Thesis

Conservation of β2-adrenergic signaling in rat and human cardiomyocytes during heart failure

submitted by

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in partial fulfillment of the requirements for the degree of

Doktorin der gesamten Heilkunde

(Dr.^{in} med. univ.)

at the

Medical University of Graz

executed at the **University Department of Internal Medicine**

Division of Cardiology

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Graz, Date 05.04.2024

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Graz, am 05.04.2024 *Claudia Fernandes Hollnagel eh.*

Acknowledgments

I would like to express my sincere gratitude to my mentor and supervisor, Dr. Senka Holzer, for consistently believing in me and dedicating time to provide constructive feedback throughout my thesis. Special thanks to Rainer Peter for initiating the project idea, supplying essential literature, and offering incentives at the commencement of my experiments.

I am indebted to the Cardio Group, particularly Ingrid Matzer, Julia Voglhuber, and Natasa Djalinac, for introducing me to lab work, offering assistance whenever I had questions, and providing valuable data for my thesis.

Heartfelt appreciation goes to my friends, especially Anna Ammer, Thomas Brauchert (for showing me the BioRender: Scientific Image and Illustration Software program), and Esther Tuffour, for standing by me during times of frustration. Furthermore, I extend special thanks to Korax and Craig P. Teller for sharing insightful information and providing constructive feedback on my writing.

I want to acknowledge the support of my family, including my aunt Heloisa Hollnagel and grandparents Bruno and Neusa Hollnagel, for their interest, shared experiences, and valuable perspectives on my project. Finally, none of my achievements would have been possible without the unwavering support of my parents, Carlos and Rose Hollnagel, who not only allowed me to pursue my studies but also provided steadfast financial and moral support throughout my years of education.

Zusammenfassung in Deutsch (maximal 5000 Zeichen)

Einführung: Die Rolle der beta-2-adrenergen Rezeptoren (β2AR) in der Herzinsuffizienz ist nach wie vor ein aktives Forschungsgebiet. Das duale Verhalten der β2AR-Signaltransduktion, mit sowohl kardioprotektiven als auch kardiotoxischen Effekten, ist noch nicht vollständig verstanden. Darüber hinaus ist die Relevanz von Tiermodellen für die Übertragung auf den Menschen noch nicht ausreichend geklärt.

Material und Methoden: In dieser Arbeit wird die Aufrechterhaltung des β2AR in männlichen Rattenherzen und in männlichen und weiblichen menschlichen Herzen während der Herzinsuffizienz untersucht. Wir verwendeten ein hypertensives Dahl salt-sensitive Rattenmodell mit einer salzangereicherten Diät (HSD, 8% NaCl), um das Verhalten des β2AR genauer zu verstehen. Männliche Dahl salt-sensitive Ratten, die mit einer salzarmen Diät (LSD, 0,3% NaCl) gefüttert wurden, dienten als Kontrollgruppe für das Rattenmodell. Die Rattenherzen wurden sowohl zu einem frühen Zeitpunkt von 12 Wochen als auch zu einem späten Zeitpunkt von 17 oder 19 Wochen gesammelt. Das menschliche Herzzellgewebe wurde von Transplantatspendern erworben, die verschiedene Ausprägungen der Herzinsuffizienz aufwiesen. Die Charakterisierung der Ratten als Herzinsuffizienz mit erhaltener Ejektionsfraktion (HFpEF) erfolgte mittels Gravimetrie und Echokardiographie. Menschliche Probanden wurden anhand der echokardiographischen Daten in die Gruppen HFpEF und Herzinsuffizienz mit reduzierter Ejektionsfraktion (HFrEF) eingeteilt. Die Kardiomyozyten wurden nach der Langendorff-Isolationsmethode gewonnen. Zur Quantifizierung von β2AR wurden Immunzytochemie (ICC), Immunoblotting und Microarray eingesetzt. ICC-Zellen wurden zusätzlich mit dem β-adrenergen Agonisten Isoprenalin stimuliert.

