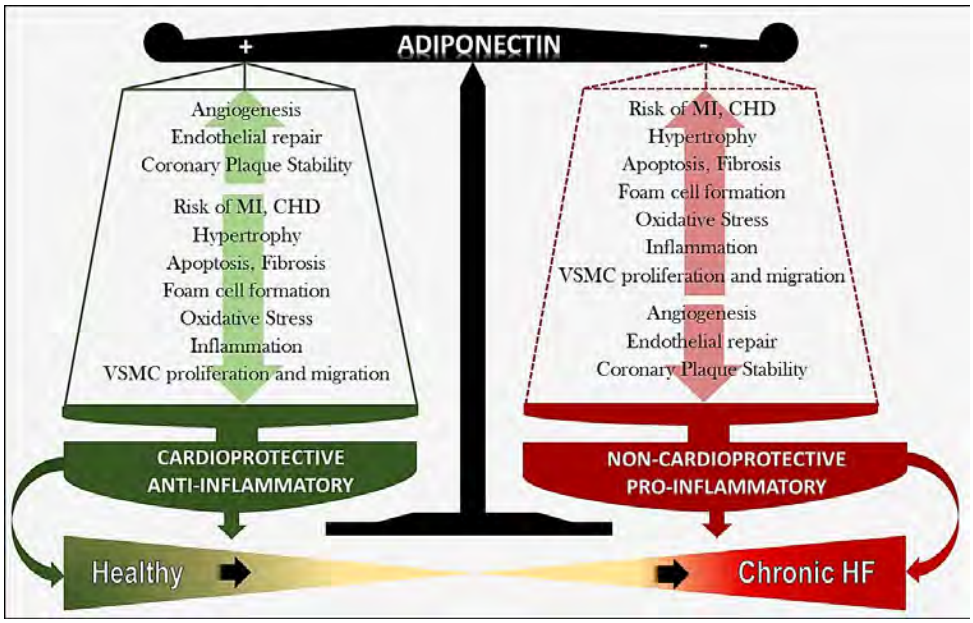

Figure 2. Dual role of adiponectin in heart failure

Adiponectin offers a variety of myocardial and vascular protective effects and is an important anti-inflammatory adipokine in healthy individuals. In the early and acute settings, high adiponectin concentrations likely confer cardioprotection and exert anti-inflammatory actions. However, recent evidence indicates that circulating adiponectin loses its functional predominance in the chronic phase of HF, contributing to adverse cardiac remodelling over time. Therefore, it seems that adiponectin plays a dual role in HF: protective and anti-inflammatory in early and acute HF and harmful and pro-inflammatory towards the progression of chronic HF. *Abbreviations: CHD; Coronary heart disease, HF; Heart failure, MI; Myocardial infarction, VSMC; Vascular smooth muscle cell.*

A TANGLED THREESOME: ADIPONECTIN, INFLAMMATION AND MUSCLE WASTING

Besides the search for underlying mechanisms of increased adiponectin levels and its negative connotation in HFrEF, attention is increasingly focused on the contribution of adiponectin in the disturbed skeletal muscle metabolism among patients with HFrEF (62). As mentioned in **chapters 2 and 6**, a functional skeletal muscle adiponectin resistance is apparent in HFrEF patients (4, 63, 64). Moreover, adiponectin also seems to play a major role in the wasting process in HFrEF (65-67). These facts, together with the assumption that increased circulating adiponectin is a compensatory action to overcome inflammation and disturbed energy metabolism, led us to focus on the differential regulation of adiponectin and the mechanisms

of muscle adiponectin resistance in chronic HFrEF, and thereby taking into account the role of inflammation.

As highlighted in **chapters 3 and 4**, primary skeletal muscle cell cultures are an important supportive tool to study changes in skeletal muscle function and phenotype, including energy metabolism. In the present thesis, we established for the first time primary muscle cell cultures from satellite cells of skeletal muscle biopsies taken from patients with HFrEF. By doing so, we were able to examine the role of adiponectin, and the underlying mechanism of adiponectin resistance, at the level of the skeletal muscle in HFrEF.

Impaired myoblast proliferation: the hidden culprit of muscle wasting in heart failure?

We first examined if there were any phenotypical differences between primary cultures from the skeletal muscle of HFrEF origin and cultures from healthy donors. In **chapter 4**, we demonstrated that the xCELLigence real-time cellular analysis (RTCA) system is a valuable tool to monitor myoblast proliferation *in vitro*. In **chapter 5**, we observed a delay in proliferation kinetics in myoblasts of HFrEF patients compared to myoblast cultures from healthy donors, and this in the absence of increased cellular senescence.

Muscle mass maintenance is mediated by mechanisms controlling activation, proliferation, differentiation and self-renewal of satellite cells and myoblasts. (68). Satellite cell dysfunction, and subsequently impaired myoblast proliferation, is considered a major underlying factor contributing to muscle wasting (69). Clinical studies further demonstrated that loss of skeletal muscle mass in HFrEF is associated with a reduction in peak exercise pulmonary oxygen uptake (VO_2 peak) (70, 71). In addition, as described in **chapter 7**, we demonstrated that the proliferation rate of primary human myoblasts *in vitro* is strongly correlated with *in vivo* clinical parameters of functional capacity, including leg lean mass, VO_2 peak and work efficiency. However, these results do not (definitely) proof a causal link between impaired myoblast proliferation and increased muscle loss in HFrEF. Thus, these studies must be viewed as provisional at present.

Besides an impaired proliferation capacity, also a decreased rate of protein synthesis contributes to muscle wasting in HFrEF (72). This may be a consequence of the systemic inflammation that is present in patients with HFrEF. Indeed, in **chapter 8**, we observed a pro-inflammatory profile in HFrEF patients, represented by increased serum levels of high sensitivity CRP (hsCRP), TNF-α, and IL-6. Furthermore, we also documented in **chapter 5** a

reduced anti-inflammatory capacity in HFrEF myoblasts, supported by a diminished expression of the survival receptor, tumor necrosis factor-α receptor 2 (TNFR2, p75), and a lower IL-6 secretion (73). Repeated exposure of fibroblasts to TNF-α had been shown to weaken the secretion of IL-6 and hence, proliferation (74). Based on our results and findings in literature (75, 76), we speculated that persistent exposure of skeletal muscle cells to high systemic levels of pro-inflammatory cytokines, including TNF-α and IL-6, in HFrEF induces a refractory cell state, provoking a reduction in TNFR2 expression and a decrease in muscle cell secretion of IL-6, thereby attenuating skeletal muscle cell proliferation. This hypothesis was further supported by the findings in **chapter 8**, where we investigated TNFR2 expression in the skeletal muscle of HFrEF patients and its relation with immune activation. An increased systemic inflammation, as documented by increased serum TNF-α and IL-6 levels, was associated with a decreased muscle TNFR2 mRNA in HFrEF. Concomitantly, we observed a down-regulation and decreased activation of signal transducer and activator of transcription 3 (STAT3) in skeletal muscle of patients with HFrEF. Both, STAT3 and TNFR2, regulates skeletal muscle regeneration (e.g.; activation, survival and proliferation of satellite cells) and therefore, diminished skeletal muscle expression levels may possibly add to reduced satellite cell and myoblast proliferation and loss of skeletal muscle tissue (77-80). In **chapter 8**, however, we did not find a relationship between impaired STAT3/TNFR2 expression and skeletal muscle mass, strength and quality.

HFrEF is associated with multiple metabolic disturbances of which many act negatively on skeletal muscle metabolism and wasting (72, 81, 82). Evidence suggests that this disturbed energy metabolism might contribute to the impaired proliferation capacity of primary muscle cells and C_2C_{12} myoblasts (83-88). In addition, early *in vitro* work demonstrated that adiponectin stimulates porcine artery smooth muscle cell proliferation, by using DNA and protein synthesis as surrogates of this process (89). In agreement with Loncar et al., we describe in **chapter 8** that increased circulating adiponectin levels are strongly correlated with reduced quadriceps muscle strength and quality and moderate with quadriceps mass in HFrEF (65). Furthermore, we show in this chapter that adiponectin concentrations in circulation are negatively correlated with exercise capacity (VO_2 peak, maximal workload and work efficiency). Recently, Goto et al. demonstrated a positive association between skeletal muscle mass and the expression of adiponectin receptor 1 (AdipoR1) in atrophied skeletal muscles of mice and in C_2C_{12} cells (90). In **chapter 6**, we observed that the absence of AdipoR1 attenuates myoblast proliferation capacity. Hence, the etiology of peripheral muscle wasting

in HFrEF is likely multifactorial and seems to involve impairment of the proliferative and anti-inflammatory ability and energy metabolism.

