Metabolic phenotyping of the pressure-overloaded heart

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Focus on model development

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Contents

Chapter **1**

General Introduction

Pressure overload-induced heart failure

Currently, heart failure is one of the leading causes of death worldwide, affecting around 26 million people[1]. Aortic stenosis and systemic arterial hypertension, each resulting in cardiac pressure overload, belong to the most-frequent cardiovascular diseases and major risk factors of heart failure[2]. There is still no effective therapy to cure the pressure-overloaded heart and in contrast to other cardiovascular diseases, its incidence, prevalence, and economic costs are steadily increasing. Currently, over 1.13 billion people suffer from hypertension worldwide, a number that has nearly doubled since 1975 and is still on the rise[3]. Furthermore, hypertension is the underlying cause of 45% of all deaths caused by cardiac problems[4]. Also the incidence of aortic stenosis, the most serious global valve disease problem, reaches epidemic proportions. With demographic aging of the global population the incidence of pressure overload-induced heart failure is expected to increase even further, thereby stressing the urgent need for effective medical therapy.

Pressure overload is a condition in which the cardiac muscle has to contract while experiencing an excessive afterload. In response to this increased afterload, the heart will increase its muscle mass, leading to the development of cardiac hypertrophy. It has initially been thought that this hypertrophic response functioned as a compensatory mechanism to help maintain cardiac output, but current literature challenges this dogma by suggesting that hypertrophy itself is a pathological (non-compensatory) response[5]. Either way, this cardiac hypertrophy will ultimately become maladaptive and the heart will start to fail[6, 7]. It has been proposed that altered cardiac energy metabolism contributes to the development of cardiac hypertrophy and finally also to that of heart failure[8, 9].

Cardiac metabolism in the healthy heart

The heart is an organ that pumps blood throughout the entire body via the vascular system, providing oxygen and nutrients to the peripheral tissues and thereby warranting proper function of these tissues. Optimal cardiac pump function is required to maintain this

sufficient supply of oxygen and nutrients to the periphery[10, 11]. Maintenance of pump function is inevitably linked to the availability of energy in the form of ATP. This ATP can be derived from a high-energy-phosphate pool (phosphocreatine; PCr), but in the heart this pool is relatively small and can be exhausted within a few seconds[12, 13]. Therefore, the majority of ATP is generated continuously in the mitochondria via the utilization of several classes of cardiac substrates, including long chain fatty acids, glucose, amino acids, ketone bodies and lactate.

In the healthy heart, fatty acids are the predominant substrate and account for approximately 60–70% of energy provision, whereas 10–40% of the energy is generated by the utilization of glucose. Ketone body, amino acid and lactate oxidation cover the remaining contribution[12, 14]. Under a variety of physiological conditions the healthy heart is metabolically flexible, which confers the advantage of adequately supplying ATP for continual cardiac contraction. However, when exposed to pathophysiological stressors, the heart loses its ability to switch between substrates and this will eventually lead to contractile failure[15]. An example of such pathophysiological stressor is “pressure overload”.

Cardiac metabolism in the pressure-overloaded heart

At an early stage, chronic pressure overload-induced heart failure is characterized by deranged myocardial fuel metabolism, shifting from predominant mitochondrial oxidation of fatty acids towards almost complete reliance on glucose uptake and glycolysis **(Figure 1)[16, 17]**. This shift is accompanied by the re-activation of the “fetal gene program”, which is characterized by an increase in the expression of fetal genes such as α-myosin heavy chain (α-MHC), β-myosin heavy chain (β-MHC), sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and glucose transporter 1 (GLUT1), and the development of cardiac hypertrophy[18, 19]. It has been suggested that these changes in gene expression are an adaptive cardioprotective process, possibly triggered by glucose-derived metabolic signals[18]. Interestingly, the significant increase in glucose uptake in pressure overload is not concomitant with a parallel increase

in glucose oxidation, leading to uncoupling of glycolysis from glucose oxidation[20-22]. At this stage, anaplerosis is upregulated to replenish the tricarboxcylic acid cycle (TCA) flux[23], but is rather inefficient in terms of glucose metabolism. Matching the rate of glycolysis and glucose oxidation could, therefore, be key to improve contractile function. Regarding fatty acid metabolism, fatty acid oxidation was clearly diminished in animals subjected to pressure overload[24, 25], but there is no clear consensus with respect to alterations in the rate of fatty acid uptake in the pressure-overloaded heart[16, 26]. More research into this particular area is therefore required.

While these early changes induced by pressure overload generally are regarded as cardioprotective, and result in maintenance of contractile function, at a later stage during persistent pressure overload the energy supply often can no longer match energy demand, eventually leading to severe left ventricular dysfunction, and consequently heart failure. When the heart progresses towards this severe-stage or end-stage of heart failure it is characterized by (*i*) decreased fatty acid uptake and utilization, (*ii*) insulin resistance, and (*iii*) impaired myocardial glucose uptake and oxidation[24, 27-30]. This overall decrease in energy metabolism leads to an energy depleted state of the heart, accompanied with ventricular dilatation, in which the heart is no longer able to contract accordingly **(Figure 1)**.

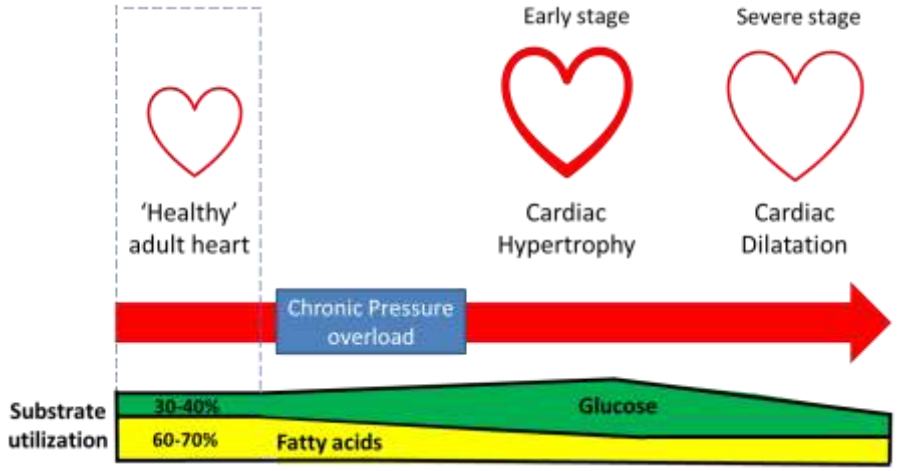

Figure 1. *Schematic overview of metabolic alterations during the development of pressure overload-induced heart failure.*

Protein turnover in the pressure-overloaded heart

Protein turnover represents the balance between protein synthesis and breakdown. In the normal heart, protein turnover helps maintain the protein pool in homeostasis through continual synthesis and degradation[31]. When the heart encounters an environment of pressure overload, this protein turnover becomes disturbed, leading to the development of cardiac hypertrophy at an early stage of heart failure. Cardiac hypertrophy is characterized by a net increase in protein synthesis[32]. It has been proposed that the activation of a key regulatory protein in protein synthesis, the protein kinase mTOR (mammalian or mechanistic Target of Rapamycin), by glycolytic intermediates, plays an important role in the development of cardiac hypertrophy[9]. Additionally, activation of the canonical protein kinase D1 (PKD1)-pathway has also been implicated in the structural remodeling of the heart. One of the key downstream substrates of PKD1 is histone deacetylase-5 (HDAC5), a member of the HDAC family that negatively regulates the acetylation status of nucleosomal histones. HDAC5 is able to bind to the transcription factor myocyte enhancer factor-2 (MEF2), thereby repressing the transcription of a number of fetal genes[33, 34]. Phosphorylation of HDAC5 by PKD1 activation results in disassociation from MEF2, and might therefore be an important contributor to the development of cardiac hypertrophy.

During the progression of heart failure towards a severe-stage, a net increase in protein synthesis exceeds the folding capacity of the endoplasmic reticulum (ER), thereby inducing ER-stress and the accumulation of damaged and misfolded proteins[35, 36]. To enhance the clearance of those large amounts of misfolded proteins, the autophagic flux is upregulated[37]. Moreover, prolonged ER-stress also triggers the activation of pro-apoptotic signaling cascades that even further contribute to cardiomyocyte dysfunction[38] and finally results in severe ventricular dilatation and cell death.

Potency for therapeutic approaches that focus on restoring the metabolic balance

Recently, several studies have shown that the metabolic changes in glucose uptake and the mitochondrial energy deficits during the development of pressure overload-induced heart failure *precede* structural and functional changes in the heart[9, 24, 39-41]. This suggests that metabolic targeting aimed at restoring mitochondrial energy provision could prevent profound structural and functional abnormalities in pressure overload-induced heart failure, which would make metabolic interventions a potential therapeutic approach. Further support that targeting metabolism (so as to obtain the metabolic profile as observed in the healthy heart) is an promising strategy, is provided by an *in vivo* study with protein kinase D1 (PKD1)-overexpressed mice[42]. On a normal chow diet, these mice showed a metabolic shift towards predominant cardiac glucose utilization, while developing cardiac hypertrophy and ventricular dilation. However, when these mice were subjected to a high fat-diet (forcing them towards increased fatty acid utilization), these abnormal metabolic, structural and functional parameters were no longer apparent. A similar protective effect on cardiac function was observed in mice with a cardiac-specific deletion of acetyl-CoA via acetyl-CoA carboxylase (ACC2). These mice showed increased fatty acid oxidation, and the deletion of ACC2 prevented metabolic reprogramming and sustained myocardial energetics (PCr/ATP ratios) and function in a model of pressure overload[43].

Outline of this thesis

Given the emerging crucial role of metabolic changes during the development of pressure overload-induced heart failure and the promising outlook of metabolic interventions as effective strategy to prevent or perhaps rescue the failing heart, in this thesis we aimed at developing experimental models for pressure overload-induced heart failure to enable the further study of the underlying molecular mechanisms as well as the study of various metabolic interventions.

To study these aims, in this thesis emphasis was put on mapping metabolic changes during the development of pressure overload-induced heart failure in both *in vitro* and *in vivo* models in an attempt to unravel the mechanism behind this specific type of heart failure. In **chapter 2**, the current state of knowledge regarding substrate metabolism during the development of pressure overload-induced heart failure has been summarized. Because substrate transporters form a main rate governing step in metabolic substrate utilization, they may offer good targets for therapeutic treatment of pressure overload-induced heart failure. Specifically, in this chapter we focused on expression levels and subcellular distribution of the substrate transporters (including those of both predominant and alternative substrates). **Chapter 3** is devoted to the generation of an *in vitro* model to mimic pressure overload-induced heart failure. This model allowed us to obtain a better insight into the molecular mechanisms behind pressure overload-induced heart failure and to search for new targets to prevent pressure overload-induced heart failure. In **chapter 4,** we additionally aimed to set-up an *in vivo* model of slowly progressing pressure overload to study metabolic alterations in relation to changes in protein synthesis during the development of pressure overload-induced heart failure. For this model rats were subjected to transverse aortic constriction (TAC) surgery.

To test whether stem cell-derived cardiomyocytes could function as a relevant human tool to study pressure overload-induced heart failure, we examined the metabolic properties of human stem cells and their derived cardiomyocytes. Because of the amply available expertise with respect to *in vitro* models of insulin resistance in our research group, for practical reasons we first tested these human-derived cardiomyocytes in a model of insulin resistance. Results of this study are presented in **chapter 5**. Based on these first data, future research may focus on the implementation of stem cell-derived cardiomyocytes in a model of pressure overload-induced heart failure, allowing us to make the translation of the findings presented in chapters 3 and 4 towards a human setting. Finally, in **chapter 6** a summary of the main results presented in this thesis is given, and based on these results new insights are discussed in a broader perspective.

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Chapter **2**

Pivotal role for membrane substrate transporters on the metabolic alterations in the pressure-overloaded heart.

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Abstract

Cardiac pressure overload (PO), such as caused by aortic stenosis and systemic hypertension, commonly results in cardiac hypertrophy and may lead to the development of heart failure. PO-induced heart failure is among the leading causes of death worldwide, but its pathological origin remains poorly understood. Metabolic alterations are proposed to be an important contributor to PO-induced cardiac hypertrophy and failure. While the healthy adult heart mainly uses long-chain fatty acids (FAs) and glucose as substrates for energy metabolism and to a lesser extent alternative substrates, i.e. lactate, ketone bodies, and amino acids (AAs), the pressure-overloaded heart is characterized by a shift in energy metabolism towards a greater reliance on glycolysis and alternative substrates. A key-governing kinetic step of both FA and glucose fluxes is at the level of their substrate-specific membrane transporters. The relative presence of these transporters in the sarcolemma determines the cardiac substrate preference. Whether the cardiac utilization of alternative substrates is also governed by membrane transporters is not yet known. In this review, we discuss current insight into the role of membrane substrate transporters in the metabolic alterations occurring in the pressure-overloaded heart. Given the increasing evidence of a role for alternative substrates in these metabolic alterations, there is an urgent need to disclose the key-governing kinetic steps in their utilization as well. Taken together, membrane substrate transporters emerge as novel targets for metabolic interventions to prevent or treat PO-induced heart failure.

1. Introduction

Heart failure remains the leading cause of death worldwide.[1] It is defined by clinical symptoms such as dyspnoea, fatigue, and ankle swelling.[2,3] Before these symptoms become apparent, the patient's heart often undergoes asymptomatic structural or functional abnormalities (left ventricular dysfunction).[2] Recognition of these changes is important as they are related to poor outcomes. There are many underlying pathologies that can lead to heart failure. Aortic stenosis and systemic arterial hypertension, each resulting in cardiac pressure overload (PO), belong to the most-frequent cardiovascular diseases and major risk factors of heart failure.[4] Upon aortic stenosis, the blood flow from the left ventricle to the aorta is restricted leading to an increased resistance to systolic ejection. During hypertension, the increased systemic blood pressure imposes an elevated afterload to the left ventricle. In response to the increased afterload and increased left ventricular wall stress in aortic stenosis or hypertension, the heart increases its muscle mass (hypertrophy). Where this hypertrophic response has initially been thought to compensate and help maintain an adequate cardiac output, current literature challenges this dogma by suggesting that the hypertrophy itself is a pathological (non-compensatory) response.[5] Either way, in the long term this concentric hypertrophy may become maladaptive and will lead to diastolic dysfunction.[6] Left ventricular hypertrophy occurs in about 10–15% of the general population,[7] in 85% of aortic stenosis patients[8] and in 40% of hypertensive patients.[9] Although several antihypertensive drugs exist, not all are equally effective. The underlying mechanism of this transition of PO towards cardiac hypertrophy and failure (from now on referred to as the pressure-overloaded heart) remains unknown. It has been proposed that in the pressure-overloaded heart altered cardiac energy metabolism—in particular substrate preference—is an important (early) contributor to the development of cardiac hypertrophy and subsequent failure and, therefore, might function as a suitable treatment target.[10]

The human adult heart is able to utilize a wide variety of substrates for energy provision, i.e. fatty acids (FAs), carbohydrates, and to lesser extent lactate, ketone bodies, and amino

acids (AAs). Under physiological conditions, the heart mainly oxidizes FAs and glucose and is metabolically flexible, i.e. is able to rapidly shift between these substrates upon changing conditions, such as availability of substrates, workload, and hormonal influences.[11] However, when chronically exposed to pathophysiological stressors, the heart loses its ability to switch between substrates and this may eventually lead to contractile failure. For instance, the pathophysiological condition of chronic PO is characterized by a shift away from the predominant utilization of FAs towards increased glycolysis.[12,13] It is suggested that this initially adaptive shift eventually becomes maladaptive and then contributes to the development of heart failure.

Substrate utilization can be determined at the level of substrate availability, cellular substrate uptake, and trapping, conversion into metabolites that can be taken up by mitochondria, and subsequent mitochondrial oxidation. Because of rapid conversion of substrates upon cellular entry (at least for the main substrates glucose and FAs) the transsarcolemmal uptake process is unidirectional, thereby presents itself as a key regulatory step. Key regulators of myocardial FA and glucose uptake are their substrate-specific transporters expressed on the cellular surface of cardiomyocytes, which therefore, present the rate-controlling site for cardiac FA and glucose fluxes.[14] FA and glucose transporters (GLUTs) have been studied comprehensively, indicating an important role for the FA transporter, CD36 (officially known as SR-B2), and the GLUTs, GLUT1 and GLUT4, in facilitating FA and glucose uptake into the heart, respectively.[15,16]

Recent data also implies an important role for the alternative substrates in the pressure-overloaded heart. Up-regulated key enzymes in the ketone oxidation pathways, and catabolism of branched chain amino acids (BCAAs) have been associated with cardiac hypertrophy and failure.[17,18] Interestingly, the transport of these alternative substrates across the sarcolemma of cardiomyocytes may also be a key factor in regulating their utilization.[17,19] The role of lactate, ketone bodies, and AAs and their specific transporters remains, however, underexplored.

This review will summarize the current state of knowledge regarding the various cardiac transmembrane substrate transporters of both the main and the alternative substrates, and their specific functioning in the healthy heart and in the pressure-overloaded heart during the development of heart failure. To sum up, substrate-specific transporters at the cardiomyocyte sarcolemma are a promising target for metabolic intervention strategies to prevent or regress the development of PO-induced heart failure or to rescue the pressure-overloaded heart.