Ergebnisse: (1) β2AR war in den Microarray- und ICC-Analysen des Dahl salt-sensitive Rattenmodells signifikant herunterreguliert, insbesondere am späten Zeitpunkt von 17 Wochen; (2) Ratten-Immunoblots konnten diese Beobachtung nicht reproduzieren; (3) β1AR und β2AR waren in vergleichbaren Ausmaß in 17 Wochen alten Ratten in ICC herunterreguliert; (4) Die ISO-induzierte schnelle Herunterregulation war in HSD Dahl salt-sensitive Ratten beeinträchtigt; (5) Die

Herunterregulation von β2AR wurde in humanen Immunoblots bei diastolischer Dysfunktion, aber nicht bei dilatativer Kardiomyopathie beobachtet.

Diskussion: Wir kommen zum Schluss, dass β2AR, bei Ratten mit hypertonieinduzierter Herzinsuffizienz während des späten kardialen Remodelings, in vergleichbarem Ausmaß wie β1AR herunterreguliert wird. Bei Patient*innen mit diastolischer Dysfunktion war die β2AR-Konzentration im Vergleich zu Patient*innen mit dilatativer Kardiomyopathie und normaler Herzfunktion reduziert. Diese Ergebnisse deuten auf ein spezifisches adrenerges Expressionsmuster hin, das von der Art, dem Stadium und der Ätiologie der Herzinsuffizienz abhängt. Zukünftige Experimente, einschließlich der Übertragung von Experimenten auf menschliches Gewebe und der Analyse nachgeschalteter β2AR-Signalmoleküle, versprechen weitere molekulare Einblicke in den β2AR-Signalweg beim Menschen während der Herzinsuffizienz. Diese Erkenntnisse könnten zu neuen Behandlungsstrategien führen, die auf bestimmte Typen, Stadien und Ätiologie der Herzinsuffizienz zugeschnitten sind und damit zum Paradigma der personalisierten Medizin beitragen würden.

Schlagwörter: β2AR, beta-2-adrenerge Rezeptoren, Herzinsuffizienz, HFpEF, Hypertension, Dahl salt-sensitive Ratten Modell, Immunocytochemie, Immunoblot, Langendorff-Isolationsmethode, Cardiomyocyten, Microarray

Abstract in English (maximal 5000 Zeichen)

Introduction: Beta-2 adrenergic (β2AR) signaling in heart failure remains an area of active investigation, presenting diverse insights. The dual nature of β2AR signaling, exhibiting both cardioprotective and cardiotoxic effects, remains incompletely understood. Moreover, the translational relevance of animal models to human data is yet to be fully established.

Material and Methods: This thesis investigates the preservation of β2AR signaling in male rat and male and female human hearts during heart failure. To discern the intricacies of β2AR behavior, we utilized a hypertensive Dahl salt-sensitive rat model fed a high-salt-diet starting at 7 weeks (HSD, 8% NaCl) for 5 (early remodeling) and 10 to 12 (late remodeling) weeks. The control group comprised male Dahl salt-sensitive rats on a low-salt-diet (LSD, 0.3% NaCl). In addition, we had valuable access to donor and explanted human heart tissue with various degrees of cardiac remodeling. Phenotyping of rats as heart failure with Preserved Ejection Fraction (HFpEF) occurred through gravimetric analyses and echocardiography. Human subjects were categorized into HFpEF and heart failure with Reduced Ejection Fraction (HFrEF) groups based on echocardiography data provided by the Department of Cardiology. Cardiomyocytes were obtained through Langendorff isolation methods. Quantification of β2AR involved Immunocytochemistry (ICC), immunoblotting, and microarray techniques. Cells used for ICC were additionally stimulated with β-adrenergic agonist isoprenaline.

Results: (1) Significant β2AR downregulation, particularly at a late remodeling time point, in the microarray and ICC analyses of the Dahl salt-sensitive rat model; (2) Conversely, rat immunoblots failed to reproduce this observation; (3) Comparable rates of β1AR and β2AR downregulation in rats in the ICC at a late remodeling timepoint; (4) Impaired rapid-ISO-induced β2AR downregulation in HSD Dahl salt-sensitive rats; (5) β2AR downregulation observed in human immunoblot for diastolic dysfunction, but not for dilated cardiomyopathy.