TNFR2 bares a dark side in heart failure

HFrEF patients are marked by elevated circulating levels of the pro-inflammatory cytokines TNF-α and IL-6, as also shown in **chapters 6 and 8** (91-93). Hence, systemic inflammation has been repeatedly described within the HF pathophysiology, but few data are available in the literature regarding skeletal muscle inflammation. Inflammation of muscle tissue has mainly been studied in the context of injury and repair (94). The downregulation of both TNFR2 and STAT3 in the skeletal muscle of HFrEF patients may result, at least in part, from the increased systemic immune activation in these patients. The association between reduced muscle STAT3/TNFR2 mRNA and increased circulating levels of TNF-α and IL-6 in **chapter 8**, support this notion. In addition, a prior study by Sasi et al. has shown that absence of TNFR2 signaling in mice subjected to hind limb ischemia results in an increased and long-term inflammation during post-ischemic recovery (80). Furthermore, decreased skeletal muscle TNFR2 expression levels may be due to tissue shedding of TNFR2, since we detected in **chapter 8** a significant increased serum concentration of soluble TNFR2 (sTNFR2) in HFrEF patients. Hence, decreasing the expression of TNFR2, is a possible compensatory mechanism to lower systemic inflammation in HFrEF (95). Hyperadiponectinemia is another interesting possibility. As described in **chapter 8**, increased serum and muscle adiponectin levels are associated with decreased muscle TNFR2 expression in HFrEF. An Offspring study of the Framingham Heart Study, a community-based, prospective epidemiologic cohort study of cardiovascular disease and its risk factors, has recently shown that circulating adiponectin is negatively correlated with circulating TNFR2 (96). The latter suggests that receptor shedding is also possibly involved as a mechanism. This view is supported by the finding that circulating levels of TNFR2 are increased in patients with both HFpEF and HFrEF and related to diastolic dysfunction and advanced HF (97, 98). In addition, Liao et al. demonstrated that adiponectin induces IL-6 production in adult mouse cardiac fibroblasts through AdipoR1, leading to STAT3 activation (99). These results together with our findings, suggest that hyperadiponectinemia may contribute to an impairment of muscle STAT3/TNFR2 signaling in HFrEF. However, suppressor of cytokine signaling 3 (SOC3) may be another factor involved in deficient muscle STAT3/TNFR2 signaling. It has been shown that IL-6 activates SOCS proteins in the skeletal muscle, including SOCS3, an inhibitor of the JAK-STAT pathway (100-102). SOCS3 negatively regulates STAT3 in human skeletal muscle via the

inhibition of STAT3 tyrosine phosphorylation (102, 103). It has been documented that overexpression of SOCS3 negatively regulates STAT3 activation and TNFR2 signaling (102-104). Recently, McKay and colleagues also demonstrated that an increased SOCS3 expression mirrors systemic and muscle IL-6 levels in satellite cells of older adults (105). In addition, satellite cell dysfunction was related to increased levels of SOCS3 and a delayed induction of IL-6 and STAT3. Collectively, these data suggest that increased SOCS3 levels may be responsible for the reduced expression of STAT3 and TNFR2 in HFrEF. Exercise intolerance in HFrEF may also affect the signaling pathway negatively, as several studies have shown that both STAT3 and TNFR2 become activated in the skeletal muscle in response to physical activity (106-108). However, it has to be stated that we observed no association between muscle STAT3/TNFR2 signalling and exercise capacity in HFrEF in **chapter 8**. Taken together, it seems that muscle loss in HFrEF is associated with increased immune activation and an accompanying down-regulation of skeletal muscle STAT3/TNFR2 signaling. Indeed, the inverse correlations between serum IL-6 and TNF-α levels with parameters of exercise capacity (VO_2 peak, maximal workload) and quadriceps features (strength, cross sectional area, quality) in **chapter 8** are consistent with this premise. Our hypothesis on skeletal muscle STAT3/TNFR2 signaling in HFrEF is summarized in **figure 3**.

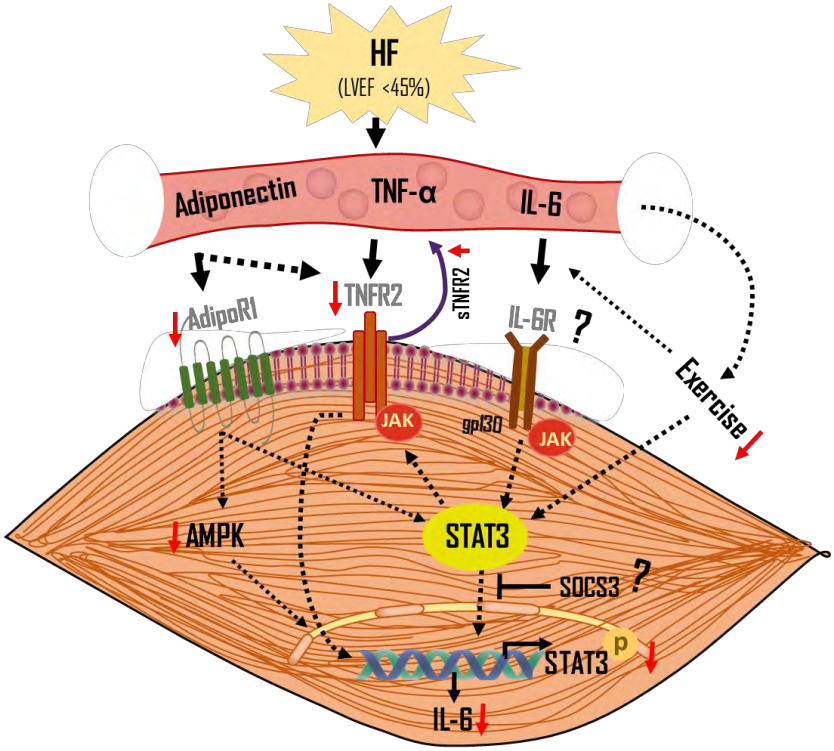

Figure 3. Hypothesis on skeletal muscle IL-6/STAT3/TNFR2 signaling in heart failure

A chronic systemic pro-inflammatory profile present in HFrEF, indicated by increased serum concentrations of TNF-α and IL-6, induces a refractory skeletal muscle cell state, provoking a decrease in skeletal muscle STAT3 and TNFR2 mRNA expression and STAT3 activation. Tissue shedding of TNFR2 occurs most likely as a compensatory mechanism to lower systemic immune activation in HFrEF. The disturbed muscle STAT3/TNFR2 signaling causes a reduction in IL-6 secretion, which hinders satellite cell and myoblast proliferation and muscle regeneration. In addition, hyper-adiponectinemia and exercise intolerance add to impairment of muscle STAT3/TNFR2 signaling.

Inflammation as an underling cause of adiponectin resistance in heart failure

The concept of a functional adiponectin resistance in HFrEF was first introduced by Kintscher et al. (109). Because adiponectin levels in circulation increased with the severity of HF, the authors assumed that adiponectin at high concentrations loses its functional activity. This results in a reduced adiponectin signalling and hence, deactivation of the underlying signalling cascade. This hypothesis was supported by a decreased AdipoR1 expression in the LV of infarcted mouse hearts (110). In addition, Skurk et al. reported a decreased expression of both adiponectin and AdipoR1 mRNA in endomyocardial biopsies of patients with dilated cardiomyopathy, suggesting a local deregulation of the adiponectin system independent of

circulating adiponectin levels (111). The absence of an association between serum adiponectin levels and skeletal muscle adiponectin mRNA expression in HFrEF in **chapter 8**, supports this presumption. The amount of adiponectin found within skeletal muscle is considerably lower compared to its serum levels in HFrEF (63, 64, 109). Interestingly, as described in **chapter 5**, there was no difference in the amount of adiponectin secreted by primary myoblast cultures of HFrEF patients and controls. These data indicate that the concentration of adiponectin in circulation is not reflected by the amount of adiponectin present within the skeletal muscle suggesting that muscle adiponectin is differentially regulated compared to serum adiponectin. In addition, recent data indicate that adiponectin exerts tissue-specific biological functions and activates distinct signaling pathways (112, 113). Furthermore, a skeletal muscle adiponectin resistance was observed in mild to moderate HFrEF (114). The latter was illustrated by an increased skeletal muscle mRNA and protein content of adiponectin in HFrEF patients, whereas the underlying signalling cascade was deactivated, including down-regulation of AdipoR1 (114). In **chapter 6**, we demonstrated that the features representing the defects in adiponectin signalling *in vivo* in the skeletal muscle from HFrEF patients are adequately preserved in primary cultures from skeletal muscle *in vitro*.