2. Substrate transporters in the healthy heart

2.1 *Glucose transporters*

The hydrophilic properties of the glucose molecule limit its diffusion through the lipid bilayer of the sarcolemma. Therefore, glucose transport across the sarcolemma is facilitated by glucose-specific transporters. In the heart glucose functions as a substrate for energy provision, but also plays an important role in cell signalling and biomolecule synthesis.[20,21]

The most abundant glucose transporters (GLUTs) expressed in the heart are the GLUT family, particularly the Class I isoforms GLUT1 and GLUT4 (*Table 1*). These passive uniporters facilitate glucose transport via diffusion based on the glucose concentration gradient. In the adult heart, GLUT4 is expressed to a subsequently higher extent than GLUT1.[22] Other members of the GLUT family (GLUT3, GLUT8, GLUT10, GLUT11, and GLUT12) are also present in the heart, but to a lesser extent (*Table 1*). While these additional GLUTs are involved in cardiac foetal development and the transport of other hexoses, their involvement in glucose uptake in the adult heart remains unknown.[16,20,21,23] Next to the GLUTs, six members of the family of sodium-glucose linked transporters (SGLT) are present in the heart, of which SGLT1 and SGLT2 are the most abundant (*Table 1*).[21] This family, consisting of 12 isoforms, uses the sodium gradient to actively transport glucose across the plasma membrane into the cell. Studies with SGLT1$^{-/-}$ and SGLT2$^{-/-}$ mice do not show alterations in cardiac glucose uptake.[24] These data seem at odds with the well-known

beneficial effects of SGLT inhibitors on cardiac function in human patients with various cardiac metabolic diseases.[25] However, it has also been proposed that the effects of SGLT inhibitors on the heart are indirect, i.e. via haemodynamic improvements.[25] Hence, it is concluded that SGLTs are of minor importance in regulation of cardiac glucose uptake.

The relative contribution of GLUT1 and GLUT4 transporters to the regulation of glucose uptake is not only dependent on differences in their expression but also determined by differences in subcellular localization. GLUT1 is constitutively present at the cellular membrane to mediate basal (non-stimulated) glucose uptake. GLUT4 on the other hand is retained in endosomal compartments and is able to quickly translocate towards the sarcolemma upon various physiological and pharmacological stimuli.[16] Short-term regulation involves vesicular GLUT4 translocation towards the cell membrane mediated by the phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K)–protein kinase B (PKB/Akt) insulin signalling pathway, or by the AMP-activated protein kinase (AMPK) contraction-induced signalling pathway (*Table 1*). Key points of convergence of these signalling pathways are the Rab GTPase activating proteins AS160 (also known as TBC1D4) (*Figure 1*) and its homologue TBC1D1. Phosphorylation of both Rab GTPase activating proteins releases the break on the activity of a set of Rab GTPases (Rab8a, Rab10, Rab11, and Rab14). Activation of these Rabs drives the unidirectionality and specificity of trafficking, which is additionally supported by a variety of Rab effector proteins.[26] Like any other subcellular vesicular trafficking process, GLUT4 translocation obeys to the principles of the SNARE theory in which pairs of cognate v-SNAREs at the donor compartment and t-SNAREs at the acceptor compartment confer the specificity of vesicular trafficking. VAMP2 is the v-SNARE for insulin-stimulated GLUT4 translocation in the heart, and VAMP3 mediates contraction-stimulated GLUT4 translocation.[27] Long-term regulation of glucose transport involves alterations in activity of transcription factors and is associated with alterations in gene expression, such as the hypertrophic transcription factor myocyte enhancer factor-2 (MEF2) which is a central regulator of GLUT4 expression.[28,29] GLUT1 expression in the heart can be regulated by the transcription factors activator protein 1 (AP1) and the hypoxia-induced

factor-1α (HIF-1α).[29–31] The subcellular distribution of additional GLUTs in the heart and the mechanisms involved in short-/long-term regulation of their expression levels remain to be elucidated. Additionally, there are some indications that GLUT transporters are able to enhance substrate uptake via augmented intrinsic activity of GLUT transporters independent of transporter recruitment towards the sarcolemma.[32]

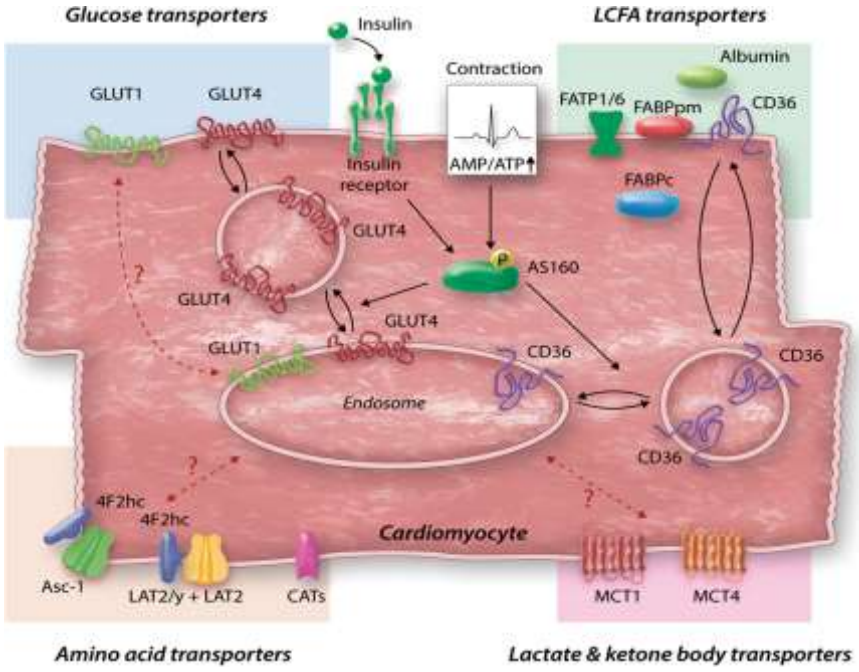

Figure 1. *Schematic presentation of cardiac specific membrane substrate transporters and their distribution (endosomes vs. sarcolemma) within cardiomyocytes. GLUT 1 and GLUT4 are the predominant glucose transporters in the heart. Under basal conditions GLUT1 is constitutively present at the sarcolemma, whereas GLUT4 is mainly located in endosomal compartments where it is able to rapidly translocate towards the sarcolemma upon hormonal (insulin) or contractile (AMP/ATP ratio) stimuli, both involving phosphorylation of AS160 and/or TBC1D1 (not indicated; both Rab GTPase activating proteins). Whether GLUT1 also has translocation ability under specific stimulation conditions remains to be elucidated. The predominant LCFA transporter in the heart is CD36. Similar to GLUT4, CD36 is situated in endosomal compartments and is able to translocate towards the sarcolemma upon hormonal or contractile stimuli. For lactate and ketone body transport MCT1 and MCT4 transporters appear most abundant in the heart. MCT1 is primarily localized on the sarcolemma, while the exact localization of MCT4 and the translocation ability of both transporters are unknown. For amino acid transport, three types of bidirectional L-type transporter systems (Asc-1, LAT2, and y⁺LAT2) and cationic amino acid transporters (CATs) have been identified in the heart, but their subcellular distribution and their translocation ability remain to be investigated. The question mark (?) indicates that it is unknown if these transporters translocate between endosomes and sarcolemma.*

2.2 *FA transporters*

Short-chain FAs and medium-chain FAs both combine favourable hydrophilic properties with relatively good lipophilic properties so that these substrates do not need any carrier or transporter to be taken up into cells. For comparison, long-chain FAs (from now on referred to as FAs) have increased lipophilic properties and much lower aqueous solubility. To increase their aqueous solubility, FAs require binding-proteins, such as extracellularly available albumin and intracellular cytoplasmic fatty acid binding protein ($FABP_c$).[33] Related to their lipophilic properties, FAs readily partition into biological membranes, but their desorption from the lipid bilayer into the cytoplasm is limited. To overcome this barrier, a protein-mediated mechanism is required to direct the FAs into metabolic pathways. In this respect, several membrane-associated FA transporters, including plasma membrane fatty acid-binding protein ($FABP_{pm}$), integral fatty acid transporter proteins 1–6 (FATP 1–6), and fatty acid translocase (FAT/CD36) have been identified in the heart (*Table 1*).[15,34] FA uptake by these transporters is driven by a steep FA concentration gradient across the sarcolemma (high extracellular; low intracellular). In the heart FAs play a major metabolic role as energy source but also function as cellular signalling molecules and as incorporated components of the phospholipid bilayer in cellular membranes.[33]

CD36 is currently known as the predominant membrane protein facilitating cardiac FA transport.[15] Studies with CD36-specific inhibitors as well as in CD36 knockout mice have convincingly shown that CD36 quantitatively contributes to >70% of cardiac FA uptake (depending on the metabolic status).[35,36] Within the FATP family, FATP1 and FATP6 are the main expressed isoforms in cardiomyocytes, but their role might be limited to uptake of very-long-chain FAs. In contrast to CD36, these cardiac FATPs are exclusively targeted to specific areas of the sarcolemma.[37] Moreover, CD36 seems to act synergistically with $FABP_{pm}$, as FA uptake is inhibited in a non-additive manner by CD36-specific inhibitors and by anti-$FABP_{pm}$ antibodies (*Table 1*, *Figure 1*).[37,38] Short-term regulation of FA uptake is mediated by hormonal (insulin) and mechanical stimuli (increased AMPK-induced contraction signalling), which trigger translocation of CD36 from intracellular storage

compartments (endosomes) to the sarcolemma via membrane vesicles within minutes in a manner analogous to short-term regulation of GLUT4 translocation (*Table 1*, *Figure 1*). This latter analogy extends to the v-SNAREs involved with VAMP2 and VAMP3 mediating insulin and contraction-induced CD36 translocation, respectively. In contrast, VAMP4 is specifically involved in CD36 translocation, and not in that of GLUT4.[27] Furthermore, all Rab members mediating GLUT4 translocation are also involved in CD36 translocation,[39] but at least one of the Rab effector proteins, i.e. Rab 11 effector Rip11, appears specific for the CD36 trafficking machinery. Long-term regulation of cardiac FA uptake involves alterations in gene expression, such as oestrogen-related receptor α,[40] key peroxisome proliferator-activated receptor-α (PPARα), and its co-activator PPAR-gamma co-activator-1α.[15]

2.3 Lactate and ketone body transporters

The role of lactate and ketone bodies in metabolically active organs has been understudied. For a long time, lactate was considered a metabolic waste product, derived from glycolysis. However, in recent decades it became clear that lactate also plays a small but marked role as metabolite when energy demands are high.[41,42] Ketone bodies are synthesized in the liver from where they are transported towards extra-hepatic tissues for oxidation (acetoacetate, AcAc; β-hydroxybutyrate, β-OHB) or are excreted by exhalation (acetone). They can serve as circulating energy source for tissues, like brain, heart, and skeletal muscle in times of fasting and prolonged exercise.[43,44]

In the human body, lactate transport across the sarcolemma is facilitated by a group of H^+-coupled or Na^+-coupled monocarboxylate transporters (MCTs). Transmembrane transport of ketone bodies is facilitated by this same group of transporters.[45,46] Currently, there are four well-known H^+-coupled MCT (MCT1, MCT2, MCT3, and MCT4) with distinct affinities for specific substrates and different expression levels between tissues.[42,47] While the above-mentioned transporters are all expressed in the heart, the MCT1 and MCT4 proteins appear most abundant, although there are species differences (*Table 1*, *Figure 1*). This expression pattern indicates a potentially bigger role for MCT1 and MCT4 in cardiac lactate and ketone

body uptake and/or efflux compared to other transporters.[48,49] Sarcolemmal expression, transporter activity, delivery of transporters from the endoplasmic reticulum to the cellular membrane, and stability of these transporters are regulated by basigin (CD147) and embigin (gp70) which function as glycoproteins, anchoring MCTs (particularly MCT1 and MCT4) to the sarcolemma.[50–52] Myocardial MCT1 is primarily localized on the sarcolemma and rarely associated with internal membrane compartments of cardiomyocytes, implying that there is a constitutive functional expression of this transporter.[53] This also would suggest that enhanced lactate or ketone body fluxes under physiological or pathological conditions are mainly due to altered substrate gradients rather than changes in MCT translocation to the sarcolemma. At long term, MCT expression can be regulated by a variety of stimuli. Under hypoxic conditions, MCT4 expression can be up-regulated through a HIF-1α-dependent mechanism. HIF-1α is able to bind to two hypoxia response elements which are present in the MCT4 promotor, but absent in MCT1 and MCT2 promotors.[49,54] Moreover, exercise enhances MCT1 expression in the heart and skeletal muscle.[47,48] Although the exact mechanism still remains to be elucidated, there might be a role for AMPK, which stimulates the MCT1 promotor upon activation. An additional mechanism is elevated Ca^{2+} levels that are able to activate the MCT1 promotor via the transcriptional factor nuclear factor of activated T-cells.

2.4 Amino acid transporters

AAs are essential building blocks for proteins, but they also play an important role in cell signalling and as metabolite precursors for a wide variety of cells. In mammalian cells, a broad range of AA transporter systems (System A, ASC, and L) is present to facilitate the movement across the cellular plasma membrane. System A and system ASC transporters mainly facilitate active influx of short-chain AAs across their concentration gradient, whereas the bidirectional L-type transport system utilizes the intracellular AA pool, generated by the A and ASC transporter systems, to actively facilitate the influx of large, neutral, branched-chain, or aromatic AAs.[55,56] These transporter systems can be further classified into distinct categories based on their substrate specificity, transport

mechanisms, and regulatory properties (reviewed by Hyde *et al*.[56]). For the L-type transporter system-specific membrane glycoproteins (rBAT or 4F2hc) are required to form a functional transporter system.[56,57]

Yet, the exact transport mechanism of AAs into the heart remains poorly understood. There are some indications for a pivotal role for the bidirectional L-type transporter systems.[18,58] At least three types of cardiac L-type amino acid transporters are identified (y^+LAT2, LAT2, and Acs-1). Whereas y^+LAT2 requires Na^+, LAT2 facilitates the exchange of AAs in a Na^+-independent manner.[56,57] Na^+-independent transport of glycine, L-alanine, L-serine, L-threonine, L-cysteine, α-aminoisobutyric acid, and β-alanine is mainly mediated by alanine-serine-cysteine transporter 1 (Asc-1),[59] whereas y^+LAT2 preferentially mediates the efflux of L-arginine in exchange of L-glutamine plus Na^+.[60] In addition to the L-type transporter systems, some studies report the presence of the cationic amino acid transporters (CATs) in cardiomyocyteks.[61,62] Interestingly, mRNA expression of CAT1 and CAT2B is enhanced by insulin and cytokines, thereby increasing L-arginine uptake into the heart (*Table 1*, *Figure 1*).[61] The latter indicates that insulin and cytokines have the potential to regulate AA transport. Insulin-induced AA transport has previously been shown in skeletal muscle, whereby the insulin action includes translocation of the System A transporter 2 (SAT2) from an intracellular storage pool towards the sarcolemma.[67] However, whether the cardiac LATs and CATs also can be recruited towards the cellular membrane via a translocation process, and whether this can be regulated by specific hormonal stimulation, remains to be studied.

3. The role of substrate transporters in the pressure-overloaded heart

Chronic PO can result in the development of cardiac hypertrophy and eventually heart failure. The transition from a healthy towards a hypertrophic and finally failing heart is accompanied by alterations in cardiac energy metabolism. Whereas the healthy heart predominantly utilizes FAs (60–90%) and glucose (10–30%) as substrates for oxidative energy provision and to a lesser extent lactate, ketone bodies, and AAs, the pressure-overloaded heart is characterized by a switch from FAs towards glycolysis.[10] This shift

towards glycolytic metabolism is initially thought to be an adaptive response to gain higher oxygen efficiency (more ATP generation per consumed oxygen molecule) (*Figure 2*).[68] There is, however, no conclusive evidence that a shift towards glucose utilization or towards utilization of alternative substrates, such as ketone bodies which are more oxygen-efficient than FAs is beneficial to cardiac function. Studies with epinephrine[69,70] suggest that the advantage of a switch to preferred glucose utilization relates to a higher ATP flux through creatine kinase, rather than an increased ATP content or increased PCr/ATP ratio. Interestingly, a reduction in ATP flux through creatine kinase was seen in patients[71,72] and animal models[73,74] with PO-induced hypertrophy and failure. A causal relationship between altered energy metabolism and contractile dysfunction has however not been proven so far.

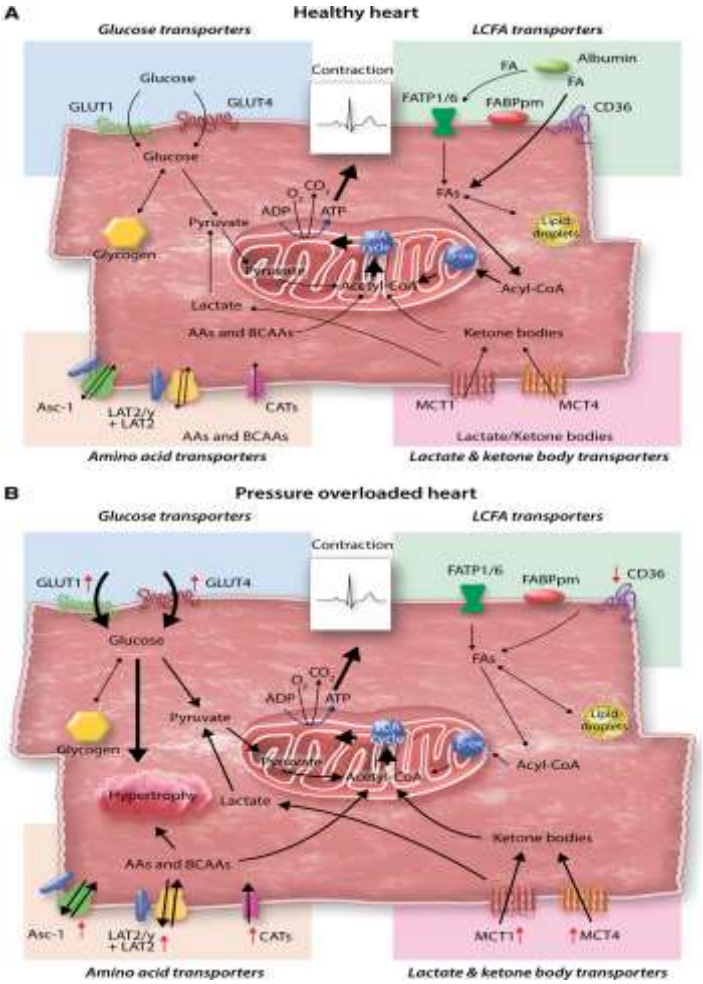

Figure 2. *Proposed mechanism by which alterations in sarcolemmal substrate transporter presence—thereby alterations in substrate uptake—contribute to the development of PO. (A) In the healthy heart, the preference for FA utilization is driven by substrate-specific transporter content at the sarcolemma (a key-governing kinetic step in cellular FA utilization). Transporter-mediated influx of FAs is high, whereas the influx of glucose, lactate, ketone bodies, and amino acids, via their specific transporters, is relatively low. (B) In the pressure-overloaded heart, sarcolemmal presence of GLUT1 and GLUT4 increases, whereas sarcolemmal CD36 presence decreases. This increase in sarcolemmal glucose transporters (thereby enhanced glucose uptake/glycolysis) triggers hypertrophic growth.⁵ Additionally, the heart increases the uptake of lactate, ketone bodies, and amino acids, either by enhancing sarcolemmal presence of their specific transporters or by increased transmembrane gradients, to serve as alternative substrates for mitochondrial energy provision. Thickness of the black arrows indicates the relative rate of substrate influx. Red arrows indicate either an up-regulation (↑) or down-regulation (↓) of sarcolemmal substrate-specific transporters under the condition of PO. AAs, amino acids; ADP, adenosine diphosphate; BCAAs, branched-chain amino acids; BCKAs, branched-chain keto acids; KAs, keto acids; TCA, tricarboxylic acid; β-ox, β-oxidation.*

On the other hand, the increased glycolytic flux in the pressure-overloaded heart does not seem to be matched by a commensurate increase in glucose oxidation, leading to inefficient glucose metabolism.[75–77] The tricarboxylic acid (TCA) cycle was shown to remain normal in the pressure-overloaded heart despite decreased FA oxidation, due to increased anaplerosis of glycolysis-derived pyruvate.[78] Anaplerosis is energetically less efficient than pyruvate oxidation because it bypasses oxidative decarboxylation and other ATP-generating steps of the TCA cycle.[75–77] Matching the rate of glycolysis and glucose oxidation, rather than increasing glucose oxidation *per se* could, therefore, be key in improve contractile function. Such matching could be achieved by regulating glucose uptake via the GLUTs, thereby restoring the maladaptive increased flux towards glycolysis under pathological conditions.