Discussion: We conclude that β2AR downregulation occurs at a comparable rate to β1AR during late-stage cardiac remodeling in rats with systemic hypertension heart failure. In humans, superior downregulation was evident in patients with

diastolic dysfunction compared to dilated cardiomyopathy. These insights suggest a specific adrenergic expression pattern depending on heart failure type, stage, and etiology. Future experiments, including translation to human tissue and analysis of β2AR downstream targets, promise additional molecular insights into β2AR signaling in humans during the development and progression of heart failure. Such insights may inform novel treatment strategies tailored to specific heart failure types, stages, and etiologies, contributing to the paradigm of personalized medicine.

Topic Tags: β2AR, beta-2-adrenergic receptor, heart failure, HFpEF, hypertension, Dahl salt-sensitive rat, Immunocytochemistry, immunoblot, Langendorff-isolation method, cardiomyocytes, Microarray

Information on previous publications

A part of this Thesis has been published in:

Matzer, I. (2021) 'Role of β1-adrenergic signalling in early- and late-stage hypertensive cardiac remodelling, Master thesis submitted at the Master Degree Course Mass Spectrometry and Molecular Analytics for the degree Master of Science in Engineering (MSc)'.

Matzer, I. *et al.* (2022) 'beta-Adrenergic Receptor Stimulation Maintains NCX-CaMKII Axis and Prevents Overactivation of IL6R-Signaling in Cardiomyocytes upon Increased Workload', *Biomedicines*, 10(7), p. 1648. Available at: https://doi.org/10.3390/biomedicines10071648

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Abbreviations

AC ..... *Adenylyl cyclase*
ACE-I..... *Angiotensin-converting enzyme inhibitor*
ADH..... *Antidiuretic hormone*
ARB..... *Angiotensin II receptor blocker*
ARNI..... *Angiotensin receptor-neprilysin inhibitor*
ATP ..... *Adenosine triphosphate*
BCA..... *Bicinchoninic acid*
BDM ..... *Butanedione monoxime*
BMI……...*Body Mass Index*
BNP..... *Brain natriuretic peptide*
BP ..... *Blood pressure*
BSA..... *Bovine serum albumin*
BW ..... *Body weight*
Ca^{2+} ..... *Calcium $^{2+}$ ions*
CamKII ..... *Calmodulin-dependent kinase II*
cAMP..... *Cyclic adenosine monophosphate*
CCB..... *Calcium channel blockers*
CRP..... *C-reactive protein*
DAGK ..... *Diacylglycerol kinase*
DCM ..... *Dilated cardiomyopathy*
E/e' ..... *Mitral velocity/septal velocity*
ECG……..… *Echocardiography*
EF..... *Ejection fraction*
EGFR ..... *Epidermal growth factor receptor*
eNOS ..... *Endothelial nitric oxide synthase*
EPac1..... *Exchange protein directly activated by cAMP 1*
ERK1/2..... *Extracellular signal-regulated kinase 1/2*
ESC..... *European society of cardiology*
GDP ..... *Guanosine diphosphate*
GPCR..... *G-protein-coupled receptors*
GRK ..... *G-protein-coupled receptor kinases*
GRK2 ..... *G-protein-coupled receptor kinase 2*

GTP ..... *Guanosine triphosphate*
HDAC5 ..... *Histone deacetylase 5*
HFmrEF ..... *Heart failure with moderately reduced ejection fraction*
HFpEF ..... *Heart failure with preserved ejection fraction*
HFrEF ..... *Heart failure with reduced ejection fraction*
HSD ..... *High-salt-diet*
ICC ..... *Immunocytochemistry*
IL6R *Interleukine-6-receptor*
IP2 ..... *Inositol-4,5 bisphosphate*
IP3 ..... *Inositol 1,4,5-triphosphate*
ISO ..... *Isoprenaline*
LCC ..... *L-type calcium channels*
LSD ..... *Low-salt-diet*
LVEF ..... *Left ventricular ejection fraction*
MRA ..... *Mineralocorticoid receptor antagonist*
NCX ..... *Sodium/calcium exchanger*
NF ..... *Not failing*
NHERF ..... *Na+/H+ exchange regulation factor*
NT-ProBNP ..... *N-terminal prohormone of brain natriuretic peptide*
NYHA ..... *New York Heart Association*
p38 MAPK ..... *Mitogen-activated protein kinase*
PDEs ..... *Phosphodiesterases*
PI3K-Akt ..... *Phosphoinositide 3-Kinase- serine/threonine kinase*
PI4P *Phosphatidylinositol-4-phosphate*
PIP2 ..... *Phosphatidylinositol 4,5-bisphosphate*
PKA ..... *cAMP dependent protein kinase*
PKC ..... *Protein kinase C*
PKD ..... *Protein kinase D*
PLB ..... *Phospholamban*
PLC ..... *Phospholipase Cβ*
RAAS ..... *Renin-angiotensin-aldosterone-system*
RyR ..... *Ryanodine receptors*
SERCA ..... *Sarco/Endoplasmatic reticulum calcium ATPase*
SGLT2I ..... *Sodium-glucose cotransporter 2 inhibitors*