As mentioned in **chapter 2**, different hypotheses have been proposed to identify the causes underlying muscle adiponectin resistance in HFrEF (4). However, when this thesis was initiated, a major pending question was, whether an abnormal expression of AdipoR1 depends on the activation of pro-inflammatory mediators and, whether a disturbed AdipoR1 signaling contributes to skeletal muscle adiponectin resistance in HFrEF. Several studies have suggested that the presence of skeletal muscle adiponectin resistance in HFrEF is, at least partly, due to down-regulation of AdipoR1 (115-117). In particular, it has been shown that phosphorylation of AdipoR1 in cardiomyocytes isolated from failing mice hearts resulted in receptor desensitization and subsequent down-regulation, as well as in impaired adiponectin signalling and cardio-metabolism (118). On the other hand, also chronic inflammation has been implicated in the development of adiponectin resistance. Increased presence of inflammatory mediators in HFrEF (e.g.; TNF-α, IL-6) contributes to immune activation and, in that, can have detrimental effects on adiponectin signaling (97, 98, 119-121). In **chapter 6** we evaluated the effect of AdipoR1 silencing in primary cell cultures and demonstrated that inhibition of AdipoR1 leads to a significant decrease in AMPK activation, but has no effect on adiponectin expression. In the presence of TNF-α, however, adiponectin signalling was

disrupted, and myogenesis and mitochondrial biogenesis impaired. Moreover, we investigated adiponectin secretion in culture supernatant from primary myotubes cultures from healthy controls and we found that TNF-α treatment leads to increased adiponectin in the culture supernatant. These findings are consistent with the results of **chapter 8**. Here, we found a positive correlation between serum adiponectin and the pro-inflammatory cytokines hsCRP and TNF-α as well as a strong association of serum adiponectin with the mRNA expression of TNF-α in the skeletal muscle of HFrEF patients. Therefore, it can be speculated that an increased inflammatory constitution contributes to muscle adiponectin resistance in HFrEF. While we were the first to recognize the interaction of AdipoR1 silencing in the presence of TNF-α, about the same time, Kahn et al. published the results of their study in which they confirmed our findings (122). Normalization of myocardial as well as systemic inflammatory processes by mechanical unloading through ventricular assist device (VAD) implantation reversed the down-regulation of AdipoR1 and reduced serum levels of adiponectin in advanced HF (122). These data concur with our assumption that adiponectin is upregulated in HFrEF to counteract the elevated concentrations of pro-inflammatory cytokines.

Contrariwise, last year, the group of Wang et al. shed new light on the mechanism of myocardial adiponectin resistance in HFrEF (118). They demonstrated that adiponectin resistance develops in the heart after myocardial infarction through AdipoR1 phosphorylative desensitization in cardiomyocytes. Interestingly, preventing AdipoR1 phosphorylation by genetic inhibition of the G-protein–coupled receptor kinase 2 (GRK2) restored adiponectin's metabolic, anti-inflammatory, and cardioprotective functions (118). However, these results add significantly to the underlying pathophysiology of adiponectin resistance in HFrEF. Yet, it might be that different molecular mechanisms are involved in cardiac and muscle adiponectin signaling. It seems that impairment of adiponectin signaling in cardiomyocytes directly affects cardiac remodeling and HF progression, whereas, skeletal muscle adiponectin resistance contributes to HF progression via indirect mechanisms, probably related through a systemic metabolic dysfunction.

Overcoming adiponectin resistance is a major challenge and holds great opportunities to alter the progression of HFrEF. Although not yet completely resolved, inflammation seems to be a pivotal mediator in the process of skeletal muscle adiponectin resistance. In this respect, and given the evidence reported to date, it is pertinent to determine whether restoration of the inflammatory cascade will abrogate adiponectin resistance at the skeletal muscle and lead to metabolic and functional improvements in HFrEF.

EXERCISE TRAINING AS A PANACEA FOR ADIPONECTIN RESISTANCE AND MUSCLE WASTING IN HEART FAILURE

To date, exercise training is the most efficacious treatment modality to ameliorate exercise capacity and quality of life in both HFrEF and HFpEF patients (123-130). The mechanisms responsible for these benefits include, at least in HFrEF, anti-inflammatory effects, decreased neuro-hormonal activation and restored insulin resistance (123-126, 131). The study in **chapter 8**, suggests that an increased inflammatory constitution leads to reduced physical performance. Available data demonstrate that exercise is able to attenuate mitochondrial, histological, and functional alterations in the skeletal muscle of patients with HFrEF and HFpEF (132). In addition, it has been demonstrated that physical activity positively influence satellite cell content and activation status and myoblast proliferation (133-135). Resistance exercise training, for instance, stimulates muscle cell proliferation by IL-6 induced activation of STAT3 signaling (136-139). Furthermore, in HFrEF, exercise was shown to down-tune the activation of the adiponectin pathway at the circulatory and at the myocyte level (125, 127). In particular, besides improvements in work efficiency and muscle strength, combined endurance and resistance exercise training lowered the circulating adiponectin concentration and normalized the muscle-specific expression of adiponectin, AdipoR1 and of genes involved in lipid and glucose metabolism towards healthy control levels (125). Consistent with these data, we observed in **chapter 8** a negative correlation between serum adiponectin levels and parameters of exercise capacity (e.g.; VO_2 peak, maximal workload and work efficiency) in HFrEF patients. To date, however, no information is yet available regarding the influence of exercise on adiponectin signaling in HFpEF patients.

FUTURE SCIENTIFIC PERSPECTIVES

Adiponectin as a target in search of another BNP

Adiponectin has not been officially recognized as a biomarker in HFrEF. Several results from studies, though, suggest that adiponectin might be useful as a marker for monitoring treatment and predicting prognosis, or that adiponectin may act as a valuable adjunct to natriuretic peptides in the sub-classification of patients with HF. Some clinical studies, however, have also questioned the utility of adiponectin as a biomarker in HFrEF. Here, we discuss the current evidence available.

Of the myriad of biomarkers that have been studied in HFrEF, natriuretic peptides are the best characterized. BNP and NT-proBNP are the gold standard biomarkers in assessing the diagnosis and prognosis of HFrEF, but does adiponectin have additive biomarker value? Cavusoglu and colleagues demonstrated that high adiponectin is an independent predictor of LV systolic dysfunction in patients undergoing coronary angiography (n= 389) (140). In HFpEF patients, decreased adiponectin levels have been associated with hypertension, CAD, and degree of diastolic dysfunction. HFrEF patients with serum adiponectin concentrations above the 75th percentile were shown to be at an increased risk of death, irrespective of baseline clinical (e.g.; age, sex, diabetes, NYHA class) or laboratory findings (e.g.; serum TNF-α, IL-6, hsCRP) (13). The rise in adiponectin level was proportional to the extent and worsening of HFrEF. Also, the number of risk factors was shown higher in HFrEF patients with the highest level of adiponectin (19). In addition, in a study by Dieplinger et al. in patients with acute decompensated HFrEF (n=137), total adiponectin level was shown to act as a significant prognostic marker for 1 year all-cause mortality, and this independently of the presence of clinical confounders (e.g.; older age, renal dysfunction, arterial hypertension, low systolic blood pressure, NYHA class III/IV) CRP, body mass index (BMI) and BNP (141). Accordingly, serum adiponectin may seem useful as an additive biomarker for monitoring patients with HF and predicting their future disease course. Yet, this prognostic value of circulating adiponectin may not hold true after adjustment for other important clinical characteristics of patients like age, exercise capacity, type of HF, and medication use (i.e.; beta-blocker treatment) (127, 128, 142, 143). Moreover, interactions between risk factors including diabetes mellitus type 2 may also affect adiponectin concentration in patients with HFrEF, making it difficult to ascertain the role of circulating adiponectin in the development and progression of HFrEF. In addition, adiponectin was not reliable as a marker for the diagnosis of acute decompensated HF in patients with shortness of breath (144). These latter findings could weaken the clinical utility of circulating adiponectin in HF. These latter findings could weaken the clinical utility of circulating adiponectin in HFrEF. However, its biphasic serum level, as mentioned in **chapter 1**, may put adiponectin forward as a useful biomarker during the transition from diastolic to systolic dysfunction in HF. The study of Fu and colleagues, examined whether total serum adiponectin is associated with onset of HF from hypertension through LV hypertrophy in spontaneously hypertensive rats, and indicated that The adiponectin level in rats decreased with increasing hypertension during the first three months, and continued to decline when diastolic dysfunction became overt, whereas at month 15, levels started to rise again prior to the appearance of systolic dysfunction at month 18

(145). In this respect, adiponectin may turn out helpful for sub-classifying HF patients, especially those with preserved LVEF. However, the comparative prognostic and diagnostic significances of the different isoforms of adiponectin, its therapeutic meaning *(vide infra)* and potential to monitor the efficacy of HFrEF therapy are all still unclear. Consequently, more research is needed before adiponectin can be considered of additive value in the clinical management of HFrEF patients.

Looking at the crystal ball: Is there a therapeutic perspective for adiponectin in heart failure?