Recently, more studies point towards an important role for lactate, ketone bodies and AAs as alternative energy source in the pressure-overloaded heart (*Figure 2*).[17,18] Below, the changes in utilization that occur in the pressure-overloaded heart for each of the substrates are discussed with emphasis on the role of substrate transporters (summarized in *Table 2*, taking into account the type, stage, and severity of heart failure). Where relevant, other heart diseases related to PO are included. Not for every substrate sufficient information about the respective transporter was present, so that in those instances we focused on changes in substrate concentrations and in substrate metabolism. Of note, the majority of data on substrate transporters in the pressure-overloaded heart has been generated from animal studies, whereas limited research has been conducted in humans. Though, one study in patients with aortic stenosis showed that the development of cardiac hypertrophy is correlated positively with total cardiac GLUT4 protein levels and negatively with total CD36 protein levels.[79]

3.1 Glucose transporters

On the level of GLUTs, an up-regulation of GLUT1 mRNA and protein has been observed in cardiomyocytes *in vitro*-stimulated with hypertrophic agents.[80] Moreover, two studies[81,82] in spontaneous hypertensive rats, a naturally occurring model of PO, provide evidence for

repressed GLUT4 expression, in one case concomitantly with increased GLUT1 expression. These changes can be explained by reactivation of the foetal gene programme during the development of cardiac hypertrophy. It has been reported that hypertrophy is accompanied by reduced PPARα levels.[83] Inactivation of PPARα is associated with increased expression of foetal-like-genes,[83] such as GLUT-1. Additionally, it has been reported that cardiac hypertrophy is accompanied by increased expression of HIF-1α, which is known to be an important transcription factor involved in GLUT1 transcription.[30] These observations are unfortunately only based on total mRNA and protein levels, whereas it is to be expected that intrinsic activity of these transporters as well as their distribution between intracellular storage and cell surface localization is additionally relevant in terms of substrate utilization.[26,32,84] A study in rats that specifically distinguished between total GLUT protein expression and sarcolemmal distribution showed increased sarcolemmal GLUT1 and GLUT4 protein contents after ascending aortic constriction, whereas total GLUT1 protein expression was increased but total GLUT4 protein expression decreased.[85] These findings are in line with *in vitro* studies with isolated cardiomyocytes where increased glucose uptake was accompanied with an increase in GLUT4 translocation to the sarcolemma.[86,87] Increased GLUT4 translocation in the heart exposed to hypertension could be due to activation of protein kinase-D1 (PKD1) via signalling initiated by the hypertensive hormones angiotensin-II and endothelin-I.[88] PKD1 stimulates GLUT4 translocation via a yet unknown pathway involving endosomal lipid kinases.[89] On the other hand, both hormones are reported to inhibit upstream insulin signalling.[90] Hence, increased basal GLUT4 translocation may be accompanied by decreased insulin-induced GLUT4 translocation. Controversial to these findings, reduced GLUT4 expression at the sarcolemma concurrently with increased GLUT4 expression in the cytoplasm has been reported in mice subjected to transverse aortic constriction (TAC).[91] These apparently discrepant findings remain unexplained.

To further investigate the role of specific GLUTs with respect to changed glucose uptake in the pressure-overloaded heart, several studies explored the effect of GLUT overexpression

and of knockout models. Based on the above findings, the possibility exists that the increase in GLUT1 expression observed in PO could be cardioprotective. This theory is supported by studies with cardiac-specific short-term and life-long overexpression of GLUT1, where mitochondrial function is preserved and adverse pathological remodelling is prevented in mice with aortic constriction.[92,93] Interestingly, loss of GLUT1 did not accelerate the transition from compensated hypertrophy to heart failure when subjected to TAC,[94] suggesting that GLUT1 is not the most critical player in cardiac adaptations in the pressure-overloaded heart. On the other hand, mice with cardiac-specific GLUT4 deletion developed significantly greater hypertrophy and more severe contractile dysfunction after TAC compared to their wild-type littermates.[95] This latter study suggests that GLUT4 may be required (and therefore more important than GLUT1) for maintenance of cardiac structure and function in response to PO.

Differences in outcome between studies could be attributed to choice of species, specific models used to study PO, time after initiation of PO, and the specificity of experimental read-out parameters, so caution is probably required while drawing conclusions. Specifically, it has been suggested that changes on the level of transporters may depend on the stage of the disease. For example, in a model of myocardial infarction (MI) the transition from compensated hypertrophy to heart failure was associated with an up-regulation of GLUT1,[96] whereas a down-regulation of GLUT1 and GLUT4 has been observed in end-stage failing human hearts.[97] Discrepancies between *in vitro* and *in vivo* studies could also be attributed to non-cardiomyocyte cell-types contributing to GLUT expression in *in vivo* models, especially when full heart homogenates are studied. Additionally, a study in dogs reported that the regional expression pattern of GLUT4 changes from predominant expression in right atrial appendages in the healthy heart, towards prominent expression in the left ventricle during the development of heart failure,[98] indicating that the regulation of glucose uptake is not only cell, but also regionally dependent. In conclusion, increased GLUT1 expression in combination with increased GLUT4 translocation may be important contributing factors on the road towards cardiac hypertrophy and failure.

3.2 FA transporters

The functioning of FA transporters in the pressure-overloaded heart is still underexplored. Although clear associations have been made among the development of cardiac hypertrophy, increased glucose utilization and suppressed FA oxidation in spontaneous hypertensive rats and in animals undergoing aortic constriction,[85,99–101] less conclusive data are available on FA uptake. *In vivo* 14-[18F]-fluoro-6-thia-heptadecanoate positron emission tomography imaging showed increased FA uptake prior to a reduction in cardiac function in spontaneous hypertensive rat hearts,[102] while *ex vivo* studies in Dahl salt-sensitive rats (a frequently used rodent model of cardiac hypertension) found a contradictory decrease in FA uptake concordantly with the progression of heart failure.[76] After TAC surgery in mice, there is a modest reduction in mRNA expression of CD36 and $FABP_c$, and a markedly decreased protein expression of CD36.[65,66] A similar pattern for alterations in CD36 expression was observed in another study where rats underwent suprarenal abdominal aorta banding, while no effect on FATP was found.[103] Together, these data would indicate that FA uptake is reduced in the pressure-overloaded heart; however, it has to be noted that subcellular distribution of CD36 was not studied.

To explore the role of CD36 in the pressure-overloaded heart more closely, both CD36 overexpression and knockout models have been studied. It has been shown that cardiac-specific overexpression of CD36 ameliorates cardiac dysfunction in mice undergoing TAC surgery.[66] Additionally, a study in spontaneously hypertensive rats observed a reduction in CD36 levels that contributed to the development of cardiac hypertrophy.[104] Moreover, an *in vivo* study on CD36-specific knockdown mice showed decreased fractional shortening and increased left ventricular end-diastolic diameter after TAC.[105] This reduced cardiac function was also confirmed *in vivo*, where cardiac-specific ablation of CD36 accelerated the progression from compensated hypertrophy to severe heart failure in mice subjected to TAC. When this same group of mice was fed a diet containing medium-chain FAs, which can enter into the cardiomyocyte independently from CD36, these mice were protected from TAC-induced heart failure.[106]

Additional studies on cytoplasmic FA transporters, i.e. $FABP_c$'s, showed that mice doubly deficient for FABP4 and FABP5 developed more severe heart failure after TAC compared to their wild-type littermates.[107] However, another study revealed that FABP4 could be a possible positive regulator of cardiac hypertrophy, because mice specifically overexpressing cardiac FABP4 were more sensitive to develop cardiac hypertrophy after TAC when compared to wild-type animals.[108] As a result, the exact role of cytoplasmic FABPs in the pressure-overloaded heart remains to be determined.

3.3 Lactate and ketone body transporters

Whereas FA and glucose transport in the pressure-overloaded heart have been studied in more detail, the relative importance of lactate and/or ketone bodies and their membrane transporters have remained unclear. Aubert *et al*.[17] measured gene expression of MCT1 and of MCT2 in mice with TAC (as a model of compensated hypertrophy) and TAC plus small apical MI (as a model of heart failure) as compared to sham control animals. They found similar transporter expression levels in compensated hypertrophy or heart failure myocardium compared to control after 4-h fasting, but significantly increased MCT2 mRNA levels in both surgery groups as compared to controls after 24-h fasting (of note, fasting increases circulating ketone bodies). Their data suggest increased ketone body utilization in heart failure due to increased delivery of ketone bodies and gene regulatory reprogramming of ketone uptake and oxidation. Other studies focussed on the expression levels of these transporters in other types of cardiac failure. MCT1 protein levels were increased in models of chronic MI induced-congestive heart failure[109] and volume overload induced-heart failure.[46] A study in a rat model of acute myocardial ischaemia/reperfusion injury noted increased MCT4 mRNA and protein expression following global ischaemia and increased MCT1 mRNA and protein expression during the early stage of reperfusion.[49] Interestingly, a hypoxic environment is able to activate the MCT4 promotor (but not MCT1 or MCT2) via HIF-1α.[54] In general, expression of MCTs thus seems to be increased during heart failure, although the type of MCT might be depending on the type of heart failure.

While not focusing on the transporter level specifically, other studies took a closer look at the effect of PO on myocardial lactate and ketone body utilization. In contrast to what has been shown in hypoxia,[110] studies with mice subjected to transverse[91] and abdominal[111] aortic banding showed reduced lactate oxidation. This observation could either indicate that lactate oxidation is repressed in the pressure-overloaded heart, independent of substrate influx, or that less intracellular lactate is available to be oxidized due to decreased lactate uptake or plasma availability.

In contrast to the observed reduction in lactate oxidation, ketone body oxidation seems to be increased in heart failure. Increased blood ketone body levels were found in non-diabetic congestive heart failure patients.[112] In addition, a group of non-diabetic, lean, predominantly non-ischaemic advanced heart failure patients had increased serum βOHB and decreased myocardial βOHB levels, implicating increased myocardial ketone body utilization. This was accompanied by increased myocardial gene expression of several genes involved in ketone oxidation, D-β-hydroxybutyrate dehydrogenase (BDH) 1 and 2, and 3-oxoacid CoA-transferase 1 (*OXCT1*, the gene encoding the rate limiting enzyme for myocardial oxidation of βOHB and AcAc, *OXCT1*).[113]

Interestingly, cardiac-specific overexpression of BDH1 in a mouse model of PO ameliorated cardiac remodelling.[114] Additionally, cardiac-specific knockout of *OXCT1* resulted in adverse left ventricular remodelling under PO.[115] These data suggest that enhanced ketone body utilization might be cardioprotective in the pressure-overloaded heart. Whether this enhanced ketone body oxidation is also linked to increased expression of ketone body transporters on the cellular surface of cardiomyocytes, or simply to differences in concentration gradient across the sarcolemma still needs to be determined.

3.4 Amino acid transporters

Little is known about the role of AA transporters in the pressure-overloaded heart. One of the scarce studies on this topic involved patients with congestive heart failure (CHF), and

reported a reduction in myocardial L-arginine uptake, which was associated with 38% lower ventricular CAT1 mRNA expression.[116] Despite these limited data on AA transporters, several studies investigated other aspects of AA metabolism in relation to heart failure. Clear associations between altered AA metabolism and pathological remodelling are found. However, data on these associations are 'divergent' and seems to be depending on type of AAs and type and stage of heart failure.

Elevated levels of plasma AAs are found in CHF patients[116,117] and in animals subjected to TAC surgery.[118] In contrast to these studies, Aquilani *et al*.[119] observed decreased plasma AA levels in patients with CHF. These differences in AA levels among published studies could possibly be attributed to the fact that Aquilani *et al*. excluded patients with diabetes and/or insulin resistance, because it is known that these latter conditions are independently associated with increased plasma AA concentrations. In addition to increased plasma AA levels, increased intramyocardial (total free) AA concentrations are found in human failing right ventricles[120] and in tissue of animals subjected to TAC[121] or to a high-salt diet.[76] These findings suggest that cardiac AA uptake is increased in the (pressure-overloaded) failing heart. Genes involved in AA degradation pathways were found down-regulated in TAC-subjected mice, and impaired BCAA catabolism and intramyocardial accumulation of branched-chain alpha-keto acid (BCKA) were suggested to promote cardiac dysfunction in the pressure-overloaded heart.[118,122] Reciprocally, pharmacological enhancement of BCAA catabolic pathways was able to preserve cardiac function in TAC-induced pressure-overloaded hearts.[123] Moreover, when Dahl salt-sensitive rats received drinking water supplemented with a mixture of BCAAs, their cardiac systolic function was preserved compared to rats receiving normal water.[124] This suggests that increased availability of BCAAs, BCAA supplementation, and utilization of the pressure-overloaded heart could be cardioprotective, and that catabolic pathways could function as a potential therapeutic target.

Most likely differences in altered AA homeostasis upon heart failure development relate to the type of AA, the type of heart failure, and the developmental stage. It has for instance

been shown that plasma levels of different AAs are reduced at different severity stages of heart failure.[119] Moreover, intramyocardial BCAA levels and several other AA concentrations were significantly increased 1 week after TAC surgery, whereas this was no longer prominent after prolonged duration of TAC.[121] A similar trend of increased BCAAs was found in the Dahl salt-sensitive rats, however, a distinct pattern in other AAs was described.[76] Divergent AA levels have also been found in different studies of patients with heart failure. Patients with stage C heart failure had elevated plasma levels of phenylalanine, tyrosine, taurine, glutamate, ornithine, spermine, and spermidine as well as reduced citrulline, glutamine, and histidine levels,[125] while in other studies on patients with severe heart failure reduced methionine, phenylalanine, tyrosine, histidine, threonine, homoserine, glutamine, and alanine levels were reported.[77] Taken together, to provide accurate conclusions with respect to the role of specific AAs and their metabolism in the development of specific types of heart failure more research is required. Although the exact role of specific AA transporters in relation to these altered cardiac and plasma AA levels in distinct types of heart failure (including PO-induced) is yet to be defined, studies have at least indicated that AA metabolism plays a crucial role in the development of heart failure.

4. Concluding remarks

Altered cardiac energy metabolism is an important early contributor to the development of cardiac hypertrophy and failure and emerges as a suitable treatment target in PO. For glucose and FA, substrate availability and transport of substrates into cardiomyocytes are key-governing kinetic steps in their metabolic utilization. Therefore, regulating substrate-specific transporter expression and/or their presence at the sarcolemma could be an interesting approach to manipulate substrate preference, restore altered energy metabolism, thereby prevent cardiac dysfunction or help rescue cardiac function in the pressure-overloaded heart. Available data suggest that GLUT1, GLUT4, and CD36 each play a pivotal role in the pressure-overloaded heart. Whereas the sarcolemmal presence of GLUT1 and of GLUT4 is increased, total GLUT4 and CD36 protein levels seem to be decreased in the pressure-overloaded heart (*Figure 2*, *Table 2*). However, it should be noted

that most of the underlying studies did not discriminate between PO-induced hypertrophy and PO-induced heart failure and that studies on CD36 expression did not make a distinction between subcellular and cell surface (sarcolemmal) content (*Table 2*). Moreover, it should be emphasized that, in addition to cardiomyocytes, metabolism is also altered in other cell types of the diseased heart (such as endothelial cells and fibroblasts). These distinct cell types actively crosstalk with cardiomyocytes and therefore should be considered as possible additional contributors to the development of failure in the pressure-overloaded heart.[126] Nonetheless, recovering an optimal substrate preference by specifically targeting processes involved in the regulation of the sarcolemmal localization of these specific transporters could be beneficial. The latter may comprise manipulation of subcellular transporter translocation or of their transcriptional regulation. For the heart exposed to PO, it can be deduced that CD36 expression and/or translocation should be stimulated to specifically increase FA uptake, and that GLUT4 expression and/translocation should be inhibited. For this, specific differences in the GLUT4 and CD36 translocation machineries, related to e.g. VAMPs and Rab effector proteins, may be exploited to manipulate cardiac substrate preference. Specifically, up-regulation of VAMP4 and down-regulation of Rip11 would stimulate CD36 translocation without affecting that of GLUT4.[27]

It should be noted that rebalancing substrate uptake would only be advantageous when the pressure-overloaded heart is not simultaneously faced with periods of oxygen shortage, i.e. when it is solely relying on glycolysis as energy providing pathway, and that the effectiveness of such manipulation might depend on the stage of heart failure development. Additionally, with respect to the translation of the available findings on substrate transporters to humans, it should be kept in mind that most of the presented data originates from animal models. Whereas the clinical definition of heart failure was historically based on measurement of left ventricular ejection fraction, it currently restricts itself to stages at which clinical symptoms are present.[2] This complexity makes animal modelling of heart failure more difficult, and translational aspects must, therefore, be interpreted with caution. Moreover, individuals with PO often present with comorbidities like obesity and

Type 2 diabetes. These comorbidities are known to affect cardiac metabolism in patients; for instance, obesity and Type 2 diabetes result in elevated FA oxidation rates.[127,128] Further insight into what happens to FA transporters and other membrane transporters in these individuals may provide important information for early diagnostics and novel therapeutic treatment strategies to rescue the pressure-overloaded heart.