SR .....*Sarcoplasmatic reticulum*

TBST .....*Tris-buffered saline with Tween 20*

VEGF .....*Vascular endothelial growth factor*

β1AR .....*Beta 1 adrenergic receptor*

β2AR .....*Beta 2 adrenergic receptor*

β3AR……..…….*Beta 3 adrenergic receptor*

βAR .....*Beta adrenergic receptors*

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1. Introduction

For heart failure, understanding the intricacies of beta 2 adrenergic receptor (β2AR) signaling is critical to fully understand its pathophysiology. This signaling pathway presents a complex duality, with both protective and detrimental effects on cardiac function. Yet, despite extensive research efforts, gaps persist in comprehending its role. Against this backdrop, this thesis delves into the conservation of β2AR signaling in rat and human cardiomyocytes during heart failure, aiming to unravel its behavior across species and disease states. In the following clinical, cellular, and molecular aspects of heart failure will be explored, elucidating the rationale behind our investigation, and delineating the objectives of our study.

1.1 Clinical insights

1.1.1 Definition and epidemiology of heart failure

The European Society of Cardiology defines heart failure as a syndrome characterized by various symptoms such as shortness of breath, edema, and fatigue that can be attributed to structural changes in the heart (McDonagh *et al.*, 2021). This condition is progressive, initially presenting symptoms only under stress, but advancing to the point where symptoms occur at rest (Silbernagl *et al.*, 2020).

Heart failure is a prevalent condition, affecting 1-3% of the global adult population. It is associated with a substantial mortality rate, ranging from 50-75% within 5 years (Savarese *et al.*, 2023). In 2010 in Germany, 61.4% of treated heart failure patients were female most likely because of their higher life expectancy (Klauber *et al.*, 2014). It is notably widespread among older individuals; the Spanish PRICE study conducted in 2004/2005 revealed that 16.1% of individuals aged >74 years screened positive for heart failure (Anguita Sánchez *et al.*, 2008). As the population ages, healthcare costs related to heart failure are escalating, reaching up to 25,500€ per patient per year in Germany (Savarese *et al.*, 2023). Due to demographic changes and improved survival rates in acute myocardial infarction, valvular heart diseases, cardiomyopathies, or secondary myocardial conditions, the number of patients with heart failure is expected to continue rising (Arzneimittelkommission Der Deutschen Apotheker *et al.*, 2023).

1.1.2 Types of heart failure, etiology, risk factors

Heart failure can manifest as an acute incident, for example, through acute ischemia or arrhythmias (Severino *et al.*, 2020), (McDonagh *et al.*, 2021) or as a chronic disorder. The affected part of the heart can be described as left, right, or global. Furthermore, it can be categorized based on functional disorders, such as systolic dysfunction with compromised contraction that leads to reduced ejection fraction, diastolic dysfunction with reduced filling capacity and impaired relaxation, or a combination of both (McDonagh *et al.*, 2021).

The etiologies for this clinical syndrome are highly diverse. In the Western hemisphere, predominant causes of heart failure include coronary heart disease and hypertension, along with their comorbidities. The remaining cases develop through various rarer conditions, such as non-ischemic cardiomyopathies, valve disorders, arrhythmias, or diseases of the pericardium. Additionally, toxins such as alcohol or chemotherapies play a role in the development of heart failure (McDonagh *et al.*, 2021), (Arzneimittelkommission Der Deutschen Apotheker *et al.*, 2023).