At present, there is only limited data from animal studies regarding the potential therapeutic effect of adiponectin in HF. Very recently, a study by Wang et al. investigated the role of adiponectin in cardiomyocyte contractile function in a rat model of volume overload induced HF (146). An inverse correlation between adiponectin levels and ventricular remodeling was observed. In particular, ventricular myocyte contractility and intracellular Ca^{2+} transients were impaired under chronic volume overload due to reduced AMPK phosphorylation, which was restored by administration of adenoviral adiponectin, indicating a protective role for adiponectin in volume overload-induced HF. Furthermore, absence of adiponectin in mice with angiotensin II-induced cardiac hypertrophy resulted in increased cardiac fibrosis, increased levels of ROS and inflammatory cytokines, and up-regulation of the β-catenin signalling pathway which is centrally involved in cardiac hypertrophy; these impairments could be reversed by adiponectin supplementation (147). A recent study by Tanaka et al. further demonstrated that adiponectin overexpression and/or supplementation in aldosterone-induced HFpEF mice ameliorates LVH, myocardial oxidative stress, lung congestion and diastolic function without affecting blood pressure or LVEF (148). Moreover, Essick et al. showed that adiponectin protects against oxidative stress-mediated autophagic induced cardiomyocyte death by modulating mammalian target of rapamycin (mTOR)/AMPK signalling in mice subjected to chronic angiotensin-II infusion (149). These data demonstrate the anti-oxidant potential of adiponectin in oxidative stress-associated CVD, amongst which is atherosclerosis and HFpEF.

All results considered, modulation of adiponectin or its signaling pathway might be a promising therapeutic avenue in the treatment of HF. However, the exact mechanisms by which adiponectin affects cardiac remodelling, its temporal nature in HFpEF and HFrEF and its interaction with confounding factors have to be clarified. Moreover, as previously

suggested, changing the ratio of total adiponectin to HMW adiponectin could be a better therapeutic approach than targeting total adiponectin levels (150, 151).

Furthermore, if adiponectin eventually enters clinical trial testing, there will be several hurdles which need to be overcome, as was recently meticulously described by Mather et al (152). In brief, oral administration of adiponectin may not be possible due to its degradation by digestive enzymes, its rapid clearance from circulation and therefore relatively short serum half-life. As such, it will be difficult for adiponectin to reach the bloodstream in sufficiently high concentrations. In addition, the production of adiponectin might be challenging because of its posttranslational modifications and oligomeric structures. Furthermore, long-term supplementation of recombinant adiponectin would be very expensive. Pharmacological treatment (e.g.; peroxisome proliferator-activated receptor, PPAR agonists) may offer a solution to modify the adiponectin levels in circulation (153-155). However, to avoid unwanted side effects, we believe that life style interventions, especially exercise training, will be an efficient and easy way to influence adiponectin levels in HF patients (127). Alternatively, selective compounds targeting adiponectin receptor responses could be considered. A small-molecule AdipoR agonist (AdipoRon) was recently found to bind both AdipoR1 and AdipoR2 *in vitro*, and demonstrated similar effects as adiponectin in muscle and liver such as activation of the AMPK and PPARα pathways in mice fed a high fat diet (156). Another promising approach might be the modification of adiponectin secretion by biological compounds. In this regard, Hino et al. developed a high-throughput screening assay using insulin-resistant-mimic adipocytes and identified six compounds that promote adiponectin secretion in a conditioned medium of 3T3-L1 adipocytes without having PPARγ agonistic activity (157).

Opening the black box: Where should we focus on in the near future?

Up to now, in most studies of HFrEF, circulating levels of total adiponectin have been measured. Recent evidence, however, indicates that total and high-molecular weight (HMW) adiponectin have equal potency in assessing incident HF and mortality risk in chronic HFrEF (16, 158). Ample data suggest that the HMW adiponectin is the most bioactive isoform of adiponectin with close relevance to cardioprotection (159). Moreover, the HMW isoform is a more sensitive marker of metabolic abnormality than total adiponectin (160-162). Hence, this underscore the need to consider HMW adiponectin levels and functions in HFrEF. Although HMW adiponectin has been difficult to detect in the past and only semi-quantitative

determinations were available, a novel sandwich enzyme-linked immunosorbent assay (ELISA) method has been developed to accurately determine serum HMW adiponectin levels (163). We did not undertake measurement of HMW adiponectin nor ratio of HMW to total adiponectin, and therefore, warrants closer scrutiny.

Insulin resistance has been previously reported as an intrinsic feature of HFrEF (164). Insulin resistance progresses in parallel with the severity of HFrEF and is considered instrumental in the development of skeletal muscle wasting (165). In particular, insulin sensitivity is worse in cachectic HFrEF patients (166). Adiponectin regulates insulin-sensitizing effects at the level of the skeletal muscle partly via activation of AMPK (167, 168). In HFrEF, however, adiponectin concentrations are elevated despite a profound resistance to insulin, suggesting a disconnection between increased circulating adiponectin and improvement of insulin sensitivity in HFrEF (169). However, a major pending question is to what comes first in HFrEF: hyperadiponectinemia or hyperinsulinemia? It is increasingly assumed in literature that insulin resistance emerge prior to adiponectin resistance. Wang et al. suggested that an impaired insulin sensitivity represent a causal link between adiponectin resistance and skeletal muscle wasting in HFrEF (165). Lack of insulin signaling in an adipocyte-specific insulin knock-out (KO) mouse model results in hyperadiponectinemia (170). In addition, the interaction between adiponectin and insulin was investigated in mice with muscle-specific insulin resistance, and showed that insulin resistance leads to hyperadiponectinemia and adiponectin resistance (171, 172). In contrast, Mullen et al. found evidence for adiponectin resistance to emerge prior to the initiation of insulin resistance in high-fat fed rats (173). The interaction between adiponectin and insulin in HFrEF is complex and, therefore, should be addressed in the near future. Moreover, it has been demonstrated that insulin induced proliferation, differentiation and survival of C_2C_{12} myoblasts (86, 174). Insulin-like growth factor-1 (IGF-1) also serves as an important regulator of skeletal muscle growth and homeostasis (175). Keeping in mind that HFrEF patients are characterized by an impaired myoblast proliferation (**chapter 5**) and decreased muscle IGF-1 expression levels, it would be interesting to investigate the importance of an impaired insulin signaling in the initiation of muscle wasting in HFrEF.

The finding of a disturbed skeletal muscle STAT3/TNFR2 signaling in HFrEF patients (**chapters 5 and 8**) deserves further attention. We admit that it might be interesting to investigate skeletal muscle SOCS3 expression in HFrEF patients. SOCS3 is transcriptionally upregulated by STAT transcription factors. In line with lower STAT3 activity, expression

levels of SOCS3 also seem to be disturbed in failing human hearts (176-178). Increased knowledge of the underlying mechanisms involved could restore skeletal muscle STAT3/TNFR2 expression and hence, proliferative capacity of myoblasts. Additionally, the decreased myoblast IL-6 secretion needs to be explored *in vivo* by immunohistochemical evaluation of IL-6 levels in skeletal muscle fibers *(m. vastus lateralis)* of HFrEF patients. The elaboration of IL-6 regulation in the skeletal muscle of HFrEF patients will be the subject of a forthcoming study. Subsequently, experimental studies are needed to untangle the association between adiponectin and TNFR2 signaling in HFrEF and their importance in the process of muscle wasting. In this respect, it has been illustrated that soluble TNFR2 (sTNFR2) is increased in patients with HFrEF and associated with peak oxygen consumption (VO_2 peak) (179, 180). Moreover, an offspring study of the Framingham Heart Study, a community-based, prospective epidemiologic cohort study of cardiovascular disease and its risk factors, has recently shown that adiponectin is negatively correlated with circulating TNFR2 (96). We are currently investigating the relation between adiponectin and TNFR2 (systemic and local) in HFrEF patients.

While the role of adiponectin in chronic HFrEF has been intensively evaluated in recent years, little is known about its function in HFpEF. Preclinical studies have demonstrated that adiponectin is protective against major hallmarks of HFpEF such as hypertension, LVH and diastolic dysfunction. However, the group of Negi et al. is the only one to date who has studied circulating adiponectin levels in HFpEF (181). They demonstrated that total serum adiponectin levels are diminished in chronic HFpEF patients and related to the severity of diastolic dysfunction (181). In addition, it was also shown that in a murine model of HFpEF, hypoadiponectinemia exacerbated hypertension, diastolic dysfunction and even HF (148, 182). Whether adiponectin contributes to the pathophysiology of HFpEF needs further exploration. As BNP is less accurate in predicting outcomes in HFpEF patients, other biomarkers are urgently needed in the context of HFpEF (183). Adiponectin could play an important role as an additive biomarker in HFpEF, but clinical evidence is currently lacking. Also, as HFpEF is considered a heterogeneous syndrome with several phenotypes, adiponectin could aid in distinguishing different subtypes (184). Furthermore, data regarding muscle adiponectin signalling in HFpEF is not yet available. In this context, it will also be worthwhile to study adiponectin levels during the different stages of HF, including early and end-stage HF as well as acute HF.