Recent data also implicate a role for alternative substrates, such as lactate, ketone bodies, and AAs, in the pathophysiology of the pressure-overloaded heart. Increased availability and enhanced utilization of alternative substrates seem to be cardioprotective (*Figure 2*). Whether this enhanced utilization of ketone bodies and AAs is triggered by increased expression of their specific transporters at the cellular membrane, or simply related to differences in concentration gradients of these substrates needs to be determined. Future research focusing on the role of transporters of the alternative substrates in PO might lead to new therapeutic strategies to combat PO-induced cardiac hypertrophy and failure.

Table 1. *Cardiac membrane substrate transporters and their expression in pressure-overloaded hearts.*

Transporters	Genes	Predominant substrates	Physiological triggers for translocation		Pressure overload	References
			Contraction	Insulin		
Glucose transporters						
GLUT 1 (predominant)	SLC2A1	Glucose, galactose, mannose, glucosamine	+	+	↑	
GLUT 3	SLC2A3	Glucose, galactose, mannose, xylose	n.k.	n.k.	n.k.	
GLUT 4 (predominant)	SLC2A4	Glucose, glucosamine	++	+++	↑	
GLUT 8	SLC2A8	Glucose, fructose, galactose	n.k.	+	n.k.	(Schwenk et al. 2010)[27]
GLUT 10	SLC2A10	Glucose, galactose	n.k.	n.k.	n.k.	(Waller et al. 2013)[63]
GLUT 11	SLC2A11	Glucose, fructose	n.k.	n.k.	n.k.	(Maria et al. 2015)[64]
GLUT 12	SLC2A12	Glucose	n.k.	-	n.k.	(Szablewski et al. 2017)[21]
SGLTs	SLC5As	Glucose, galactose, mannose, fructose, urea, water	n.k.	n.k.	n.k.	
Fatty acid transporters						
FAT/CD36 (predominant)	CD36	LCFAs and VLCFAs	++	++	↓*	
FATP1	SLC27A1	LCFAs and VLCFAs	-	-	n.k.	(Schwenk et al. 2010)[27]
FATP6	SLC27A6	LCFAs and VLCFAs	n.k.	n.k.	n.k.	(Dong et al. 2015)[65]
$FABP_{pm}$	GOT2	LCFAs	+	-/+	↓*	(Guo et al. 2018)[66]
Lactate and ketone body transporters						
MCT1 (predominant)	SLC16A1	Lactate, pyruvate, ketone bodies	n.k.	n.k.	n.k.	
MCT2	SLC16A7	Pyruvate, Lactate, ketone bodies	n.k.	n.k.	n.k.	(Halestrap et al. 2012)[47]
MCT3	SLC16A8	Lactate	n.k.	n.k.	n.k.	(Adeva-Andany et al. 2014)[42]
MCT4 (predominant)	SLC16A3	Lactate, ketone bodies	n.k.	n.k.	n.k.	
Amino acid transporters						
LAT2	SLC7A8	Neutral L-amino acid	n.k.	n.k.	n.k.	
y^+LAT2	SLC7A6	Cationic amino acids; Na+/ large neutral amino acids	n.k.	n.k.	n.k.	(Fotiadis et al. 2013)[57]
Acs-1	SLC7A10	Small neutral amino acids	n.k.	n.k.	n.k.	(Hyde et al. 2003)[56]
CATs	SLC7As	Cationic-amino-acid (and Na+-dependent neutral-amino-acid)	n.k.	n.k.	n.k.	

*For glucose, fatty acid, ketone body, and lactate transporters, the most predominant cardiac transporters are indicated. For the majority of other (less important) substrate transporters only total cardiac mRNA levels have been investigated. Moreover, the expression levels were often relatively low when compared with the expression levels of the predominant transporters, were investigated in foetal heart, or were determined in other areas of the heart than left ventricle. –, no response; –/+, translocation disputed; +, moderate response; ++, strong response; +++, very strong response; ↑, up-regulation of sarcolemmal substrate specific transporters under the condition of pressure overload; ↓, down-regulation of sarcolemmal substrate specific transporters under the condition of pressure overload; n.k., translocation not known. *, translocation of the specific transporters was not investigated.*

Table 2. *Overview of available animal studies reporting on transporter levels in PO-induced hypertrophy and heart failure.*

Pressure overload-induced heart failure models	Cardiac hypertrophy	Time Course PO	Needle size (TAC)	Cardiac functional changes		Transporter expression changes						Gene or protein expression analysis	Ref.
Reduced cardiac function models				**EF (%)**	**FS (%)**	**GLUT1**	**GLUT4**	**CD36**	**FATP**	**FABP**	**MCT2**		
SHR model	n.d.	n.a.	n.a.	n.d.	n.d.	↑	↓	n.d.	n.d.	n.d.	n.d.	mRNA	(Paternostro et al.)[81]
TAC model (Rats)	Yes	17-19 wks	0.6 mm clip	n.d.	n.d.	↑	↓	n.d.	n.d.	n.d.	n.d.	Protein (Total)	(Tian et al.)[85]
SHR model	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	↓	n.d.	n.d.	n.d.	Protein (Total)	(Lauzier et al.)[104]
TAC model (Mice)													
(Comp. hypertrophy)	CH	1 month	26-28 G	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	↑	mRNA	(Aubert et al.)[17]
(Heart Failure)	HF	1 month	26-28 G + MI	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	↑	mRNA	(Aubert et al.)[17]
TAC model (Mice)	Yes	4 wks	30 G clip	9% ↓	7% ↓	↑	↑	n.d.	n.d.	n.d.	n.d.	Protein (Total)	(Pereira et al.)[92]
AAB model (Rats)	Yes	9 wks	20 G	9% ↓	9% ↓	n.d.	n.d.	↓	=	n.d.	n.d.	Protein (Total)	(Dobrzyn et al.)[103]
TAC model (Mice)	Yes	8 wks	27 G	21% ↓	14% ↓	=	=	↓	n.d.	n.d.	n.d.	Protein (Total)	(Dong et al.)[65]
TAC model (Mice)	Yes	8 wks	27G	21% ↓	14% ↓	↑	↓	↓	n.d.	n.d.	n.d.	mRNA	(Dong et al.)[65]
Dahl Salt model *(Rats 17W)*	CHF	n.a.	n.a.	n.d.	19% ↓	↑	↓	↓	n.d.	n.d.	n.d.	mRNA	(Kato et al.)[76]
TAC model (Mice)	Yes	2 months	27 G	40% ↓	n.d.	n.d.	n.d.	↓	n.d.	n.d.	n.d.	Protein (Total)	(Guo et al.)[66]
TAC model (Rats)	Yes	17-19 wks	0.6 mm clip	n.d.	n.d.	↑	↑	n.d.	n.d.	n.d.	n.d.	Protein (PM)	(Tian et al.)[85]
SHR model	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	=	n.d.	n.d.	n.d.	Protein (PM)	(Lauzier et al.)[104]
TAC model (Mice)	Yes	6 wks	27 G	15% ↓	n.d.	n.d.	↓	n.d.	n.d.	n.d.	n.d.	Protein (PM)	(Zhabyeyev et al.)[91]
Unaltered cardiac function models													
	Yes	n.a.	n.a.	4% ↑	6% ↑	↑	↓		n.d.	n.d.	n.d.	Protein (Total)	(Wang et al.)[82]
SHR model	Yes	4 wks	30 G clip	=	=	↑	=	n.d.	n.d.	n.d.	n.d.	Protein (Total)	(Pereira et al.)[94]
ACC model (Mice)	Yes	6 wks	27 G	n.d.	=	n.d.	n.d.	n.d.	↑	=	n.d.	Protein (Total)	(Steinbusch et al.)[105]
TAC model (Mice)	Yes	5 wks	27 G	=	n.d.	n.d.	n.d.	↓	n.d.	n.d.	n.d.	Protein (Total)	(Sung et al.)[106]
TAC model (Mice)	Yes	n.a.	n.a.	n.d.	=	=	↑	↓	n.d.	n.d.	n.d.	mRNA	(Kato et al.)[76]
Dahl Salt model *(Rats, 11W)*								=					
In vitro models													
Neonatal-rat-CMs + AngII	Yes	n.a.	n.a.	n.a.	n.a.	↑	↑		n.d.	n.d.	n.d.	Protein (PM)	(Stuck et al.)[86]
Neonatal-rat-CMs + PE	n.d.	n.a.	n.a.	n.a.	n.a.	↑	↑	n.d.	n.d.	n.d.	n.d.	Protein (PM)	(Fischer et al.)[87]
Adult-rat-CMs + PE	Yes	n.a.	n.a.	n.a.	n.a.	n.d.	↓	n.d.	n.d.	n.d.	n.d.	mRNA (GLUT1/GLUT4 ratio)	(Montessuit et al.)[80]
								n.d.					

AAB, abdominal aortic banding; ACC, ascending aortic constriction; AngII, angiotensin II; CH, compensated hypertrophy; CHF, congestive heart failure; CM, cardiomyocytes; HF, heart failure; n.a., not applicable; n.d., not determined; PE, phenylephrine; PM, plasma membrane; SHR, spontaneous hypertensive rats; TAC, transverse aortic constriction.

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Chapter **3**

Metabolic interventions to prevent hypertrophy-induced contractile dysfunction *in vitro*.

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Chapter **4**

Metabolic remodeling in an *in vivo* rat model of advanced pressure overload-induced heart failure.

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Chapter **5**

Human embryonic stem cell-derived cardiomyocytes as an *in vitro* model to study cardiac insulin resistance

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Abstract

Patients with type 2 diabetes (T2D) and/or insulin resistance (IR) have an increased risk for the development of heart failure (HF). Evidence indicates that this increased risk is linked to an altered cardiac substrate preference of the insulin resistant heart, which shifts from a balanced utilization of glucose and long-chain fatty acids (FAs) towards an almost complete reliance on FAs as main fuel source. This shift leads to a loss of endosomal proton pump activity and increased cardiac fat accumulation, which eventually triggers cardiac dysfunction. In this review, we describe the advantages and disadvantages of currently used in vitro models to study the underlying mechanism of IR-induced HF and provide insight into a human in vitro model: human embryonic stem cell-derived cardiomyocytes (hESC-CMs). Using functional metabolic assays we demonstrate that, similar to rodent studies, hESC-CMs subjected to 16h of high palmitate (HP) treatment develop the main features of IR, i.e., decreased insulin-stimulated glucose and FA uptake, as well as loss of endosomal acidification and insulin signaling. Taken together, these data propose that HP-treated hESC-CMs are a promising in vitro model of lipid overload-induced IR for further research into the underlying mechanism of cardiac IR and for identifying new pharmacological agents and therapeutic strategies.

1. Introduction

Type 2 diabetes (T2D) is a growing health problem worldwide. Fueled by life style changes, such as increased intake of energy-rich food and sedentary lifestyle, the diabetes epidemic has grown parallel with the obesity epidemic[1]. The global prevalence of diabetes among adults has increased dramatically in recent decades, with 4.7% of adults reported with T2D in 1980 and 8.5% in 2014[2]. This major increase in T2D is associated with an increased risk for the development of heart failure (HF) and other cardiovascular diseases, emphasizing the serious need for research in this particular field[3-6]. Insulin resistance (IR), a hallmark of T2D[7,8], is a complex metabolic disorder that occurs upon persistent exposure of the respective organ or tissue to fat. The IR heart has lost the ability to respond adequately to normal plasma levels of insulin, *i.e.*, the rate of insulin-stimulated (GLUT4-mediated) glucose uptake is reduced[9,10]. The consequent alterations in substrate metabolism eventually lead to impaired cardiac function. Despite improvements in primary and secondary prevention over the past years, patients with T2D or IR still have a 2–4 times higher risk of developing HF[11,12]. This highlights the importance of further elucidating the pathophysiological mechanism behind IR-induced HF. Development of representative model systems to investigate the molecular mechanism will facilitate the search for new targets to replace or support the current treatment strategies.

In recent decades, several animal models have been developed to investigate the underlying mechanism of IR-induced HF. For further in depth unraveling of this molecular mechanism, cellular models of the hearts of these animals would be preferred, since they have the additional advantage of allowing precise control of the experimental environment, including pharmacological manipulation[13,14]. However, the use of cellular models obtained from experimental animals also comes with limitations, as they do not perfectly correlate to normal human cardiac physiology. To closer reflect the human situation, human *in vitro* models would be particularly beneficial in exploring the underlying mechanisms of cardiac disease. However, primary human cardiac cells or human heart tissue cannot be obtained easily. Therefore, the emerging human stem cell-derived cardiomyocytes could be a useful tool for drug discovery and the development of personalized treatment strategies.

In this review, we provide an overview of the current knowledge and use of human stem cells to study IR and the potential application of human stem cell-derived cardiomyocytes (hSC-CMs) for studying cardiac IR. First, we summarize the available human cardiomyocyte sources after which we provide more details about the basic characteristics of stem cell-derived cardiomyocytes (including human induced pluripotent and embryonic stem cell-derived cardiomyocytes (hiPSC-CMs and hESC-CMs, respectively)) and the use of cell models to study IR. Finally, we present novel metabolic data on hESC-CMs as a promising human *in vitro* model for the study of cardiac IR.

2. Sources of human cardiomyocytes

2.1. Primary cardiac tissue

Cardiac tissue biopsies, obtained during cardiac surgery, can be used for the isolation of cardiomyocytes. These primary sources directly represent the situation in the human heart. However, when isolating cardiomyocytes, tissues need to be immersed into a collagenase-containing bath as quickly as possible, since snap-frozen or fixated tissue is unsuitable for cardiomyocyte isolation[15]. A major drawback of this collagenase treatment to digest the heart tissue is the low yield. Moreover, cardiomyocytes are terminally differentiated cells, making it difficult to obtain sufficient cells for metabolic measurements. Additionally, biopsies are usually taken at a localized area of the heart, during surgery or with catheters, and might not be representative for the characteristics and function of the left ventricle (LV). Removing cardiac tissue from healthy individuals is not ethically approved and there is a limited availability of unused donor hearts, which prevents us from comparing healthy individuals to patients with specific cardiac disease. Moreover, cardiac biopsies are mainly obtained from patients with end-stage HF and are therefore not representative of the early pathological changes in the heart[16].

2.2. Commercially available cell lines

Alternatives to these cardiac biopsies are the commercially available proliferating human cell lines, such as AC16 and SV40 cells. The AC16 cell line was gained by fusing primary

cultures of adult ventricular heart tissue with SV40 transformed human fibroblasts[17]. This cell line is stable, expresses cardiomyocyte specific markers (α-MHC, β-MHC, α-Actin, troponin I), and displays electrophysiological properties, but detailed information on its metabolic characteristics is not available. A number of other immortalized human SV40 cardiomyocyte lines are also commercially recommended. However, literature on their cardiomyocyte specific characteristics is lacking. Before these cell lines can be deemed suitable for the study of cardiac metabolism, more research into their cardiac and metabolic properties has to be conducted[18,19].

2.3. Human stem cell-derived cardiomyocytes

In the last decade, great efforts have been made to identify less-invasive and more representative sources of human cardiomyocytes for various applications, especially for drug cardiotoxicity screening, myocardial regeneration, and drug discovery[20-23]. The human pluripotent stem cell appears to be a suitable candidate, because of its potential to proliferate and differentiate into various cell types including cardiomyocytes[24-26]. Human stem cell-derived cardiomyocytes, such as hiPSC-CMs and hESC-CMs have attracted considerable attention. Specifically, based on their myogenic gene profile and contractile properties they appear to be functional cardiomyocytes[27-29]. hiPSCs are generated by overexpressing embryonic transcription factors in somatic cells, such as fibroblasts[30]. The major advantage of hiPSCs is that they can be obtained *via* minimally invasive procedures; they can usually be isolated from skin biopsies. Besides the skin biopsies, new methods have become available to obtain hiPSCs, namely from urine and blood[31-33]. These methods appear to be non-invasive, relatively simple, cost-effective, and a reasonably quick process compared to isolation from skin biopsies and hold great promise[33,34]. During fibroblast reprogramming and differentiation of fibroblast-derived hiPSCs, the patient-specific genetic profile is retained. In this way, cardiomyocytes from patients with specific metabolic or cardiac disease related mutations can be produced, thus opening a window for personalized, patient-specific drug discovery[33,35]. However, patient specific epigenetic markers are also maintained during cellular reprograming to hiPSCs, which might influence the differentiation process. Conversely, hESCs are derived from human healthy blastocysts

that are surplus to the requirements of *in vitro* fertilization, without the influence of genetic mutations and epigenetic differences. They are commercially available, thus a standardized cell line can be obtained on a regular basis. Although hESCs are less suitable for a personalized-medicine approach, as they are obtained from a healthy donor, they have proven useful as *in vitro* model for testing the safety and efficacy of human drugs and for studying underlying mechanisms in the development of cardiac disease, independent of patient-specific genetic abnormalities.