Heart failure is often associated with various comorbidities, including cardiovascular conditions like atrial fibrillation, coronary heart disease, valve disorders, hypertension, and stroke. It is also linked to other diseases such as diabetes, electrolyte disorders, depression, arthritis, renal disorders, and erectile dysfunction. Considering and treating these comorbidities is essential (McDonagh *et al.*, 2021), (Arzneimittelkommission Der Deutschen Apotheker *et al.*, 2023).

As heart failure is not a distinct disease entity but rather a consequence of one or multiple underlying diseases, risk factors for heart failure are closely linked to their causes. These risk factors include hypertension, coronary heart disease, smoking, alcohol abuse, physical inactivity, obesity, as well as genetic predispositions. Patients with multiple risk factors and nonspecific symptoms, such as weakness that may be attributed to heart failure, should be screened for the condition. (Arzneimittelkommission Der Deutschen Apotheker *et al.*, 2023), (McDonagh *et al.*, 2021).

1.1.3 Symptoms of heart failure

Symptoms of heart failure vary depending on which side of the heart is affected. Left heart failure can cause fatigue and shortness of breath as pulmonary venous pressure increases and cardiac output decreases. This condition may progress to pulmonary edema, leading to dyspnea and respiratory alkalosis (Silbernagl *et al.*, 2020). Right heart failure results in increased systemic venous pressure and peripheral edema, commonly seen as ankle swelling and nocturia. Hepatic hypertrophy and malabsorption are also one of the many consequences of increased fluid in the systemic circulation (Silbernagl *et al.*, 2020).

Symptoms are classified according to the New York Heart Association (NYHA) Classification, where NYHA I denotes mild symptoms, and NYHA IV represents the highest severity of symptoms (Arzneimittelkommission Der Deutschen Apotheker et al., 2023).

1.1.4 Clinical classification and diagnosis of heart failure

To diagnose heart failure, clinical evaluation is essential, including the cardinal symptoms of heart failure, such as dyspnea, ankle edemas, and weakness, along with signs observed during clinical examination and objective evidence of heart dysfunction. Screening involves laboratory parameters involving the hormones brain natriuretic peptide (BNP) and N-terminal prohormone of brain natriuretic peptide (NT-ProBNP), which rise significantly under cardiac stress. Electrocardiography (ECG) may reveal signs of hypertrophy, high filling pressures, disorders in repolarization, or may even appear normal. Figure 1 describes the diagnostic algorithm used by the European Society of Cardiology (ESC) Guidelines of 2021.