CONCLUSIONS

Skeletal muscle wasting and adiponectin resistance are two important features of HFrEF. In this thesis we introduced the use of primary cultures from the skeletal muscle of HFrEF patients as a tool to investigate the underlying mechanisms of adiponectin resistance and muscle wasting. We show that myoblasts from HFrEF patients have a diminished proliferative activity, which is strongly correlated with *in vivo* clinical parameters of functional capacity, yet myoblasts from HFrEF patients also exhibit a reduced anti-inflammatory activity. In addition, an impaired STAT3/TNFR2 signalling in the skeletal muscle of patients with HFrEF was indicated and associated with increased systemic inflammation. We demonstrate that exposure of primary cell cultures from healthy subjects to the pro-inflammatory cytokine TNF-α leads to deterioration of adiponectin signalling, myogenesis and mitochondrial biogenesis, impairments which are similar to those seen in patients with HFrEF. Silencing of AdipoR1 in healthy donor cultures attenuates myoblasts proliferation and AMPK activation. Overall, our findings indicate that adiponectin resistance and skeletal muscle dysfunction in HFrEF primarily emanate, albeit not necessary exclusively, from an increased inflammatory constitution. Further research is needed to fully elucidate and understand the interrelations between inflammation, adiponectin and muscle wasting in HFrEF. In the end, new insights may open up new avenues in the treatment of patients with HFrEF.

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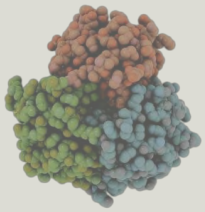


Chapter 10

Summary of the thesis

HEART FAILURE: MORE THAN A CARDIAC ISSUE

Chronic heart failure (HF) has been singled out as growing clinical and public health problem, associated with significant mortality, morbidity, and healthcare expenditures, particularly among those aged ≥65 years. Despite improvements in therapy and progress in reducing HF-related mortality, hospitalizations remain frequent and rates of readmissions continue to rise. Exercise intolerance, manifesting as dyspnoe and fatigue during exercise, is the cardinal symptom of HF and severely impacts quality of life. Research conducted during the last two decades has learned that HF is a multi-system disease which, beyond the impairment of cardiac function, also affects the functional capacity of other organs and physiological systems that ultimately contribute to its symptomatology and progression. Adaptations in the peripheral musculature, such as loss of muscle quantity and functionality and a disturbed energy metabolism, are now generally accepted as important predictors of exercise intolerance in HF. Whereas the clinical relevance of HF myopathy is beyond doubt, there is a compelling need for scientific progress in the field of metabolic alterations in HF and the relation with skeletal muscle wasting in order to develop strategies that tackle muscle abnormalities in HF. In this setting, the adipocytokine, adiponectin has recently turned up to be a specific protein of interest.

SKELETAL MUSCLE WASTING AND ADIPONECTIN RESISTANCE

With its fundamental role in skeletal muscle energy metabolism, a major role was indicated for adiponectin in muscle metabolism dysregulation among patients with HF, especially HF patients with reduced ejection fraction (HFrEF). In strong contrast to patients at risk for cardiovascular diseases, circulating adiponectin levels are increased in patients with HFrEF and related with metabolic impairment and advanced disease state. These elevated adiponectin levels likely act as a protective and compensatory mechanism for the energy metabolic alterations and increased immune activation present in the early stage of HFrEF. In addition, HFrEF patients are characterized by a functional skeletal muscle adiponectin resistance. Overcoming muscle adiponectin resistance is, however, a major challenge and holds great opportunities to alter the progression of HFrEF.

The relevance of skeletal muscle myopathy and muscle metabolic disturbances, comprehensive information regarding the complex role of adiponectin in HF, as well as the assumption that adiponectin functions as a marker of skeletal muscle wasting, are provided in

chapter 1. Furthermore, **chapter 2** gives an overview of the evidence and plausible pathophysiological mechanisms underlying skeletal muscle adiponectin resistance in HFrEF.

The general objectives of this thesis are to investigate growth and phenotype of HFrEF skeletal muscles, the role of adiponectin and the mechanisms of skeletal muscle adiponectin resistance in HFrEF. The composition of the different research studies are outlined in **chapter 3**.

MAIN FINDINGS OF THE THESIS

In order to be able to explore the differential regulation of adiponectin at the level of the skeletal muscle in patients with HFrEF, we were drawn back *'from the bedside to the bench'*. More specifically, we isolated satellite cells from human skeletal muscle biopsies (*m. vastus lateralis*) from HFrEF patients and established primary HFrEF myoblasts and myotubes cultures. In the first part of this thesis (**chapters 4 and 5**), we report the development and the characterization of primary HFrEF skeletal muscle cell cultures. The second part of this thesis (**chapters 6 to 9**), provides further mechanistic insight into skeletal muscle adiponectin resistance and wasting in HFrEF.

In **chapter 4**, we describe the methods used throughout the different studies to initiate primary HFrEF skeletal muscle cell cultures and to recover sufficient amounts of high-quality intact RNA from primary myoblast cultures. In addition, this chapter presents the results of a comparative study between the conventional MTT assay and the xCELLigence RTCA system to evaluate myoblast proliferation. A strong linear relationship was noticed between both techniques, indicating that the xCELLigence RTCA system is a valuable tool for monitoring HFrEF myoblast proliferation *in vitro*, supporting the study of skeletal muscle wasting in HFrEF patients.

In **chapter 5**, the phenotypic and proliferative characteristics of skeletal muscle cells from HFrEF patients are analyzed in relation to healthy donor cell cultures. The study demonstrates that myoblasts and myotubes derived from HFrEF patients are not different from healthy donors in terms of morphology, such as myoblast size, shape and actin microfilament. Myogenic differentiation capacity, viability and senescence were identical between both groups. However, myoblasts of HFrEF patients exhibit an altered proliferative and reduced anti-inflammatory activity, represented by the diminished expression of the survival receptor

TNFR2 and lower IL-6 secretion. This study indicates for the first time that specific *in vitro* cultured skeletal muscle cells from HFrEF patients represent a promising tool that could open a path to greater insight in the process of muscle wasting in HFrEF and may lead to important insights in the further exploration of this intriguing issue.

Chapter 6 was designed to investigate the adiponectin resistance in cultured primary skeletal muscle cells from HFrEF patients. Similar to skeletal muscle biopsies of HFrEF patients, adiponectin expression is increased in primary skeletal muscle cell cultures from HFrEF patients, whereas the expression of the main skeletal muscle adiponectin receptor, AdipoR1, is reduced together with several downstream adiponectin-related genes (e.g.; AMPK, HK2) with impaired cellular energy metabolism signalling as a prominent feature. This indicates that the features representing defects in adiponectin signalling *in vivo* in skeletal muscle from HFrEF patients are adequately preserved *in vitro* in primary muscle cell cultures.

Additionally, insight into the underlying mechanism of skeletal muscle adiponectin resistance is presented. A major pending question was whether an abnormal expression of AdipoR1 and/or inflammation contribute to an increased skeletal muscle adiponectin expression and, ultimately muscle adiponectin resistance in HFrEF. We demonstrate that silencing of AdipoR1 attenuates myoblasts proliferation and activation of AMPK in primary cultures from healthy controls, but, was insufficient to induce skeletal muscle adiponectin resistance. TNF-α exposure of healthy donor myoblasts impairs adiponectin signalling illustrated by reduced expression levels of AdipoR1, AMPK, PPARα and ACADM. In line, exposure of TNF-α, decreases mitochondrial metabolism, myogenic differentiation capacity and polarizes cytokine secretion toward a growth-promoting phenotype. Moreover, the siAdipoR1-induced reduction in myoblast proliferation was partially restored by TNF-α. These results suggest that an increased inflammatory constitution contributes to skeletal muscle dysfunction and adiponectin resistance in HFrEF. In this respect, and given the evidence reported to date, it is pertinent to determine whether restoration of the immune activation will abrogate skeletal muscle adiponectin resistance and lead to metabolic and functional improvements in HFrEF.

Satellite cell dysfunction is considered a major underlying factor contributing to muscle wasting. In **chapter 5**, we demonstrate that satellite cell-derived myoblasts from HFrEF patients are characterized by a time delay in proliferative activity. The results in **chapter 7** extend this finding by demonstrating that the proliferation of primary human myoblasts *in vitro* is strongly correlated with *in vivo* clinical parameters of functional capacity, including

leg lean mass, VO_2 peak, maximal workload and work efficiency. However, these results do not definitively proof a causal link between impaired myoblast proliferation and muscle wasting in HFrEF and hence, must be viewed as provisional at present.