3. Basic characteristics of human stem cell-derived cardiomyocytes

3.1. Structural, molecular and electrophysiological characteristics

hSC-CMs differ from adult human cardiomyocytes with respect to shape, sarcomeric organization and receptor expression. Adult human cardiomyocytes have a large and cylindric shape, aligned sarcomeres and large numbers of mitochondria, whereas hSC-CMs have a smaller and oblong morphology, sarcomeres are short and disorganized and less mitochondria are present. It has however been pointed out that maturity can be achieved by prolonged culture of these hSC-CMs (up to 100 days), thereby enhancing structural organization and functional performance over time[36-38]. Additionally, the transcriptional or molecular profile of hSC-CMs differs from undifferentiated stem cells. Important differences include the loss of pluripotency markers (OCT4, NANOG, klf4, POU5F1, SOX2) and the upregulation of mesodermal and cardiac myofilament markers[29,36,39-41]. During the differentiation process, an expression pattern can be distinguished of early and late cardiomyocyte markers. After 5–6 days of differentiation the early markers (GATA-4, ISL1 and KDR) become highly expressed. The expression level of late markers (NKX2–5, TBX5, MEF2C, HAND1/2) peaks around day 8–9, followed by an upregulation of myofilament genes (TNNT2 and MYH6) at day 8–10[42,43]. Twenty to 30 days differentiated cardiomyocytes showed homogeneity among cells based on expression levels of the above-mentioned markers. Furthermore, hSC-CMs are generally characterized as spontaneously, synchronic beating cells derived from human stem cells. The capability of a cell to contract depends on its capability to generate action potentials[44,45]. Whether action potential (AP) phenotype,

ion channels and calcium handling in hSC-CMs are similar to the adult heart has been investigated extensively. Similar to human adult cardiomyocytes, a heterogeneous population of atrial-, nodal-, or ventricular-like cells can be distinguished in hSC-CMs based on AP phenotypes[26,46]. However, these AP characteristics seem to vary between culture conditions and culture time, possibly undermining the correctness of this classification[38,47,48]. To what extent hSC-CMs represent the human adult heart in terms of structural, molecular and physiological characteristics remains debatable.

3.2. Metabolic properties

In terms of metabolism, adult cardiomyocytes are highly active, and for the synthesis of ATP, these cells are mainly (50–70%) dependent on oxidative FA metabolism. In comparison, embryonic and fetal cardiomyocytes mainly rely on glycolysis. For the uptake of glucose the class 1 facilitative transporters (GLUT1, GLUT2, GLUT3 and GLUT4) are the most abundant. Throughout the embryonic period, GLUT1 is expressed ubiquitously and is considered responsible for basal glucose uptake. In all forms of hESCs as well as in fibroblasts glucose uptake is mainly facilitated by the constitutively expressed GLUT1 transporter[49]. The human adult heart, conversely, abundantly expresses GLUT4, a high-affinity insulin sensitive glucose transporter, next to GLUT1[50]. FA uptake in the adult heart is mainly facilitated by fatty acid translocase (CD36)[51]. Whether these transporters are present in hSC-CMs and to what extent they contribute to substrate uptake in hSC-CMs has not been studied so far, but will be further discussed in Section 6. These changes in transporter content (at least as studied so far for the GLUTs) are expected to occur in concordance with changes in metabolism. Whereas stem cells are mainly dependent on glycolysis, a switch towards mitochondrial oxidative metabolism occurs upon differentiation into cardiomyocytes. Compared to hSCs, hSC-CMs have been shown to have increased mitochondrial oxygen consumption and reduced glycolytic reliance[28,52,53]. Moreover, this switch is accompanied by a substantial increase in mitochondrial biogenesis, higher expression of genes and proteins related to oxidative phosphorylation, FA oxidation and lipolysis, which changes continue during the maturation of cardiomyocytes in culture. Furthermore, these unique metabolic properties of cardiomyocytes can be used as a

selection method to generate purified cardiomyocytes. For instance, treatment of 20 day-differentiated hiPSC-CMs with lactate, which can only be metabolized *via* oxidative phosphorylation, was shown to result in 99% purification of hSC cultures[53]. These data suggest that hSC-CMs have the potential to use oxidative metabolic pathways, like adult cardiomyocytes[54-57], and therefore could potentially be useful to study the underlying molecular mechanisms of human metabolic diseases.

4. *In vitro* and *ex vivo* rodent models of lipid-induced insulin resistance

In recent decades, several animal models have been developed to investigate the underlying mechanism of HF in T2D. Many of these models are based on lipid overload. For instance, the chronic exposure of rodents to a HP diet is often used to achieve lipid overload-induced IR in skeletal muscle[58,59] and heart[60-63]. These animal models allow the metabolic changes to be studied in relation to cardiac structural and functional changes. Additionally, *ex vivo* primary cell culture models, such as neonatal rat cardiomyocytes, isolated adult rat and mouse cardiomyocytes, as well as *in vitro* immortal mouse atrial cardiomyocyte cell lines, such as HL-1 cells, are commonly used to study cardiac metabolism[14]. Together, these *in vitro*, *ex vivo* and *in vivo* animal models were instrumental in unraveling the mechanism of the onset of lipid-induced IR. This mechanism includes (i) a rapid CD36 translocation to the sarcolemma and chronically increased FA uptake, (ii) subsequent accumulation of toxic lipid intermediates (*e.g.*, ceramides), (iii) inhibition of insulin signaling (as determined by decreased phosphorylation of protein kinases in the canonical insulin signaling cascade, such as Akt, upon insulin treatment), (iv) decreased insulin-stimulated glucose and FA uptake, ultimately leading to cardiac dysfunction.

The use of these cellular models allows full control of the experimental environment including pharmacological manipulation[13,14]. Though, isolated cells lack the inter-cellular and intra-organ contacts, are not subjected to a preload or afterload and hormonal/neuronal changes, and therefore do not perfectly represent the situation on the whole organ. Furthermore, cardiac cells from animal models are genetically not equivalent to their human counterparts, and subcellular/organellar differences in these cells occur

between species[16,64]. Primary human cardiac cells and human heart tissue can, however, not be obtained easily. Therefore, the emerging hSC-CMs could be a useful new tool for drug discovery and the development of personalized treatment strategies.

5. Insulin resistance in human induced pluripotent stem cells

As described earlier, considerable research has been conducted on hSCs and their derived cardiomyocytes. However, detailed information on their functional metabolic properties and whether IR can modulate metabolism in these cells is scarce. As recently shown[65,66], iPSCs and iPSC-derived skeletal myotubes possess the intrinsic ability to acquire IR, including the expected metabolic changes. In iPSCs from patients with a genetic insulin receptor defect, IR can lead to reduced mitochondrial oxidative capacity, decreased expression of glucose metabolism related genes, and increased reliance on anaerobic metabolism. Interestingly, after differentiation, the skeletal myotubes, derived from these patient iPSCs still showed impaired insulin signaling and insulin-stimulated glucose uptake, decreased mitochondrial function, impaired glycogen synthase activity, and glycogen accumulation when compared to myotubes of control subjects. These data indicate that the insulin resistant phenotype is conserved after reprogramming and re-differentiation into human skeletal myotubes. Moreover, a decrease in gene expression of insulin-regulated genes (RAD1, HK2 and GLUT4) was found[65-67]. Whether these findings on hiPSC-derived skeletal myotubes also apply to hiPSC-CMs or hESC-CMs remains unknown.

Another study[68] aimed at developing an *in vitro* model of diabetic cardiomyopathy, using hiPSC-CMs from healthy donors. hiPSC-CMs were cultured without glucose to force the cells towards FA utilization. The investigators hypothesized that additional exposure to a diabetic environment would promote features similar to diabetic cardiomyopathy. A diabetic environment was mimicked through culture in a maturation medium including 10 mM glucose and relevant concentrations of endothelin-1 and cortisol. Compared to cells on standard maturation medium, cells exposed to this diabetic environment showed disorganized sarcomere structures, altered calcium transients, cellular hypertrophy, intracellular lipid accumulation and oxidative stress. Further, an upregulation was shown of

genes involved in the Krebs cycle, mitochondrial electron transport chain, and glucose metabolism, whereas a downregulation was observed of genes controlling protein synthesis and the cellular response to dysfunctional protein production. These data indicate that hiPSC-CMs exposed to a diabetic milieu recapitulate a diabetic cardiomyopathic phenotype *in vitro*[68].

The above findings[65,66,68], each in different hiPSC models of IR and with distinct outcomes, may relate to differences in genetic background between the individual patients and maintained residual epigenetic differences during cellular reprogramming. Consequently, the establishment of an *in vitro* model without genetic variation would allow us to study the underlying mechanism of IR independent of genetic mutations.

6. Insulin resistance in human embryonic stem cell-derived cardiomyocytes

To our knowledge, it has not been investigated whether hESC-CMs can function as a suitable *in vitro* model to study cardiac IR. In previous *in vitro* studies on rodent cell lines, prolonged HP treatment triggered IR with defects in insulin signaling, substrate metabolism, and also diminished contractile function[60,61,63]. In the current experimental study, we differentiated hESCs into functional cardiomyocytes to study the impact of HP-induced IR on insulin signaling and cardiac metabolism in hESC-CMs. We aimed at establishing a human *in vitro* model to study the underlying mechanism of cardiac IR. Promising data on this new human *in vitro* model are described in more detail in the next paragraphs.

6.1. Differentiation of hESCs towards hESC-CMs

hESCs were differentiated into cardiomyocytes for 30 days. It has been described that hSC-CMs at this point already express various features specific for human cardiomyocytes even though they might not be fully matured yet[42,43,69]. After 30 days of differentiation, > 30% of the cells showed spontaneous contraction and a robust induction (50 to 600-fold) of the expression of key cardiac sarcomeric markers (Fig. 1A), thus confirming differentiation of hESCs towards functional cardiomyocytes. Although these results are in agreement with reports from other groups[29,36,42,43], it should be noted that the observed levels are not

closely comparable to the levels measured in human fetal or LV heart tissue. Nevertheless, these data indicate that we were able to differentiate hESCs into hESC-CMs, keeping in mind that the contractile properties still depict an incompletely matured phenotype.

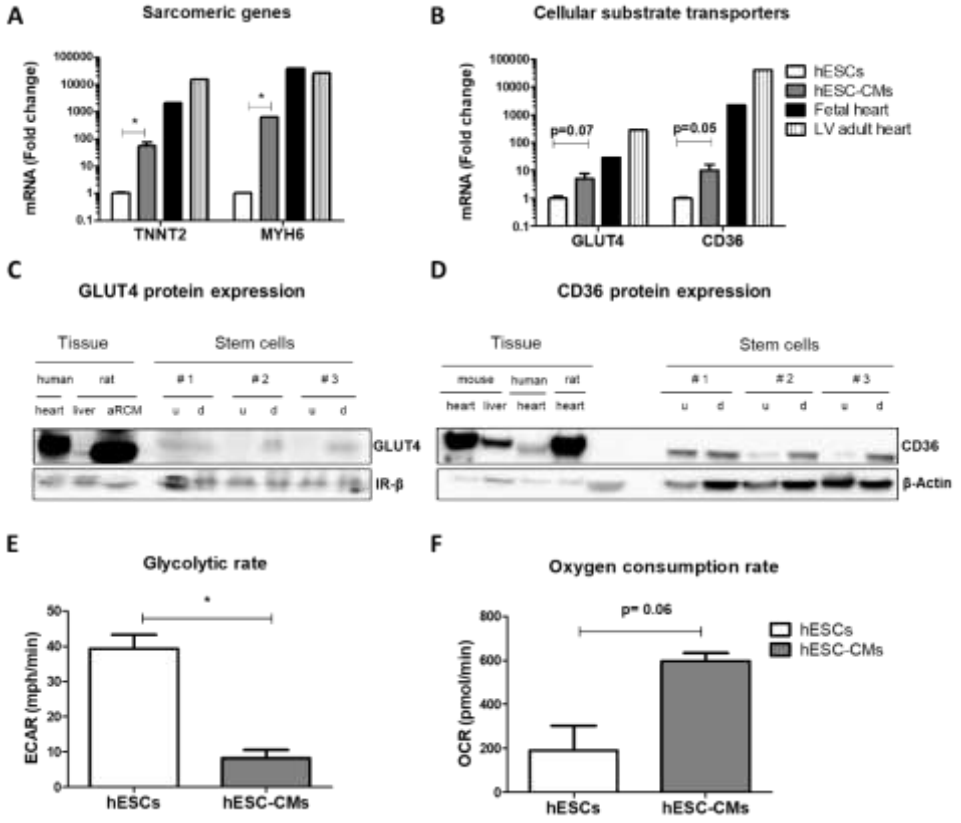

Figure 1. *Differentiation of human embryonic stem cells into cardiomyocytes. Human embryonic stem cells (hESCs) were kindly provided by the Wicell Research Institute (Madison, WI) and subsequently maintained and differentiated for 30 days into hESC-CMs according to manufacturer's protocol (Gibco) as described previously[81]. Quantitative RT-PCR analysis of cardiomyocyte specific genes (A) and substrate transporter genes (B) was performed upon differentiation towards hESC-CMs. TATA box binding protein was used as housekeeping gene and data were normalized against undifferentiated cells, whereas human fetal whole heart (pooled from 4 hearts) and adult left ventricle (LV) tissue (pooled from 3 hearts) were used as positive controls. Immunoblot images of GLUT4 (C) and CD36 (D) are shown for hESCs (u) and hESC-CMs (d). Human, mouse and rat heart tissues and adult rat cardiomyocytes (aRCM) were used as positive controls whereas mouse and rat liver tissues served as negative controls. Cellular acidification rates (ECAR) (E), as a measure for glycolytic rate, and oxygen consumption rates (OCR) (F) were determined with a Seahorse device (Agilent; Seahorse, North Billerica, MA, US), according to the manufacturer's XF Glycolysis Stress protocol, using glucose (10 mM), oligomycin (1 µM), and 2-deoxy-d-glucose (50 mM). Values are displayed as mean ± S.E.M. (n = 3). Data were analyzed by using two-sided Student t-test and considered statistically significant at $p < 0.05$. *$p < 0.05$ compared to hESCs.*

To obtain information about the specific metabolic properties of the 30 days differentiated hESC-CMs, we examined gene and protein expression levels of the specific glucose- and fatty acid transporters (GLUT4 and CD36, respectively), glycolytic capacity, and oxygen consumption rate in biological replicate samples. GLUT4 and CD36 mRNA levels were 5.1-fold and 10.1-fold increased, respectively, in hESC-CMs as compared to hESCs, suggesting that the differentiating cells are switching towards a more mature phenotype (Fig. 1B). These findings on mRNA level were confirmed by western blot analysis where hESC-CMs showed increased expression of both GLUT4 (Fig. 1C) and CD36 (Fig. 1D) compared to hESCs, although it should be noted that GLUT4 and CD36 levels are several orders of magnitude lower than human fetal and adult cardiac tissue levels. Mitochondrial respiration measurements (Seahorse analyser) showed a 73% lower glycolytic rate (ECAR activity) in the hESC-CMs as compared to hESCs (Fig. 1E) and an increased oxygen consumption rate (Fig. 1F). In this respect, our 30 days differentiated hESC-CMs more closely resemble the (oxidation-dependent) human adult heart compared to the (glycolysis-dependent) fetal heart. Taken together, the presence of substrate transporters in hESC-CMs and the switch to aerobic metabolism offers potential for studying insulin signaling and other metabolic pathways in these cells.

6.2. Lipid overload induces IR in hESC-CMs

To examine the onset of lipid overload-induced IR in hESC-CMs, these cells were cultured in media with HP and then assessed on parameters of insulin sensitivity in biological replicate samples. Using a similar protocol as we have previously established to induce IR in HL-1 cells[63], we exposed 30 days differentiated hESC-CMs to 250 µM HP and 50 nM insulin for 16 h (further referred to as HP treatment). Additionally, a short-term 100 nM insulin stimulus was given to evaluate whether the IR state has fully developed within these cells by assessing established metabolic and subcellular parameters of IR. Similar to rodent cardiomyocytes, hESC-CMs showed increased Akt and AS160 phosphorylation (2.0-fold and 1.7-fold, respectively) upon insulin stimulation (Fig. 2A–B). Additionally, uptake of [^{3}H]-deoxyglucose and of [^{14}C]-palmitate was significantly increased upon insulin stimulation. These observations indicate that 30 days differentiated hESC-CMs are insulin sensitive

under basal conditions. When cells were pre-treated with HP, both the activity of insulin signaling related proteins as well as cellular substrate uptake did not change upon insulin stimulation (Fig. 2C–D), suggesting that lipid overload induces IR in hESC-CMs. Furthermore, glycogen content was 58% lower in HP-treated hESC-CMs as compared to untreated cells (4.1 ± 0.4 and 1.7 ± 0.2 µg/10^6 cells in hESCs *versus* hESC-CMs, respectively; $p < 0.01$), suggesting that glycogen synthesis is also decreased. This is in line with other studies showing decreased glycogen synthesis in T2D patients and first degree-relatives[70,71].