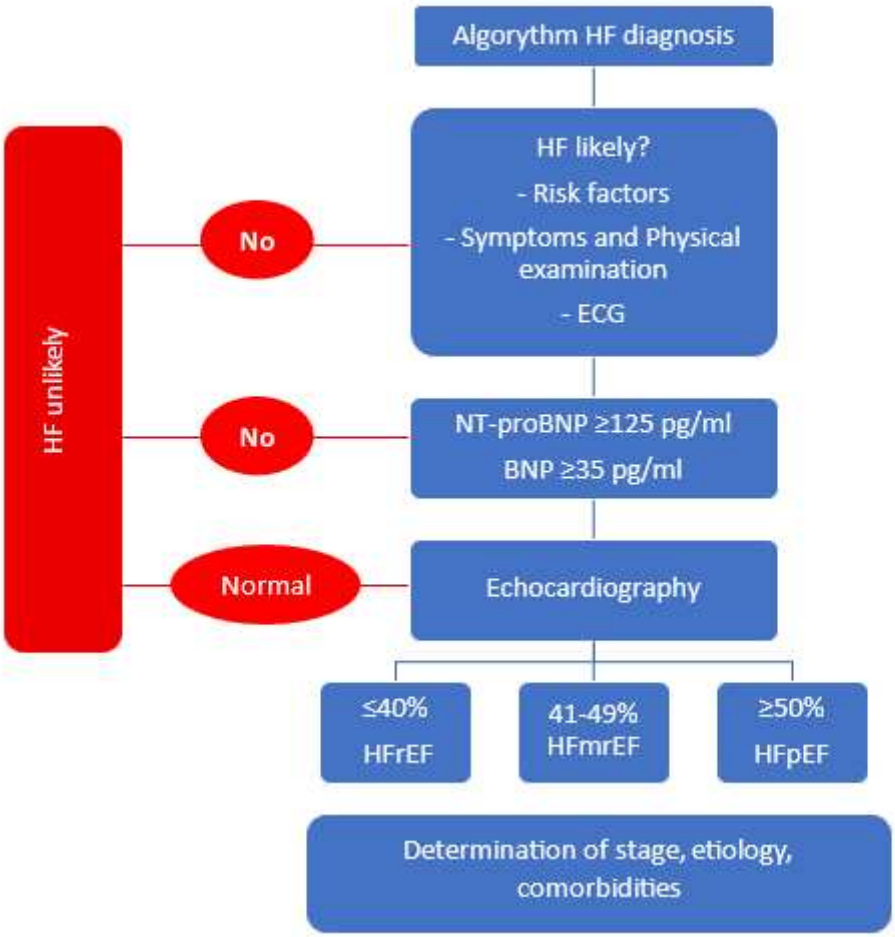

Figure 1: Heart failure diagnostic algorithm

To diagnose heart failure, commence with an anamnesis of risk factors and symptoms, along with a clinical examination. If available, perform an electrocardiogram (ECG). If the gathered information suggests heart failure, conduct screening using NT-proBNP or BNP, markers detecting heart stress. A normal result makes heart failure unlikely, while a positive result necessitates an echocardiography. This diagnostic tool categorizes heart failure into three groups: HFrEF (heart failure with reduced ejection fraction), HFmrEF (heart failure with moderately reduced ejection fraction), and HFpEF (heart failure with preserved ejection fraction). Subsequent therapy and prognosis rely on this categorization. Following the diagnosis, proceed to define the patient's stage, etiology, and comorbidities for optimal care and treatment. The scheme has been adapted and translated from the ESC 2021 Guidelines for acute and chronic heart failure (McDonagh *et al.*, 2021).

If these parameters suggest heart failure, further evaluation can be conducted using echocardiography. Heart failure is classified by measuring the left ventricular ejection fraction (LVEF), indicating the volume the left ventricle ejects per contraction in percent (McDonagh *et al.*, 2021). With this parameter, heart failure is divided into three groups: Preserved ejection fraction (HFpEF) LVEF ≥50%, moderately reduced ejection fraction (HFmrEF) LVEF 41-49%, and reduced ejection

fraction (HFrEF) LVEF ≤40% (McDonagh *et al.*, 2021). About 50% of patients with heart failure have a HFpEF (Zile *et al.*, 2015).

Additionally, diastolic dysfunction (DD) can be quantified through echocardiographic measurements (Pieske *et al.*, 2019). DD refers to a condition marked by irregularities in the relaxation and filling of the heart during the diastolic phase of the cardiac cycle. In essence, diastole denotes the interval when the myocardium loses its capacity to generate force and contract, returning to an unstressed length and force. Diastolic dysfunction manifests when these processes become prolonged, slowed, or incomplete. (Zile and Brutsaert, 2002). Following parameters such as the size of the left atrium (left atrial volume index > 32 ml/m^2), mitral velocity E > 90 cm/s, septal velocity e' < 9 cm/s, and E/e' ratio > 9 have been identified as relevant threshold values for diastolic dysfunction. Exceeding these thresholds is associated with an increased risk of cardiovascular mortality (McDonagh *et al.*, 2021).

1.1.5 Treatment of heart failure

The following treatment regimen is adopted from the 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure (McDonagh *et al.*, 2021).

Heart failure is treated with lifestyle interventions such as rehabilitation, medications, and, as a last resort, device therapy, which encompasses pacemakers and defibrillators, as heart failure is often associated with arrhythmias and cardiac assist devices**.** Drug therapy plays a central role in heart failure treatment and in the following section, the currently available and most commonly used medications for HFrEF and HFpEF will be elaborated.