In **chapter 8**, further proof is provided for the impaired anti-inflammatory capacity of HFrEF muscle cells. We investigate skeletal muscle STAT3/TNFR2 signalling in HFrEF. Both, STAT3 and TNFR2 mRNA, as well as STAT3 activation were decreased in skeletal muscle biopsies of HFrEF patients. While the exact underlying mechanism for this impaired STAT3/TNFR2 signalling need further exploration, this study provides evidence that an increased systemic inflammation is associated with skeletal muscle STAT3/TNFR2 dysregulation in HFrEF. Furthermore, both elevated serum and muscle adiponectin levels are negatively correlated with TNFR2 mRNA expression, suggesting that adiponectin is also involved in the impaired skeletal muscle anti-inflammatory capacity in HFrEF. However, muscle STAT3/TNFR2 signalling in HFrEF is not associated with exercise capacity or quadriceps parameters including strength, mass and quality, as for muscle adiponectin. In contrast, increased serum levels of adiponectin in HFrEF are associated with an impaired exercise capacity, lower quadriceps muscle strength and quality. More knowledge of skeletal muscle STAT3/TNFR2 signaling in chronic HFrEF and its association with inflammation and adiponectin will eventually contribute to novel therapeutic strategies combating muscle wasting in HFrEF.

In **chapter 9**, data gathered from this thesis are combined to illustrate the interaction between adiponectin, inflammation and muscle wasting in HFrEF. Our hypotheses are further elaborated according to recent literature. Special attention goes to the dual role of adiponectin in HF, the impaired myoblast proliferative capacity and skeletal muscle STAT3/TNFR2 signaling in HFrEF as well as the underlying cause of skeletal muscle adiponectin resistance in HFrEF. Furthermore, the role of exercise training in the treatment of skeletal muscle adiponectin resistance and muscle wasting is discussed. Finally, further perspectives and fundamental questions that were raised during the process of this thesis are given and will hopefully be answered in future studies.

HARTFALEN: MEER DAN EEN CARDIALE AANDOENING

Chronisch hartfalen (HF) is een groeiend probleem voor de volksgezondheid, kent een hoge morbiditeit en mortaliteit en vormt een aanzienlijke belasting voor de gezondheidszorg, in het bijzonder in de leeftijdsgroep ≥65 jaar. Ondanks de substantiële therapeutische vooruitgang in de behandeling van patiënten met HF wordt de ziekte nog steeds gekenmerkt door frequente ziekenhuisopnamen. Volgend op een hospitalisatie, wordt 40% van de patiënten gerehospitaliseerd of overlijdt binnen het jaar. Vermoeidheid en kortademigheid bij inspanning of zelfs in rust zijn de klassieke klachten die leiden tot inspanningsintolerantie en een verminderde levenskwaliteit bij patiënten met HF. De voorbije jaren is het duidelijk geworden dat HF een multisysteem aandoening is. Naast centraal cardiale en hemodynamische factoren, dragen ook perifere veranderingen, waaronder afwijkingen ter hoogte van de skeletspier, bij aan de symptomatologie en progressie van HF. Vanuit deze optiek heeft cachexie, een complex syndroom dat wordt gekenmerkt door een aanzienlijk en progressief verlies van spiermassa, bij patiënten met ernstig HF aandacht verworven. Perifere skeletspierafwijkingen, waaronder verlies van spierweefsel en een verstoord energiemetabolisme zijn in sterke mate verantwoordelijk voor de fysieke beperkingen van deze patiënten. Hoewel er nu voldoende bewijs is voor het bestaan van een algemene en klinisch relevante myopathie bij HF, blijft de etiologie tot op heden onvolledig opgehelderd. In de zoektocht naar onderliggende mechanismen van spieratrofie in HF en mogelijke strategieën om deze spieraantasting tegen te gaan werd recent de aandacht gericht op het adipocytokine adiponectine.

SKELETSPIERATROFIE EN ADIPONECTINE RESISTENTIE

Binnen de pathogenese van de spierafwijkingen in HF verschuift de aandacht steeds meer naar de metabole veranderingen. Hierbij wordt een relevante rol toebedeeld aan het adipocytokine, adiponectine, voornamelijk bij HF patiënten met een gedaalde ejectiefractie (HFrEF). Adiponectine speelt een belangrijke rol in de insulinegevoeligheid en reguleert het skeletspier energiemetabolisme. In tegenstelling tot patiënten die gekenmerkt worden door een verhoogd cardiovasculair risicoprofiel, zijn circulerende concentraties van adiponectine binnen de populatie van HFrEF patiënten verhoogd en bovendien ook gekoppeld aan een slechte prognose. Deze verhoogde concentraties fungeren mogelijks als een beschermend compensatiemechanisme voor de metabole veranderingen en verhoogde immuunactivatie in

de vroege fase van HFrEF. Bovendien worden HFrEF patiënten gekenmerkt door een functionele skeletspier adiponectine resistentie. Het omkeren van deze adiponectine resistentie is echter een grote uitdaging en zou op termijn de progressie van HFrEF kunnen afremmen.

In **hoofdstuk 1** beschrijven we de relevantie van de perifere spierafwijkingen bij HFrEF en gaan we dieper in op de complexe rol van adiponectine. Argumentatie dat adiponectine fungeert als een marker voor spieratrofie in deze patiëntengroep wordt eveneens besproken. In **hoofdstuk 2** behandelen we de skeletspier adiponectine resistentie in HFrEF en geven we een overzicht van de mogelijke onderliggende mechanismen.

Het doel van het onderzoeksproject, zoals voorgesteld in deze thesis, is inzicht de verkrijgen in het proces van skeletspieratrofie en adiponectine resistentie in HFrEF. De fenotypische eigenschappen van een skeletspier van HFrEF patiënten worden bestudeerd alsook de onderliggende mechanismen verantwoordelijk voor de skeletspier adiponectine resistentie in HFrEF. Een overzicht van de verschillende studies is voorgesteld in **hoofdstuk 3**.

HOOFDBEVINDINGEN VAN DE THESIS

Om de relatie tussen adiponectine en de afwijkingen gevonden in de skeletspier bij patiënten met HFrEF te bestuderen, werd de *'bedside to bench'* benadering toegepast. Meer in het bijzonder, wordt in dit onderzoeksproject gebruik gemaakt van myoblast- en myotubes culturen afgeleid van satellietcellen verkregen uit spierbiopten van de *m. vastus lateralis* van HFrEF patiënten. Het eerste deel van deze thesis (**hoofdstuk 4 en 5**) beschrijft de fenotypering van primaire HFrEF skeletspierculturen. Een twee deel (**hoofdstukken 6 tot 9**) omvat mechanistisch inzicht in de skeletspier adiponectine resistentie en spieratrofie in HFrEF.

Hoofdstuk 4 levert een overzicht van de methoden die in de verschillende studies gebruikt werden om primaire HFrEF spiercelculturen te initiëren en vervolgens voldoende hoeveelheden van hoogwaardige intact RNA te verkrijgen. Daarnaast worden in dit hoofdstuk twee technieken, meer bepaald, de conventionele MTT test en het xCELLigence RTCA systeem, vergeleken voor de bepaling van primaire myoblast proliferatie. Een sterke lineaire relatie werd opgemerkt tussen beide technieken wat aangeeft dat het xCELLigence RTCA

systeem een waardevol instrument is voor de evaluatie van HFrEF myoblast proliferatie *in vitro* en eveneens aangewend kan worden voor de studie van skeletspieratrofie in HFrEF.

In **hoofdstuk 5** worden de fenotypische en proliferatieve eigenschappen van de skeletspier cellen afkomstig van HFrEF patiënten geanalyseerd in relatie tot gezonde donor spiercelculturen. Deze studie toont aan dat primaire myoblasten en myotubes afgeleid van HFrEF patiënten niet verschillend zijn van gezonde donoren wat betreft morfologie, waaronder myoblast grootte, vorm en actine microfilament. Myogene differentiatie capaciteit, viabiliteit en senescentie zijn eveneens vergelijkbaar tussen beide groepen. Myoblasten van HFrEF patiënten vertonen, echter, een vertraagde proliferatieve capaciteit en een gedaalde anti-inflammatoire activiteit, weergegeven door een verminderde expressie van de receptor TNFR2 en een verlaagde IL-6 secretie. Deze studie laat voor de eerste maal zien dat primaire *in vitro* skeletspierculturen van HFrEF patiënten een veelbelovende techniek is welke kan resulteren in belangrijke nieuwe inzichten in het proces van spieratrofie in HFrEF.

In **hoofdstuk 6** wordt dieper ingegaan op de adiponectine resistentie ter hoogte van de skeletspier in HFrEF patiënten. In overeenkomst met de verhoogde expressie van adiponectine in skeletspier biopten van HFrEF patiënten, is de expressie van adiponectine ook verhoogd in spiercelculturen van HFrEF patiënten, terwijl de expressie van de voornaamste skeletspier adiponectine receptor, AdipoR1, alsook enkele adiponectine-gerelateerde genen van de onderliggende signaalweg, waaronder AMPK, verlaagd zijn. Dit wijst erop dat de adiponectine resistentie aanwezig *in vivo* in de skeletspier van HFrEF patiënten behouden blijft *in vitro* in primaire skeletspiercelculturen.