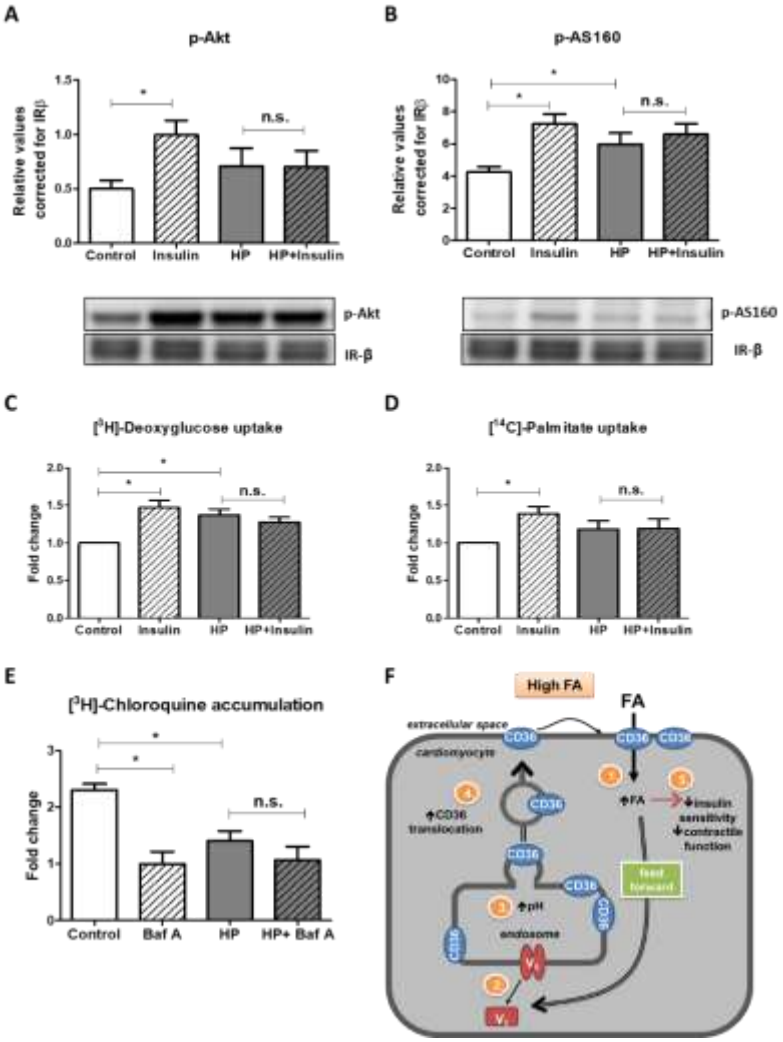

Figure 2. *Lipid overload leads to insulin resistance (IR) and impairment of vacuolar-type H⁺-ATPase (vATPase) function in human embryonic stem cell-derived cardiomyocytes (hESC-CMs). hESC-CMs were cultured under control or high palmitate conditions (250 μM palmitate; 50 nM insulin) for 16 h to induce IR, followed by short-term (30 min) insulin stimulation (100 nM) on day 31 for measurements of insulin sensitivity. A–B) Protein expression levels of insulin signaling related proteins, phospho-Akt-Ser473 (p-Akt) and phospho-AS160-Thr642 (p-AS160). Insulin receptor beta (IRβ) was used as loading control. C–D) Glucose- and fatty acid uptake in hESC-CMs, determined by means of [³H]-deoxyglucose and [¹⁴C]-palmitate, respectively (n = 4). E) v-ATPase function is measured by means of cellular chloroquine accumulation[60]. hESC-CMs were treated with [³H]-chloroquine with/without 100 nM Bafilomycin A (Baf A, a potent v-ATPase inhibitor) for 25 min. The Baf A sensitive component of the total cellular chloroquine accumulation reflects v-ATPase activity (n = 3). F) Schematic presentation of lipid-induced v-ATPase inhibition and its consequences for insulin sensitivity and contractile function (adapted with permission from [60]). When long-chain fatty acid (FA) supply is low, the FA*

transporter CD36 is primarily found in endosomes. The v-ATPase V_0 sub-complex, which is integral to the endosomal membrane, is assembled with the cytosolic V_1 sub-complex allowing for acidification of the endosomal lumen. Upon chronic lipid oversupply, when FA uptake surpasses the cellular metabolic needs, a series of events is triggered: [1] Increased CD36-mediated FA uptake results in elevated intramyocellular FA levels [2]. FAs cause the V_1 and V_0 sub-complexes to dissociate. Therefore, V_1 is shifted into the soluble cytoplasm [3]. The disassembly of v-ATPase leads to endosomal alkalinization [4]. Increased endosomal pH triggers the translocation of CD36 vesicles to the cell surface [5]. Palmitate overload eventually culminates into loss of insulin sensitivity and contractile function. Data are expressed as mean ± S.E.M. Data were analyzed by the Mann-Whitney U *test and considered statistically significant at *p < 0.05. Data were normalized to control (A–D) or Baf A (E).*

Our group has recently unveiled a novel mechanism underlying lipid overload-induced IR. This includes inhibition of vacuolar H^+-ATPase (v-ATPase), a resident protein in membranes of acidic organelles, such as endosomes, which are the storage compartments for CD36[60]. V-ATPase is responsible for maintaining endosomal acidification by means of its proton pumping activity. We have shown that lipid overload directly impairs v-ATPase function, causing loss of endosomal acidification. Since proper endosomal acidification is necessary for intracellular CD36 retention[72], the alkalinized endosomes cannot harbor CD36 anymore, so that it translocates to the cell surface to increase fatty acid uptake, the key step in lipid overload-induced IR. The degree of endosomal acidification can be assessed by cell-associated radioactivity upon incubation of cells with tracer amounts of the divalent weak base [^{3}H]chloroquine, as previously established[72]. Fig. 2E shows that hESC-CMs exposed to HP display a reduction of cell-associated [^{3}H]chloroquine accumulation compared to untreated control cells, indicating impairment of v-ATPase function (Fig. 2E). This novel mechanism underlying lipid overload-induced IR is depicted in (Fig. 2F). In conclusion, all tested hallmarks of lipid-induced IR in these cells are in close agreement with the described mechanism of lipid-induced IR (see Section 4), thereby establishing the suitability of these cells for cardiac IR-related studies.

There are some limitations to the presented IR-model. In particular, the 30 days differentiated hESC-CMs do not fully represent the phenotype of the human adult cardiomyocyte. It has been pointed out that structural and functional maturity can be achieved by adjusted culture conditions (such as oxygen concentration)[73], alternate medium composition (such as galactose and FA-rich medium)[74], electrical stimulation[75], and prolonged culture[36-38,76]. Furthermore, we used a 100 nM insulin concentration, as it is

known that cells in culture are less responsive to insulin stimulation than *in vivo* animal or *ex vivo* isolated heart models. Perhaps, further maturation of hESC-CMs may lead to a higher insulin response, as neonatal cardiomyocytes compared to adult, have high basal glucose uptake levels due to a relatively high GLUT1 expression, which makes them less responsive to insulin-stimulated glucose uptake[77]. Nevertheless, the 30 days-differentiated hESC-CMs show signs of metabolic differentiation and the ability to become insulin resistant thereby providing promising future outlook for the use of hESC-CMs as a model to study metabolic diseases.

7. Concluding remarks

In summary, human *in vitro* cell models appear useful to obtain metabolic insight into human disease pathogenesis, especially in case of IR in the heart. On a large scale the molecular mechanisms in these human models seem to be mostly comparable to the currently used animal models, although it remains necessary to exclude whether fundamental differences, due to genetic, structural and physiological interspecies variation, are present. Currently, several human *in vitro* cell models are available, with hSC-CMs showing a good potential for application in future research. The ability to generate cardiomyocytes from human stem cells provides an unlimited source of cells without the need of invasive procedures. In our present study, we have developed a human *in vitro* model to study lipid-induced IR in human cardiomyocytes. We observed that upon 16 h HP treatment hESC-CMs acquire impairment in v-ATPase function and lose their sensitivity to insulin, suggesting that hallmark features of IR in the heart are conserved between rodents and humans. Although these 30 days differentiated cells might not fully display the cardiomyocyte-specific morphology and sarcomere organization[43,78-80], their metabolic characteristics show a maturing metabolic phenotype as seen in the adult human heart. Therefore, HP treated hESC-CMs provide a promising, valuable human *in vitro* cell model to study cardiac IR. Moreover, functional measurements such as cardiac contractility and electrophysiological properties could be of additional value to confirm that the observed IR phenotype upon HP treatment also affects contractile function in this model. Finally, this model may be important for identifying novel therapeutic targets to combat T2D-induced heart failure.

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Chapter **6**

General Discussion

Main findings of this thesis

The aim of this thesis was to develop experimental models so as to enable the further study of molecular mechanisms underlying the development of pressure overload-induced heart failure and to explore the therapeutic potential of metabolic targeting. In this chapter, the most essential findings of this thesis are summarized and discussed in the context of currently available knowledge. In addition, an outlook towards future research and applications is provided. The main findings of this thesis are as follows:

1. Stimulation of adult rat cardiomyocytes with phenylephrine (PE) for 24h provides an *in vitro* model that closely displays the main characteristics of pressure overload-induced heart failure (i.e. increased glucose uptake, increased protein synthesis, development of cardiac hypertrophy and contractile dysfunction).
2. This PE *in vitro* model indicates that metabolic changes precede changes in protein synthesis and functional changes during the development of pressure overload-induced heart failure.
3. Metabolic targeting, in favor of decreased glucose utilization and/or increased fatty acid utilization, is beneficial for the pressure-overloaded heart.
4. The induction of pressure overload in our young male Lewis rat model is accompanied by a relatively rapid development of severe heart failure and profound myocardial metabolic abnormalities which are more representative for end-stage heart failure than for early-stage heart failure.
5. We developed a human *in vitro* model with embryonic stem cell-derived cardiomyocytes that is suitable to study lipid-induced insulin resistance.

These main findings are further discussed in the following 3 sections. Findings 1 and 5 are summarized in the section “Models to study pressure overload-induced cardiac hypertrophy”, findings 2 and 4 in the section “Time course of metabolic, structural and functional changes during the development of pressure overload-induced heart failure”, and finding 3 in the section “Metabolic targets to rescue to pressure-overloaded heart”.

Models to study pressure overload-induced cardiac hypertrophy

Dysfunctional energy metabolism plays an important role in the development of pressure overload-induced hypertrophy and heart failure. To study these metabolic impairments in relation to the development of heart failure, human cardiac biopsies can be obtained from patients. Unfortunately, however, these biopsies are often restricted to patients with end-stage heart failure and/or derived after heart transplantation[1]. This limited access to patient and control tissue makes it difficult to study metabolic and structural changes over time during development. Nowadays, clinical studies therefore often/ideally focus on non-invasive *in vivo* imaging (PET-imaging) of substrate metabolism in patients at different stages in the development of heart failure (i.e. hypertensive patients or patients with hypertension and left ventricular hypertrophy)[2, 3]. Such imaging forms a unique platform to identify metabolic changes in relation to cardiac structural and functional changes in patients before and during the development of pressure overload-induced cardiac hypertrophy and failure, and will open a new window for treatment strategies to prevent the development of heart failure. For further detailed investigation of the molecular mechanisms involved in the development of pressure overload-induced hypertrophy and failure, animal models are still desired.

Animal models of pressure overload provide the great advantage to study the disease phenotype in a complex multicellular, interactive organ environment, which is not easily done in *in vitro* cell culture models. An ideal *in vivo* or *in vitro* model for cardiac pressure overload should accurately recapitulate the physiological and pathological conditions of the human heart. At the moment, the most widely used animal procedure to study the changes that occur during the development of pressure overload-induced heart failure *in vivo* is via constriction of the mid transverse aortic arch (TAC-surgery), by tightening a suture against a needle with a certain diameter[4]. Since this is a relative robust occlusion of the aortic arch this model closely represents the human disease phenotype of aortic stenosis. This method initially leads to the development of compensated cardiac hypertrophy, but it is suggested that this thickening of muscle wall eventually becomes maladaptive and results in cardiac dilatation and heart failure[5]. However, several

parameters, such as animal strain, age, sex, and needle size have been reported to affect study outcome[6-8]. Moreover, the degree of occlusion is highly operator-dependent and technically demanding, which may lead to considerable variation from study to study. Therefore, one must be careful when comparing results from different studies. To generate high precision and standardized degrees of aortic constriction, several new models have been developed, including transverse aortic constriction by the use of fixed diameter titanium clips and O-rings[9, 10]. In order to minimize variation in our study we choose to use the titanium clip approach to induce aortic constriction, as described in **chapter 4.** We specifically choose to use young rats (3 weeks of age) instead of adult rats, since it has previously been described that aortic banding in adult rats only leads to the development of compensated hypertrophy, whereas young rats do develop severe heart failure with decreased ejection fraction within a period of 15-20 weeks after TAC[11]. The fact that our results deviate from what has been described earlier by Doenst et al.[12], even though the same protocol has been applied, may be explained by differences in rat strain[13], i.e. Lewis versus Sprague-Dawley, respectively.

Finally, one must take into account that the TAC-model, which resembles aortic stenosis, does not reflect the main characteristics of hypertension-induced pressure overload, which shows slowly progressing development of cardiac hypertrophy and finally failure. To study the gradual increase in pressure overload, less acute models are required. Widely used models in this perspective are the spontaneously hypertensive and dahl salt-sensitive rats, which are often used to study compensated left ventricular hypertrophy and its transition to systolic heart failure[14-17].

In addition to the above-described *in vivo* models, animal-free *in vitro* studies with primary cells (adult or neonatal cardiomyocytes) or cell lines (rat ventricular H9c2 or mouse atrial HL-1 cells) provide an intriguing alternative. *In vitro* cell culture studies provide a valuable complement to *in vivo* experiments, allowing for a more controlled manipulation of cellular functions and processes, to study the underlying molecular mechanisms behind diseases and to relatively easy test pharmacological compounds. The benefit of immortalized cell lines is that they have the capacity to proliferate, whereas primary cardiomyocytes, which

are isolated directly from the hearts of rats or mice, have a finite lifespan (up to approximately 48h) and do not have any proliferation capacity[18]. When having a closer look into the metabolic properties of the various cardiomyocytes used to perform *in vitro* studies, it however appears that metabolic profiles differ tremendously between cell types. Whereas neonatal cardiomyocytes and the immortalized H9c2 cells and HL-1 cells are mainly dependent on glycolysis (similar to the fetal heart), primary adult cardiomyocytes are more dependent on fatty acid utilization, similar to the human adult heart[19]. This difference in metabolic properties among cells is important to keep in mind when metabolic alterations during the development of heart failure are the main focus of the study.

To simulate pressure overload-induced cardiac hypertrophy in these cell models, an often-used approach is cellular stimulation with hormonal activators/compounds such as endothelin-1 (ET-1), angiotensin-2 (AT-II), catecholamines and growth factors, which activate signaling pathways involved in cellular growth/hypertrophy[20-24]. Next to the well-known release of ET-1 and AT-II from endothelial cells and kidney, respectively, chronic hypertension also leads to a local release of ET-1 and AT-II from cardiac tissue[25-30]. These locally available hormones trigger the activation of their G-protein coupled receptors (GPCRs), the endothelin receptor (ETA), and the angiotensin-1 receptor (AT-1)[27, 31], thereby activating the hypertrophic program. Interestingly, in our study in **chapter 3** we tested the above-mentioned stimuli, but did not observe an effect of ET-1 and Ang-II on hypertrophic gene expression (ANF and BNP) in adult rat cardiomyocytes. This might be related to contaminating factors in the collagenase buffer used during cell isolations, resulting in malfunctional receptor-ligand interactions. PE, on the other hand, was able to elicit a hypertrophic response, and therefore PE was used in our further studies. PE, is an α-1-adrenergic receptor agonist, a pharmacological compound that mimics the effects of endogenous catecholamines (epinephrine, norepinephrine)[32]. Although this compound is non-physiologic it is able to activate several complex signaling molecules downstream of the protein kinase C pathway, thereby triggering a hypertrophic phenotype in cardiomyocytes and therefore a useful alternative to physiological compounds[21, 33]. It remains however important for understanding of the model to complement and properly

compare the findings of such *in vitro* models with more physiologic *in vivo* models of pressure overload.

A new emerging model to study human heart failure is the human stem cell-derived cardiomyocyte. As described in **chapter 5**, human embryonic stem cell (hESC)- and human induced-pluripotent stem cell (hiPSC)- derived cardiomyocytes could provide the first step in the translation towards a human setting. So far, only few studies have focused on eliciting a hypertrophic response in hESC-derived cardiomyocytes, by showing increased cell size and increased hypertrophic gene expression after exposure to PE, ET-1, Ang-II, or mechanical stretch[34-37]. It remains, however, to be explored whether these current models are also able to display the main metabolic (i.e. increased glucose uptake) and functional characteristics (i.e. impaired contractile function) of pressure overload, since we **(chapter 5)** and others noted that the metabolic and structural properties of these stem cell-derived cardiomyocytes are currently not fully representative to the phenotype of the adult human heart[38-40]. Similar to rodent *in vitro* cell lines, stem cell-derived cardiomyocytes predominantly rely on glycolysis, rather than fatty acid utilization, thereby more closely representing a neonatal phenotype[18, 41]. Current research focusing on the improvement of metabolic, structural and functional maturation of this human cell model, forms a first step in the use of these cells to study cardiac diseases from a molecular perspective[40, 42, 43]. Once these improvements are realized, this human model would be a highly valuable tool to study pressure overload in a human setting.

Overall, a perfect model to study pressure overload-induced heart failure does not yet exist, therefore it is currently still important to combine observations from both *in vitro* and *in vivo* animal models, and (if available) human models, to provide an accurate view on possible underlying mechanisms behind pressure overload-induced heart failure.

Time course of metabolic, structural and functional changes during the development of pressure overload-induced heart failure

In this thesis we used several of the above mentioned models to study the underlying myocardial changes that occur during the development of pressure overload-induced heart failure. As described in the introduction of this thesis, chronic pressure overload, as a consequence of increased workload, induces cardiac hypertrophy and ultimately severe heart failure. It is well established that cardiac left ventricular hypertrophy and heart failure are associated with metabolic alterations (i.e. increased glycolysis for energy production)[44, 45], and it has been proposed that these metabolic changes form a trigger in the pathogenesis of the disease[46, 47]. However, the exact mechanism how these metabolic abnormalities relate to changes in protein synthesis, structure and function of the heart remains unknown.

Metabolic changes during the development of cardiac hypertrophy

In **chapter 3,** our *in vitro* model (i.e. PE-stimulated cardiomyocytes) showed increased cellular glucose and fatty acid uptake, increased protein synthesis, increased expression of hypertrophic markers and contractile dysfunction when compared to control cells. Moreover, we showed that metabolic changes in glucose and fatty uptake preceded changes in protein synthesis, expression of hypertrophic markers and contractile dysfunction. This indicates that metabolic changes precede structural and functional remodeling of the heart during the development of pressure overload-induced hypertrophy. This latter finding is supported by several *in vivo* studies[12, 17, 48, 49]. The studies in models of acute (TAC)[48] and chronic (spontaneous hypertensive rats)[17] pressure overload showed that glucose uptake was increased before changes in wall thickness and/or heart-weight-to-body-weight-ratio were observed. In agreement with these animal studies, hypertensive patients showed higher myocardial glucose uptake rate compared to normotensive patients[2]. But surprisingly, hypertensive patients with cardiac hypertrophy showed reduced cardiac glucose uptake compared to hypertensive patients without cardiac hypertrophy[2], which is not completely in line with the observations in the animal models,

where a clear increase in cardiac glucose uptake was noted during the progression towards cardiac hypertrophy[16, 50]. Despite these conflicting data, overall these results, combined with our own observations in chapter 3, suggest that exposure to both acute and chronic pressure overload, firstly triggers an increase in glucose uptake, which then contributes to the development of cardiac hypertrophy (structural remodeling of the heart).

Some discripancy is observed for glucose oxidation in the pressure-overloaded heart. The increase in glucose uptake in hearts of spontaneous hypertensive rats, that were not yet hypertrophic, was paralled with an increase in glucose oxidation[17], which remained prominent even at the hypertrophic stage[17, 51, 52]. But, these findings on increased glucose oxidation were not confirmed in sprague-dawley rats undergoing TAC surgery. In these rats hypertrophy developed within 2 weeks after TAC, but glucose oxidation was not yet altered at this time point[12]. The deviating results in terms of timing in alterations in glucose oxidation could perhaps be explained by the use of different models (systemic hypertension-induced versus aortic 'stenosis'-induced) of pressure overload. In conclusion, whether glucose oxidation is already increased or unaltered at the early stage of pressure overload requires further investigation.

The above described observations on glucose oxidation are different from what has been reported for fatty acid oxidation in the pressure-overloaded heart. Namely, fatty acid oxidation was already negatively affected at the early stage of compensated hypertrophy. Fifteen week-old spontaneous hypertensive rats showed reduced oleate oxidation[53], and palmitate oxidation[52], aortic banded rats 2 weeks after TAC a dramatic decline in fatty acid oxidation[12], and dahl salt-sensitive rats a decline in β-oxidation metabolites[50]. These results show that impairment in fatty acid oxidation is already prominent at the early stage of compensated cardiac hypertrophy and precedes the development of severe heart failure. Our own data **(chapter 3)** support this idea of an early decrease in fatty acid oxidation, since ACC phosphorylation levels were decreased after 16h and 24h PE stimulation compared to control. This indicates that shuttling of fatty acids into the mitochondria, and thus most likely fatty acid oxidation, is already impaired in 16h PE-stimulated cells and thus before protein synthesis and cardiac function are affected (24h).

As described in detail in **chapter 3**, there is currently no clear consensus with respect to early changes in cellular fatty acid uptake in the pressure-overloaded heart. Our own data **(chapter 3)** which showed an early increase in fatty acid uptake after 16h PE stimulation (remaining to be present at 24h PE stimulation), is supported by two *in vivo* studies showing increased fatty acid uptake at the early stage of compensated hypertrophy[50, 54]. However, in animal models of acute and chronic pressure overload CD36 mRNA expression and total protein expression tend to be decreased **(chapter 2)**, implying a decrease in fatty acid uptake. Therefore, perhaps the observed increase in fatty acid uptake is model related. However, it has to be noted that subcellular distribution of CD36 was not studied.

Overall, the early stage of pressure overload is accompanied by an increase in glucose uptake, followed by reduced fatty acid oxidation and structural remodeling of the heart. Importantly, literature describing the metabolic changes at this early stage of pressure overload, especially changes in fatty acid uptake and in glucose oxidation, is contradictory and therefore would require more detailed investigation.

Metabolic changes during the transition of compensated hypertrophy towards severe failure

As heart failure progresses from compensated hypertrophy towards severe heart failure, the above described observations on increased glucose uptake at the early stage of pressure overload are no longer maintained. Towards the progression of severe heart failure glucose uptake was decreased in an animal model of pressure overload[49], and a decrease in glucose uptake correlated with the severity of diastolic dysfunction in hypertensive patients[2]. The fact that we found no increase in glucose uptake in our rat model of pressure overload **(chapter 4)**, points out that at 4 weeks after TAC these rats already encounter an advanced stage of heart failure in which the early increase in glucose uptake is no longer present.

Interestingly, where there was either no effect or an increase in glucose oxidation observed at the early stage of pressure overload (2 weeks after TAC), a trend towards reduced glucose oxidation was shown between 2 and 10 weeks after TAC, and a significant decline in glucose oxidation at 20 weeks after TAC[12, 55]. The unaltered ejection fraction in these rats until 10

weeks after TAC, and cardiac dilatation and impaired ejection fraction only present at 20 weeks after TAC, together suggest that glucose oxidation is only decreased at a later stage, i.e., during the progression from compensated hypertrophy towards severe heart failure[12, 55].

The decrease in glucose oxidation in rats with advanced stage of heart failure was accompanied by a decrease in fatty acid oxidation[12, 55]. This association with reduced fatty acid utilization in an advanced stage of heart failure has also been confirmed in human studies of patients with dilated cardiomyopathy (DCM)[56-58]. Beside these studies on fatty acid oxidation, an *in vivo* study in dahl salt-sensitive rats showed attenuated fatty acid uptake towards the development of severe heart failure[50], which is further supported by our own model of advanced heart failure **(chapter 4),** showing a decline in fatty acid uptake.

In conclusion, towards the development of severe heart failure, it seems that overall metabolism is affected. Both glucose uptake and oxidation, and fatty acid uptake and oxidation are declined compared to the early hypertrophic stage of heart failure.

Protein synthesis and autophagy in the early and late stage of pressure overload-induced heart failure

In addition to metabolic changes we looked at the underlying mechanisms explaining the observed structural changes in the pressure-overloaded heart. Cardiac hypertrophy is a dynamic process resulting from a disproportionate protein synthesis and protein breakdown. From other studies we know that the early increase in glucose uptake during the early development of pressure overload-induced cardiac hypertrophy is accompanied by the activation of mTOR[47, 48], a well-known positive regulator of protein synthesis and cardiac hypertrophy. A similar pattern of increased glucose utilization and increased mTOR activity was observed in our *in vitro* model of PE-induced pressure overload already after 16h PE stimulation, which was followed by enhanced protein synthesis and development of hypertrophy after 24h PE stimulation **(chapter 3)**. In line with these results, indirect inhibition of mTOR, by Akt-I, was able to prevent the PE-induced increase in protein

synthesis **(chapter 3)**. These results provide further evidence that the early activation of mTOR by pressure overload indeed plays an major role in the development of pressure overload-induced hypertrophy. This is also supported by studies in which mTOR activity was directly inhibited by rapamycin, resulting in a regression in cardiac hypertrophic response in the pressure-overloaded heart[59-61]. It is conceivable that the reduced glucose utilization at an advanced stage of pressure overload leads to the de-activation of mTOR, thereby shifting the protein turnover to favor protein breakdown, ultimately leading to ventricular dilation and the development of severe heart failure.

Autophagy is one of the well-studied protein breakdown mechanisms in the pressure-overloaded heart. In terms of autophagy at an early stage of pressure overload, there are however two contradictory hypotheses described in the literature: 1) Increased autophagy in the early stage of pressure overload as an essential mechanism to provide the heart with sufficient amounts of macronutrients, metabolites and organelles required for processes involved in ATP production (glycolysis and TCA cycle) and hypertrophic growth[62], and 2) Reduced autophagic activity during the early stage of compensated hypertrophy, leading to protein turnover imbalance in favor of protein synthesis, thereby triggering the hypertrophic phenotype[63]. In our *in vitro* model **(chapter 3),** we did not observe any prominent early changes in autophagy response in PE-stimulated cells compared to control (16h and 24h). To draw clear conclusions with respect to autophagic activity in the early stage of pressure overload, time course studies, as performed to study metabolic alterations in the pressure-overloaded heart, could be of high value.

Our *in vivo* model of advanced stage heart failure showed increased autophagic activity **(chapter 4)**. This finding is in line with reported results on increased autophagy in animal studies during the progression from compensated hypertrophy towards severe heart failure and in hearts of patients with severe heart failure[64-67]. It has been proposed that, during the progression of compensated hypertrophy towards severe heart failure, an exuberant autophagic response is triggered by endoplasmic reticulum (ER)-stress, due to accumulation of misfolded proteins that exceed the folding capacity of the ER[47, 62]. This increase in autophagy, together with the deteriorated energy status in the failing heart,

leads to excessive ROS production, degradation of non-specific essential metabolic enzymes and mitochondria, and thereby further impairment of both glucose and fatty acid metabolism and a further decline in ATP.

Taken together, these data suggest that metabolic changes precede structural changes during the development of pressure overload-induced hypertrophy and failure. Additionally, these data provide evidence for a key role of mTOR towards the development of cardiac hypertrophy, and suggest that ER-stress might be an important factor in the transition from hypertrophy towards severe failure.

Metabolic targets to rescue to pressure-overloaded heart

The fact that metabolic changes precede structural and functional changes in the pressure-overloaded heart, makes targeting metabolic abnormalities a promising therapeutic approach. Based on the above described results several metabolic interventions could be applied in the early prevention of pressure overload-induced heart failure: 1) Inhibition of glucose uptake, 2) Stimulation of glucose oxidation, 3) Stimulation of fatty acid uptake, 4) Enhancement of fatty acid oxidation. **Chapter 3** of this thesis confirms that the use of compounds (Akt-I and DPY), that intervene in the metabolic substrate preference (via inhibition of glucose uptake and enhancement of fatty acid uptake), are protective in terms of maintaining contractile function in cardiomyocytes exposed to hypertrophic stimuli. This confirms that pharmacological intervention that target metabolic pathways, thereby aiming to restore the substrate balance in favor of fatty acid utilization (so as to obtain the metabolic profile as observed in the healthy adult heart) are indeed beneficial in terms of cardiac outcome.

Targeting glucose uptake

Specific targeting of glucose uptake would be a valuable approach, given that glucose uptake is increased in the early stage of pressure overload. Compounds that inhibit glucose uptake could perhaps prevent the uncoupling between glycolysis and glucose oxidation as observed in the hypertrophic heart. We showed that specific inhibition of the Pi3k/Akt/mTOR pathway was protective in our *in vitro* study **(chapter 3).** This is most likely due to indirect inhibition of GLUT4 translocation to the sarcolemma, the main glucose transporter in the heart[68]. Interventions that specifically aim at inhibiting the GLUT4 translocation machinery could protect the pressure-overloaded heart from developing hypertrophy and failure. The proteins that make up the GLUT4 translocation machinery are further detailed in the following references;[69, 70]. Expression of GLUT4 can also be regulated by targeting specific genes such as the hypertrophic transcription factor myocyte enhancer factor-2 (MEF2), which is upregulated in cardiac hypertrophy[71]. Additionally, targeting the increased expression of GLUT1 during pressure overload should not be forgotten. It has been reported that hypertrophy is accompanied by increased expression of HIF-1α[72, 73] and activator protein 1 (AP1)[74] and reduced levels of PPARα[75], which are associated with increased expression of fetal-like-genes, such as GLUT1.

Interestingly, we also observed increased activity of the PKD-pathway upon PE stimulation **(chapter 3),** which independently from the Pi3K/Akt/mTOR pathway is also involved in GLUT4 translocation. Whether this pathway plays an important role in pressure overload-induced heart failure remains to be validated. PKD1 stimulates GLUT4 translocation via a yet unknown pathway involving endosomal lipid kinases[76]. Further unraveling the exact mechanism behind PKD1-activated GLUT4 translocation could lead to new strategies to combat pressure overload-induced heart failure.

Targeting glucose oxidation

Additionally, several studies have focused on enhancement of glucose oxidation as treatment of the pressure-overloaded heart, which (similar to inhibition of glucose uptake)

would promote a better coupling between glycolysis and glucose oxidation. Activation of pyruvate dehydrogenase (PDH), an enzyme involved in the conversion of pyruvate (the end-product of glycolysis) to acetyl-CoA, would achieve such a coupling and indeed appears to be beneficial in pressure overload-induced heart failure. Dichloroacetate (DCA) is a compound that inhibits the activity of pyruvate dehydrogenase kinase (PDK), thereby removing the inhibition of PDH and allowing the conversion of pyruvate to acetyl-CoA[77]. Treatment of dahl salt-sensitive rats with DCA prevented the progression of hypertrophy to heart failure and improved systolic cardiac function and survival[16]. However, studies in patients with congestive heart failure that were treated with DCA, reported some conflicting results. One study, with moderate and severe heart failure patients, found improved cardiac function after treatment with DCA[78], whereas a subsequent study did not show any beneficial effects in patients with congestive heart failure[79]. Nevertheless, most studies point in the direction that enhancement of glucose oxidation in the pressure-overloaded heart could ameliorate the development of cardiac hypertrophy and the progression towards severe heart failure.

Targeting fatty acid uptake

Although the exact role of fatty acid uptake in the pressure-overloaded heart remains to be investigated, targeting fatty acid uptake could be an additional promising approach. It was namely shown that cardiac-specific overexpression of CD36 (the predominant fatty acid transporter in the heart) protects the heart against pressure overload-induced heart failure[80], which indicates that stimulation of fatty acid uptake (not only oxidation) is beneficial in the pressure-overloaded heart. This enhancement of cellular fatty acid uptake will increase the intracellular fatty acid pool, and will most likely force the heart towards fatty acid utilization. Targeting CD36 expression at the sarcolemma is therefore a considerable intervention option. This can for instance be achieved by targeting compartments of the trafficking machinery, which are specifically involved in CD36 translocation from endosomal storage compartments to the cellular membrane[70] or via the upregulation of genes involved in regulating fatty acid uptake, such as estrogen-related

receptor-α (ERRα)[81], key peroxisome proliferator-activated receptor-α (PPARα), and its co-activator PPAR-gamma co-activator-1α (PGC-1α)[82]. However, it should not be forgotten that a mismatch between fatty acid uptake and fatty acid oxidation could lead to the accumulation of lipid intermediates, thereby inducing lipotoxicity and insulin resistance in the heart. Hence, it should be checked, when targeting these trafficking proteins or transcription factors in the pressure-overloaded heart, whether the increase in fatty acid uptake would be accompanied by increased fatty acid oxidation.

Targeting fatty acid oxidation

This idea of restoring the metabolic profile, towards a profile as observed in the healthy adult heart, is supported by several *in vivo* studies focusing on enhanced fatty acid oxidation in the pressure-overloaded heart. PKD1-overexpressing mice, which are predominantly dependent on glucose utilization, developed cardiac hypertrophy and ventricular dilatation, but showed improved cardiac structure and function when subjected to a high-fat diet[83]. This high-fat diet generally leads to increased fatty acid oxidation. Additionally, mice with increased cardiac fatty acid oxidation, due to a cardiac-specific deletion of acetyl-CoA via acetyl-CoA carboxylase (ACC2), showed reduced glycolytic rates and anaplerosis, and were protected against the development of cardiac dysfunction induced by pressure overload (TAC and Angiotensin-2 infusion)[84, 85]. Moreover, diabetic mice and mice subjected to a high-fat diet, showed less cardiac dysfunction after TAC surgery[49, 80]. These findings demonstrate that increased cardiac fatty acid utilization exerts cardio-protective effects after TAC. Screening for compounds that can enhance fatty acid oxidation, might be a useful future strategy to combat pressure overload-induced cardiac hypertrophy and ultimately failure.

Concluding remarks and future perspectives

Pressure overload-induced heart failure is accompanied by metabolic changes, which most likely precede structural and functional changes in the heart. At the very early stage of pressure overload – before any structural remodeling occurs – the heart encounters an increase in glucose uptake, which is then followed by structural remodeling of the heart. This structural remodeling is accompanied by enhanced protein synthesis most likely driven by one of its key regulatory proteins, mTOR. The increase in glucose uptake is also followed by a decrease in fatty acid oxidation during the subsequent development of cardiac hypertrophy, and ultimately, as heart failure progresses towards severe failure, an overall decrease in substrate utilization (both glucose uptake and oxidation and fatty acid uptake and oxidation), leaving the heart with insufficient amounts of energy sources. Unfortunately, at the early stage of pressure overload there is no clear consensus regarding changes in fatty acid uptake and/or in glucose oxidation. Nevertheless, we can conclude that metabolic changes are pivotal during the (early) development and progression of pressure overload-induced heart failure. Future research should focus on mapping these metabolic changes in a time-dependent manner.

Furthermore, unraveling the underlying mechanisms responsible for these changes in substrate metabolism will open a window for novel therapeutic targets that aim at restoring substrate metabolism in the failing heart. In order to obtain an overall picture with respect to the metabolic changes, it could be of significant value to combine and compare observations from different models (cell, animal and human models) in a longitudinal setting. However, it should be taken into account that models of acute pressure overload might differ from models of chronic pressure overload, and that animal models might not always reflect the pathophysiological situation in the human heart. Therefore, one should consider investigating all metabolic parameters within the same study to provide a coherent picture in specific models of pressure overload. Moreover, it should be kept in mind that timing is crucial for metabolic treatment in patients, due to the fact that there are different metabolic changes at different stages of heart failure. Ultimately, *in vivo* monitoring of substrate metabolism and of protein synthesis could allow for proper diagnosis and tailored

treatment of pressure overload-induced heart failure patients, including monitoring the efficacy of installed treatment.

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Summary

The general aim of this thesis was to develop experimental models for pressure overload-induced heart failure to enable the further study of the underlying molecular mechanisms contributing to the development of heart failure, as well as the study of various metabolic interventions to rescue the pressure-overloaded heart.

Chapter 1 provides a general introduction on cardiac pressure overload and the contributing role of alterations in substrate metabolism and protein turnover in the development of pressure overload-induced heart failure. Additionally, the aim and the outline of this thesis have been described in greater detail.

Chapter 2 highlights the current state of knowledge regarding substrate transporters (of both predominant and alternative substrates), and their key role in substrate utilization in the (pressure-overloaded) heart. Moreover, this chapter summarizes the main findings regarding expression levels and cellular distribution of these transporters in the pressure-overloaded heart, and emphasizes their potency as therapeutic target to combat the development of cardiac hypertrophy and heart failure.