Daarnaast wordt in dit hoofdstuk meer inzicht gegeven in het onderliggende mechanisme van de skeletspier adiponectine resistentie in HFrEF. Een prangende vraag was of een abnormale expressie van AdipoR1 en/of inflammatie kan leiden tot een verhoogde skeletspier adiponectine expressie en aldus kan bijdragen aan de adiponectine resistentie in HFrEF. *Silencing* van AdipoR1 in primaire spiercelculturen van gezonde donoren resulteert in een vertraagde myoblast proliferatie en gedaalde activatie van AMPK, maar is onvoldoende voor de initiatie van adiponectine resistentie. Blootstelling van gezonde spiercelculturen aan TNF-α leidt tot een verstoorde adiponectine signaalweg, geïllustreerd door een verminderde expressie van genen betrokken in de onderliggende signaalweg, AdipoR1, AMPK, PPARa en ACADM. Bovendien zorgt de blootstelling van TNF-α voor een gedaald mitochondriaal metabolisme en myogene differentiatie capaciteit. Echter, TNF-α oefent een positieve invloed

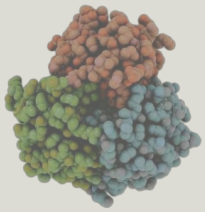
uit op de groeicapaciteit van spiercelculturen en herstelt gedeeltelijk de siAdipoR1 geïnduceerde vertraagde myoblast proliferatie. Deze resultaten suggereren dat een verhoogde inflammatoire constitutie bijdraagt tot skeletspier dysfunctie en adiponectine resistentie in HFrEF. Herstel van de inflammatoire activatie in HFrEF zou mogelijks de skeletspier adiponectine resistentie kunnen omkeren en leiden tot metabolische en functionele verbeteringen in HFrEF.

Satellietcel dysfunctie wordt beschouwd als een belangrijk onderliggend mechanisme van spieratrofie. **Hoofdstuk 7** is gewijd aan de klinische relevantie van de vertraagde proliferatieve activiteit van primaire HFrEF myoblasten, aangetoond in **hoofdstuk 5**. De proliferatiecapaciteit van myoblasten *in vitro* is sterk gecorreleerd met *in vivo* klinische parameters van functionele capaciteit, met inbegrip van de magere spiermassa ter hoogte van de onderbenen, het maximale aerobe vermogen (VO_2 piek), de maximale werklast en efficiëntie. Echter, deze resultaten leveren geen definitief bewijs van een oorzakelijk verband tussen een verminderde myoblast proliferatie en spieratrofie in HFrEF.

In **hoofdstuk 8** presenteren we de resultaten van een klinische studie die de STAT3/TNFR2 signalering in de skeletspier van HFrEF patiënten evalueert. In deze studie wordt er verder bewijs geleverd voor de verminderde anti-inflammatoire capaciteit van HFrEF spiercellen (**hoofdstuk 5**). De mRNA expressie levels van STAT3 en TNFR2 zijn verlaagd in spierbiopten van HFrEF patiënten. Verder wijst deze studie erop dat een verhoogde systemische inflammatie een belangrijke rol speelt in de verstoorde STAT3/TNFR2 signaalweg in HFrEF. Echter, er is geen verband tussen de mRNA expressie van STAT3/TNFR2, inspanningscapaciteit en eigenschappen van de quadriceps spier, waaronder kracht, massa en kwaliteit in HFrEF. De mRNA expressie van TNFR2 is negatief gecorreleerd met adiponectine (zowel circulerend als in de skeletspier), wat suggereert dat adiponectin betrokken is bij de verstoorde anti-inflammatoire capaciteit ter hoogte van de skeletspier in HFrEF. Bovendien zijn verhoogde circulerende concentraties van adiponectine in HFrEF geassocieerd met een verminderde inspanningscapaciteit en verlies van quadriceps spierkracht- en kwaliteit. Meer inzicht in de regulatie van STAT3/TNFR2 ter hoogte van de skeletspier in HFrEF en de associatie met inflammatie en adiponectine zal bijdragen tot nieuwe therapeutische strategieën voor de behandeling van spieratrofie in HFrEF.

In **hoofdstuk 9** worden alle gegevens, die verzameld werden gedurende dit onderzoek, samengelegd om de interactie tussen adiponectine, inflammatie en spieratrofie in HFrEF aan

te tonen. Onze hypotheses worden verder getoetst en uitgewerkt op basis van de recente literatuur. Speciale aandacht gaat hierbij naar de duale rol van adiponectine, de verstoorde myoblast proliferatie en de skeletspier STAT3/TNFR2 signalering in HFrEF, evenals de onderliggende oorzaak van de adiponectine resistentie. Bovendien wordt de relevantie van fysieke training voor de behandeling van spieratrofie en adiponectine resistentie in HFrEF besproken. Tot slot, worden enkele toekomstperspectieven en fundamentele vragen, die opkwamen tijdens deze thesis, geformuleerd. Toekomstige onderzoeksprojecten bieden hopelijk een antwoord op deze vragen.



LIST OF ABBREVIATIONS

A

AAD	Aminoactinomycin
ACADM	Acyl-Coenzyme A dehydrogenase, C-14 to C-12 straight chain
ACE	Angiotensin-converting enzyme
ADHF	Acute decompensated heart failure
ADN	Adiponectin
AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
AF	Atrial fibrillation
AMPK	Adenosine monophosphate activated protein kinase
ANP	Atrial natriuretic peptide
ANOVA	Analysis of variance
APPL1	Adaptor protein containing a pleckstrin homology domain, a phosphotyrosine binding domain and a leucine zipper motif 1
ATP	Adenosine-5'-triphosphate

B

B2M	Beta-2-microglobulin
BMI	Body mass index
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin

C

C1q	Complement 1q
CAD	Coronary artery disease
CaMK	Calmodulin-dependent protein kinase
CCRG	Centre for Cellular and Regenerative Therapy
CHD	Coronary heart disease
CHF	Chronic heart failure
CI	Cell index
CKD	Chronic kidney disease
CKD-EPI	Chronic kidney disease epidemiology collaboration

CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CORE	Centre for Oncological Research
COX	Cyclooxigenase
CPET	Cardiopulmonary exercise test
CRP	C-reactive protein
CSA	Cross-sectional area
Ct	Cycle threshold
CT	Computerized tomography
CV	Cardiovascular
CV	Coefficient of variance
CVD	Cardiovascular disease

D

DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DCM	Dilated cardiomyopathy
DEXA	Dual-energy X-ray absorptiometry
DI	Differentiation index
DMEM	Dulbecco's Modified Eagle Medium
DMI	Deformed myotubes index
DNA	Deoxyribonucleic acid
DP	Dual plate

E

ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
EER	Estrogen related receptors

F

FAO	Fatty acid β-oxidation
FCS	Fetal calf serum
FFA	Free fatty acid
FGF	Fibroblast growth factor

FITC	Fluorescein isothiocyanate
FOXO	Forkhead box protein O

G

gAd	Globular adiponectin
GFR	Glomerular filtration rate
GPCR	G-protein-coupled cell surface receptor
GRK	G-protein–coupled receptor kinase

H

HCM	Hypertrophic cardiomyopathy
HDL	High-density lipoprotein
HF	Heart failure
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
HK2	Hexokinase 2
HMW	High molecular weight
HMWR	HMW ratio
hsCRP	High sensitivity C-reactive protein
HOMA-IR	Homeostasis model assessment of insulin resistance
HRP	Horse radish peroxidase
HUVEC	Human umbilical vein endothelial cells

I

ICAM	Intracellular adhesion molecule
IFN-γ	Interferon-gamma
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IHD	Ischemic heart disease
IL	Interleukin
IMLC	Intramyocellular lipid content
iNOS	Inducible nitric oxide synthase
IQR	Interquartile range
IR	Insulin resistance

J

JAKs	Janus protein tyrosine kinases

K

KO	Knock-out

L

LDL	Low-density lipoprotein
LMW	Low molecular weight
LPL	Lipoprotein lipase
LV	Left ventricle / ventricular
LVEF	Left ventricular ejection fraction

M

MAFbx	Muscle atrophy F-box
MAPK	Mitogen-activated protein kinase
MetS	Metabolic syndrome
MFI	Myogenic fusion index
Min	Minutes
MMD	Medium molecular weight
MRF	Myogenic regulatory factor
MSD	Meso Scale Discovery
6MWT	6 Minute walk test
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide
MuRF	Muscle ring finger
MyoD	Myogenic differentiation

N

N	Newton
NADPH	Nicotinamide adenine dinucleotide phosphate
ND	Nanodrop
NF-κB	Nuclear factor kappa B
NO	Nitric oxide

NT-proBNP	N-terminal pro-brain natriuretic peptide
NYHA	New York Heart Association

O

oxLDL	Oxidized low-density lipoprotein
OP	Optical density

P

pAMPK	Phosphorylated AMPK
PAX	Paired box protein
PBS-D	Phosphate buffered saline
PBS-D	Dulbecco's phosphate buffered saline
PCR	Polymerase chain reaction
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator 1α
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PPARα	Peroxisome proliferator-activated receptor α

R

RAAS	Renine-angiotensin-aldosterone system
rhEGF	Recombinant human epidermal growth factor
RIN	RNA integrity number
1-RM	1-Repeated maximum
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RTCA	Real-Time Cellular Analysis
RT-PCR	Reverse transcriptase polymerase chain reaction
RV	Right ventricle

S

SA-β-gal	Senescence-associated beta-galactosidase
SD	Standard deviation
SEM	Standard error of the mean

SGBS	Simpson-Golabi-Behmel syndrome
SIRT1	Sirtuin 1
SKGM	Skeletal muscle growth medium
SOCS	Suppressors of cytokine signaling
SPSS	Statistical package for the social sciences
STAT3	Signal transducer and activator of transcription 3

T

TAC	Transverse aortic constriction
TBP	TATA box binding protein
TNF-α	Tumor necrosis factor-α
TNFR1	Tumor necrosis factor receptor receptor 1
TNFR2	Tumor necrosis factor receptor receptor 2
TZD	Thiazolidinediones

U

UA	University of Antwerp
UPS	Ubiquitin-proteasome system
UZA	Antwerp University Hospital

V

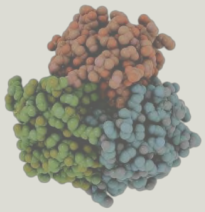
VAD	Ventricular assist device
VCAM	Vascular cell adhesion molecule
VE	Ventilation
VITO	Flemish Institute for Technological Research
VO_2	Oxygen consumption/uptake
VCO_2	Carbon dioxide production

W

WHR	Waist-to-hip ratio
WB	Western Blot
WT	Wild type

X

X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside



CURRICULUM VITAE

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Additional educational courses

Certificate of Laboratory Animal Sciences, FELASA Category C

Academic Teacher Training, Antwerp School of Education, Antwerp University

Clinical Trials Course, ESC, Brussels, Belgium

Certificate of Good Clinical Practice for the busy Investigator

English Course (Level 4-6), Institute for Language and Communication, Linguapolis

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Position

PhD Fellow, Dehousse Fellowship, University of Antwerp

Theses

PhD Thesis	"Adiponectin: Potential therapeutic target for the treatment of muscle wasting in chronic heart failure. *In vitro exploration*" Promotors: Prof. Dr. CJ. Vrints, Prof. Dr. VY. Hoymans
Master Thesis	"The role of adiponectin in patients with chronic heart failure: *in vitro* exploration" Promotor: Prof. Dr. V. Conraads (†)
Bachelor Thesis	"Spinal microglia expression in the dorsal horn after transplantation therapy using collagen-based biomatrix with olfactory ensheathing cells" Promotor: Prof. Dr. R. Deumens

Scientific activities

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Scientific Grants and Awards

ESC Travel Grant, ESC Congress 2014, Barcelona, Spain

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ESC Travel Grant, ESC Congress 2016, Rome, Italy

Grant from the Fund for Scientific Research (FWO) for participation at an international congress abroad, SHVM Congress 2015, Tarrytown, New York

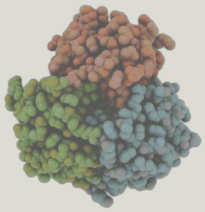
Selected for Young Investigator's Awards Poster prizes during the Heart Failure Winter Research Meeting, Les Diablerets, Switzerland, 2016

Grant of the Belgian public utility foundation VOCATIO (Laureate 2016)

International research visits

Research stay at the Pain Management and Research Center, University Medical Center Maastricht, the Netherlands
03/2009 – 06/2009

ESC Basic Science Summer School, European Heart House, Sophia Antipolis, France
European Society of Cardiology
14 - 18/06/2015



Dankwoord

Dankwoord

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Hemelse Lijdensweg…

Een doctoraat, het resultaat van een bewogen weg vol wetenschappelijke en persoonlijke avonturen. Een weg van vallen en opstaan, van verdriet en vreugde, van mislukkingen en successen. Een kronkelende weg die je links, rechts en soms achterwaarts stuurt. Een hemelse lijdensweg. Maar vooral een weg die je niet alleen bewandeld. Waar je in de scherpste bochten en gevaarlijkste kruispunten mensen tegenkomt die je de juiste richting wijzen, je moed inspreken, je hulp aanbieden. Hier past een welgemeend woord van dank.

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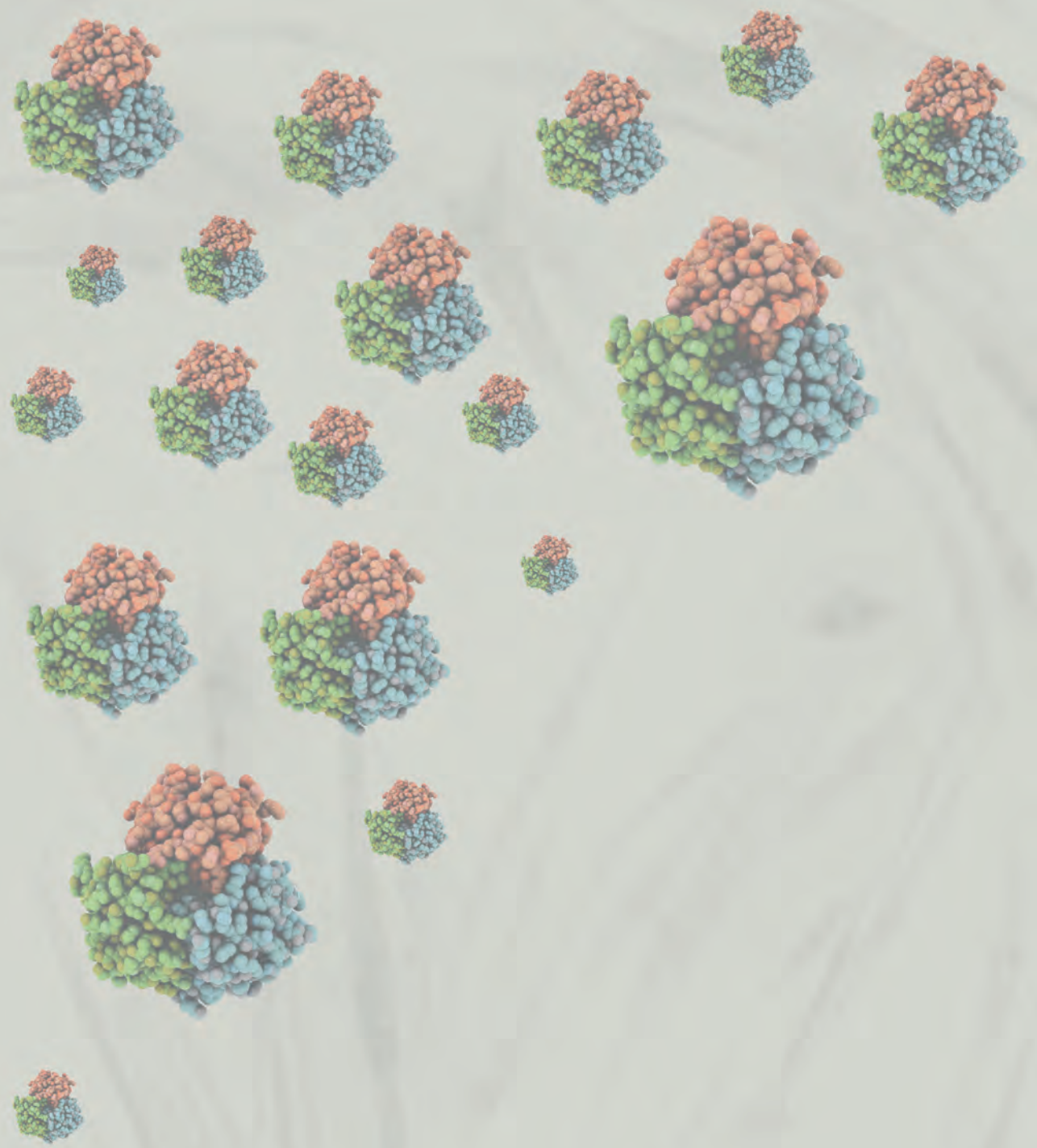
Tahnee
September 2016

Every story has an ending
Every ending is a new beginning

Chapter One…



Faculteit Geneeskunde en Gezondheidswetenschappen

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