Chapter 3 describes our developed *in vitro* cardiomyocyte model that closely mimics the main characteristics (increased glucose uptake, increased protein synthesis and expression of hypertrophic genes, and reduced contractile function) of pressure overload-induced cardiac hypertrophy. This model implies that metabolic changes occur before structural and functional changes in the pressure-overloaded heart. Moreover, this model will allow us in the future to (i) unravel the mechanisms behind this process in more detail, and to (ii) study whether metabolic interventions could prevent cardiac structural and functional changes. Our first metabolic interventions were aimed at decreasing glucose uptake. These interventions forced the cardiomyocytes to increase fatty acid utilization, resulting in restoration of contractile function, thereby supplying proof-of-concept that metabolic interventions can be used successfully to treat the hypertrophic heart.

Chapter 4 describes metabolic changes in relation to changes in protein synthesis and cardiac function in an *in vivo* animal model of advanced stage pressure overload. Although the model was originally designed to study early-stage heart failure, alterations in cardiac

function and profound metabolic abnormalities where more representative for end-stage heart failure. Therefore, future research should focus on the refinement of this model towards a more slowly progressive model of pressure overload.

Chapter 5 tested the hypothesis whether human stem cell-derived cardiomyocytes could function as a relevant human tool to study heart failure of metabolic origin. This chapter gives insight into the metabolic profile of human stem cell-derived cardiomyocytes and provides a novel cell line-derived *in vitro* model to study cardiac insulin resistance. Future research may focus on the implementation, of stem cell-derived cardiomyocytes in a model of pressure overload-induced heart failure, allowing us to make the translation of the findings presented in chapters 3 and 4 towards a human setting.

Chapter 6, the general discussion, provides an overview on currently used models to study pressure overload-induced cardiac hypertrophy and failure. Moreover, our novel findings were discussed in more detail, an elaborated view on alterations in substrate uptake, oxidation and protein synthesis during the development of heart failure was given, and possible targets for metabolic intervention were highlighted.

In conclusion, this thesis presents the importance of changes in substrate metabolism during the development of pressure overload-induced cardiac hypertrophy and heart failure. These findings extend the current knowledge on heart failure pathophysiology and may contribute to discovering new therapeutic targets.

Samenvatting

De algemene focus van dit proefschrift lag bij het ontwikkelen van experimentele modellen voor een type chronisch hartfalen waarbij de linkerventrikel van het hart een hogere druk moet genereren om bloed in de aorta te pompen (zoals aortaklepstenose en door hoge bloeddruk geïnduceerd hartfalen). Deze modellen stelde ons in staat om onderliggende mechanismen die ten grondslag liggen aan het ontstaan van deze vorm van hartfalen beter te kunnen bestuderen. Daarnaast zijn deze modellen gebruikt om verschillende metabole interventies te testen die gericht zijn op het voorkomen/behandelen van overdruk-geïnduceerd hartfalen.

De algemene introductie in **hoofdstuk 1** gaat in op de rol van veranderingen in substraatmetabolisme en eiwitsynthese tijdens de ontwikkeling van overdruk-geïnduceerd hartfalen. Daarnaast worden de algemene doelstelling en de inhoud van deze thesis in detail beschreven.

Hoofdstuk 2 gaat in op de huidige kennis met betrekking tot substraattransporters en hun functie in het substraatverbruik door het hart dat blootgesteld is aan overdruk. Daarnaast worden in dit hoofdstuk de voornaamste bevindingen met betrekking tot expressieniveaus en cellulaire distributie van deze transporters in het aan overdruk blootgestelde hart weergegeven. Tevens wordt nadruk gelegd op het feit dat deze transporters een potentieel therapeutisch target kunnen vormen in de strijd tegen hartfalen.

Hoofdstuk 3 beschrijft ons ontwikkeld *in vitro* cardiomyocyte model waarin de voornaamste karakteristieken (toegenomen glucose opname, toegenomen eiwitsynthese en expressie van hypertrofie genen, en afgenomen contractie functie) van overdruk-geïnduceerde cardiale hypertrofie worden weergegeven. Dit model impliceert dat metabole veranderingen vooraf gaan aan structurele en functionele veranderingen in het aan overdruk blootgestelde hart. Daarnaast zorgt dit model ervoor dat we in de toekomst (i) onderliggende mechanismen die ten grondslag liggen aan het ontstaan van hartfalen nader kunnen onderzoeken, en dat we (ii) metabole interventies kunnen testen die ons wellicht in staat stellen om cardiale structurele en functionele veranderingen tegen te gaan. Onze eerste metabole interventies waren gericht op het verlagen van de glucose-opname. Deze interventies forceerden cardiomyocyten om meer gebruik te maken van vetzuren als

substraat, wat resulteerde in een verbeterde contractiele functie. Deze resultaten bevestigen de hypothese dat metabole interventies nuttig kunnen zijn in het tegengaan en/of behandelen van cardiale hypertrofie.

Hoofdstuk 4 beschrijft metabole veranderingen in relatie tot veranderingen in eiwitsynthese en cardiale functie in een *in vivo* diermodel met een gevorderd stadium van hartfalen. Hoewel het model aanvankelijk was opgezet om metabole veranderingen in het vroege stadium van hartfalen te bestuderen, pasten de waargenomen veranderingen in cardiale functie en in metabolisme meer bij een eindstadium van hartfalen. Verder onderzoek zal zich moeten richten op het verfijnen van dit model, dat wil zeggen naar een model waarbij de veranderingen ten gevolge van cardiale overdruk zich meer geleidelijk ontwikkelen.

In **Hoofdstuk 5** testten we de hypothese dat uit humane stamcel-gegenereerde cardiomyocyten kunnen fungeren als een relevant humaan *in vitro* model voor het bestuderen van hartfalen met metabole origine. Dit hoofdstuk geeft inzicht in de metabole profielen van stamcellen en stamcel-gegenereerde cardiomyocyten en introduceert een nieuw *in vitro* model voor het bestuderen van cardiale insulineresistentie. Toekomstig onderzoek zou zich moeten focussen op de implementatie van deze stamcel-gegenereerde cardiomyocyten in een model van overdruk-geïnduceerd hartfalen. Dit zou uiteindelijk kunnen bijdragen aan het vertalen van onze bevindingen (opgedaan in hoofdstuk 3 en 4) naar een humane setting.

De algemene discussie in **hoofdstuk 6** geeft een overzicht van de huidige modellen die gebruikt worden om overdruk-geïnduceerde hypertrofie en hartfalen te bestuderen. In dit hoofdstuk worden onze nieuwe bevindingen uitvoerig bediscussieerd en wordt een overzicht gegeven van veranderingen in substraatopname, substraatoxidatie en eiwitsynthese tijdens de ontwikkeling van hartfalen. Daarnaast worden potentiële metabole targets in meer detail besproken.

Tot slot wordt in deze thesis het belang van veranderingen in substraatmetabolisme tijdens het ontwikkelen van overdruk-geïnduceerde hypertrofie en hartfalen aangegeven. Deze

bevindingen vullen de huidige kennis met betrekking tot de pathofysiologie die ten grondslag ligt aan het ontstaan van hartfalen aan, en kunnen hierdoor bijdragen aan het vinden van nieuwe potentiële targets.

Valorization

Socio-economic relevance and clinical potential

Heart failure is the leading cause of death worldwide, currently affecting at least 26 million people [1], and continuously increasing in its incidence, prevalence, and economic costs [1]. There are many external triggers that can lead to heart failure. Aortic stenosis and systemic arterial hypertension, each resulting in cardiac pressure overload, belong to the most-frequent cardiovascular diseases and major risk factors of heart failure[2]. Currently, over 1.13 billion people suffer from hypertension worldwide, a number that has nearly doubled since 1975 and is still on the rise[3]. Also the incidence of aortic stenosis, the most serious global valve disease problem, reaches epidemic proportions. With demographic aging of the global population the incidence of pressure overload-induced heart failure is expected to increase even further, thereby stressing the urgent need for effective medical therapy. Currently, there is, however, still no cure for pressure overload-induced heart failure, and unfortunately most available therapies are limited to symptom treatment. Before physical symptoms become apparent, the hearts of heart failure patients often already undergo asymptomatic structural and/or functional abnormalities (left ventricular dysfunction)[4]. Early detection and prevention are therefore of high importance. It has been proposed that derangements in cardiac energy substrate metabolism play a key role in the early pathogenesis of pressure overload-induced cardiac hypertrophy and eventually failure. Unravelling the underlying molecular triggers responsible for the occurring changes in substrate metabolism and protein synthesis, as seen in hypertrophying hearts, holds great promise to provide novel therapeutic strategies to prevent or treat the failing heart.

Potential target groups

The focus of this thesis is on the pathophysiology of heart failure as a consequence of pressure overload. The content of this thesis holds relevant information for (1) academic scientists working in various research fields, including cellular biology and function, substrate metabolism, protein synthesis and cardiac (patho)physiology, (2) the pharmaceutical industry working on the development of drugs/therapies against pressure overload (hypertension and aortic stenosis)-induced heart failure, (3)

clinicians/cardiologists, (4) and patients with hypertension and/or aortic stenosis, who are at high risk of developing cardiac complications. Moreover, scientists working on diseases (or patients suffering from diseases), with similar metabolic derangements as observed in the pressure-overloaded heart, such as cancer, could ultimately also benefit from our new discoveries.

To provide our obtained knowledge to the scientific community and beyond, our findings are published in scientific journals relevant to the research field, such as *Cardiovascular Research*, *BBA-Molecular Basis of Disease* and *Journal of Biological Chemistry*. Additionally, the studies described in this thesis were presented at relevant national (2nd Maastricht-Nijmegen Science day, Nijmegen, The Netherlands; CARIM day 2016, Maastricht, The Netherlands; 17th Society for Heart and Vascular Metabolism conference, Amsterdam, The Netherlands) and international (14th Society for Heart and Vascular Metabolism conference, Beijing, China; 17th Dutch-German Joint Meeting of the Molecular Cardiology Working Groups, Göttingen, Germany) scientific meetings/conferences by means of oral and poster presentations.

Activities and products

The knowledge acquired in this thesis and the established *in vitro* and *in vivo* models to study pressure overload-induced heart failure, has provided our research group with new expertise and advanced understanding in the underlying molecular mechanisms contributing to the development of pressure overload-induced heart failure, allowing them and other scientists to use these models and this new knowledge to implement in future research.

Additionally, products of great relevance for the society may be the public awareness of heart failure as a growing societal and economic burden. Connecting to cardiovascular foundations such as the Hartstichting and other health care organizations/professionals could make the knowledge described in this thesis better accessible and more useful for the benefit of the society. The main elements of public awareness campaigns by these

organizations, pay attention to signs and the sudden complications associated with heart failure, making the general public aware of heart failure symptoms and appropriate measures to take in case of an incident. Moreover, their patients education programs could help heart failure patients to make informed decisions and fully participate in all aspects of their illness, ultimately saving lives and enabling improved quality of live for patients with heart failure. Sharing information regarding research content and progress with these organizations, will not only allow these organizations to translate our findings to relevant information intelligible for layman, but will also allow foundations to assess whether funding in this particular research fields is needed.

Innovation and future directions

This thesis provides new insights into the metabolic changes during the development of pressure overload-induced heart failure. Continuing and expanding this research is highly essential if we aim to understand and further unravel the complex underlying mechanisms contributing to the development of cardiac hypertrophy and ultimately failure. In this thesis I provide evidence for the important role of changes in substrate metabolism during the development of pressure overload-induced heart failure. This fundamental research forms a first step towards the discovery of new potential therapeutic treatment targets. The facts that metabolic changes seem to precede structural and functional changes in the pressure-overloaded heart, and the fact that metabolic interventions are able to protect cardiomyocytes from contractile dysfunction, provides new target sides for early and late stage metabolic intervention studies. Although we have established a useful *in vitro* model that closely mimics the main characteristics of pressure overload-induced cardiac hypertrophy, future research will have to confirm our *in vitro* findings in animal and ultimately human disease models. Despite the projects described in this thesis being mostly of fundamental nature, these findings provide the first “building blocks” for future implementation of metabolic substrate metabolism as a non-invasive diagnostic tool in hypertensive and aortic stenosis patients. *In vivo* monitoring of substrate metabolism in pressure overload-induced heart failure patients (including determination of the disease

stage) would provide a base for tailored and patient specific treatment. Moreover, future research should focus on screening for new compounds that might be able to restore metabolic abnormalities in the pressure-overloaded heart.

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Curriculum Vitae

Curriculum Vitae

Ilvy Geraets was born on November 5, 1991 in Brunssum, the Netherlands. In September 2010, she started her Bachelor's education in Biomedical Sciences (Specialization: Molecular Life Sciences) at Maastricht University. After finishing her first internship at the Department of Toxicology, University of Maastricht, she successfully graduated for her Bachelor in July 2013. Subsequently, she participated in the Master Biomedical Sciences. During this study, she performed a 5-months internship at the department of Pathology, Maastricht University. Subsequently, she took the change to go abroad and performed her 8-months internship at the research lab of Prof. Dr. Jo G.R. De Mey, Department of Cardiovascular and Renal Research, University of Southern Denmark, Odense, Denmark. Here she investigated the vasocrine effect of human patients' pericardial and perivascular adipose tissue. From this work in Denmark she obtained 1 co-authorship publication.

After obtaining her Master's degree in July 2015, her interest in cardiovascular research brought her to join the research group of Prof. dr. Jan Glatz at the Department of Genetics and Cell Biology, section Molecular Genetics. In September 2015, she started as a Pre-AIO within this research group under the supervision of Dr. Miranda Nabben and Dr. Joost Luiken. The first year of her PhD was focussed on the use of a new human model (human stem cell-derived cardiomyocytes) to study insulin resistance in the heart. Studying the metabolic characterization of human embryonic stem cells (hESCs) and their derived cardiomyocytes formed the central topic during this period. The second part of her PhD was devoted to the development of experimental models for pressure overload-induced heart failure. These models enabled her to study underlying molecular mechanisms contributing to the development of heart failure, with the aim to derive new therapeutic targets, as well as to test metabolic interventions to rescue the pressure-overloaded heart.

Next to the design, planning and organization of research projects, she was organizer of the weekly Molecular Genetics meetings (2015-2018), supervised several bachelor and master students during their internships and participated in teaching as a tutor in a problem based learning setting. Within this 4-year period, she actively attended national and international conferences; 2nd Maastricht-Nijmegen Science day in Nijmegen (The Netherlands), 17th Society for Heart and Vascular Metabolism (SHVM) conference in Amsterdam (The Netherlands), 14th Society for Heart and Vascular Metabolism (SHVM) conference in Beijing (China), and the 17th Dutch-German Joint Meeting of the Molecular Cardiology Working Groups in Göttingen (Germany). During these conferences she presented her work by means of oral or poster presentations.

List of Publications

1. **Geraets IME**, Glatz JFC, Luiken JJFP, Nabben M. Pivotal role of membrane substrate transporters on the metabolic alterations in the pressure-overloaded heart. *Cardiovasc Res.* (2019) May 1;115(6):1000-1012.

2. **Geraets IME**, Chanda D, van Tienen FHJ, van den Wijngaard A, Kamps R, Neumann D, Liu Y, Glatz JFC, Luiken JJFP, Nabben M. Human embryonic stem cell-derived cardiomyocytes as an in vitro model to study cardiac insulin resistance. *Biochim Biophys Acta Mol Basis Dis.* (2018) May;1864 (5 Pt B):1960-1967.

3. Abdurrachim D, Nabben M, Hoerr V, Kuhlmann MT, Bovenkamp P, Ciapaite J, **Geraets IME**, Coumans W, Luiken JJFP, Glatz JFC, Schäfers M, Nicolay K, Faber C, Hermann S, Prompers JJ. Diabetic db/db mice do not develop heart failure upon pressure overload: A longitudinal *in vivo* PET, MRI, and MRS study on cardiac metabolic, structural, and functional adaptations. *Cardiovasc Res.* (2017) Aug 1;113(10):1148-1160.

4. Chanda D, Oligschlaeger Y, **Geraets I**, Liu Y, Zhu X, Li J, Nabben M, Coumans W, Luiken JJFP, Glatz JFC, Neumann D. 2-Arachidonoylglycerol ameliorates inflammatory stress-induced insulin resistance in cardiomyocytes. *J Biol Chem.* (2017) Apr 28;292(17):7105-7114.

5. Elie AG, Jensen PS, Nissen KD, **Geraets IM**, Xu A, Song E, Hansen ML, Irmukhamedov A, Rasmussen LM, Wang Y, De Mey JG. Adipokine Imbalance in the Pericardial Cavity of Cardiac and Vascular Disease Patients. *PLoS One.* (2016) May 3;11(5):e0154693.

In Preparation

1. **Geraets IME**, Coumans WA, Strzelecka A, Schönleitner P, Antoons G, Glatz JFC, Luiken JJFP, Nabben M. Metabolic interventions to prevent hypertrophy-induced contractile dysfunction in vitro.

Oral Presentations

1. June 2019: The Society for Heart and Vascular Metabolism, SHVM – Amsterdam, The Netherlands (*selected short talk*).
   Title: Metabolic interventions to treat hypertrophy-induced contractile dysfunction in vitro
2. May 2019: Genetics and Cell Biology Seminar^{+} Meeting, Maastricht, The Netherlands
   Title: Metabolic interventions to treat hypertrophy-induced contractile dysfunction in vitro
3. October 2015: Maastricht-Nijmegen Science day, Nijmegen, The Netherlands
   Title: Cardiac cilia: The role of cilia in cardiomyocytes

Poster Presentations

1. March 2019: Dutch-German Joint Meeting of the Molecular Cardiology Working, Göttingen, Germany.
2. October 2016: The Society for Heart and Vascular Metabolism, SHVM- Beijing, China.
3. November 2016: CARIM Symposium 2016, Maastricht, The Netherlands.
4. June 2015: MOSA conference, Maastricht, The Netherlands.
5. June 2015: DaCRA Summer Meeting, Sandjberg Estate, Denmark.