HFrEF drug therapy, being the most studied entity, is constituted of four pillars (Figure 2) Modulation of the Renin-angiotensin-aldosterone-system (RAAS) and the sympathetic nervous system through an Angiotensin-converting-Enzyme Inhibitor (ACE-I) or an Angiotensin Receptor-Neprilysin Inhibitor (ARNI). A beta-blocker further inhibits the sympathetic nervous system. Thirdly, a Mineralocorticoid Receptor Antagonist (MRA) blocks aldosterone from binding to the mineralocorticoid receptor in the kidneys, inhibiting further water and sodium retention and promoting potassium excretion. Lastly, sodium-glucose co-transporter 2 Inhibitors (SGLT2I) have shown a positive impact on morbidity and mortality in

patients with HFrEF and constitute the final pillar. Loop diuretics are used for symptomatic relief of volume overload, such as peripheral or pulmonary edemas, but are not shown to reduce mortality. If the therapy above proves to be insufficient drugs like Ivabradin or Digitoxin can be given.

Heart failure Drug Therapy for patients with HFrEF

Reduction of mortality			
ACE-I / ARNI	**BB**	**MRA**	**SGLT2I**
Symptomatic relief of volume overload			
LOOP DIURETICS			

Figure 2: Heart failure therapy for patients with HFrEF

This figure illustrates the primary drug pillars of heart failure therapy for patients with HFrEF. A combination of ACE-I/ARNI, BB, MRA, and SGLT2I has been proven to reduce mortality. Loop diuretics are administered to alleviate symptoms of volume overload, such as dyspnea or swelling, but do not reduce mortality rates. ACE-I = Angiotensin-converting-Enzyme Inhibitor, ARNI = Angiotensin Receptor-Neprilysin Inhibitor, BB = Betablocker, MRA = Mineralocorticoid Receptor Antagonist, SGLT2I = sodium-glucose co-transporter 2 Inhibitor. This figure was adapted and translated from the 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure (McDonagh *et al.*, 2021).

Data is scarcer in patients with HFpEF. Consequently, the guidelines are not as elaborated. It is suggested that risk factors, comorbidities, and the cause of heart failure should be the main focus of therapy. Many patients are treated with an ACE-I or an Angiotensin II receptor blocker (ARB), beta-blocker, or MRA because most of them have either coronary heart disease or hypertension. The ARB is also a RAAS inhibitor like ACE-I and is given against hypertension. There is a possibility of SGLT2I becoming a new pillar in the therapy of HFpEF. Dapagliflozin and Empagliflozin are active substances of this group. The “DELIVER” trial demonstrated that Dapagliflozin reduces cardiovascular death and hospitalization in HFmrEF and HFpEF (Solomon *et al.*, 2021). Additionally, the “Emperor Preserved” trial showed that Empagliflozin has a reduction in hospitalization and mortality in patients with HFpEF (Zinman *et al.*, 2015). Symptomatic therapy with loop diuretics is indicated for the same reasons as in HFrEF. These therapeutic

approaches have been implemented in the German heart failure guidelines (Arzneimittelkommission Der Deutschen Apotheker *et al.*, 2023).

There are many new subcellular mechanisms we are finding that counteract the development of cardiac failure. Enhanced comprehension of the molecular biology and pathophysiology underlying heart failure presents an avenue for developing more effective pharmaceuticals and improved disease management.

1.1.6 Pathophysiology of heart failure

The pathophysiology of heart failure starts with cardiac stress, for example through volume and/or pressure overload, or tissue loss. This increases ventricular wall tension, initially leading to alterations in systolic function. The calculation of ventricular wall tension, guided by Laplace's law, involves the interplay between radius, wall thickness, transmural pressure, and tension. In response, the body initiates signals promoting concentric hypertrophy of the ventricles, enhancing systolic function but impairing diastolic function due to reduced end-diastolic volume (Silbernagl *et al.*, 2020).

Additional countermeasures involve neurohumoral responses triggered by a decline in blood pressure. This results in the release of antidiuretic hormone (ADH) and activation of the sympathetic nervous system (Silbernagl *et al.*, 2020). These responses lead to water retention, vasoconstriction, increased heart rate and contractility, and centralization of blood volume. Kidney underperfusion and the sympathetic nervous system activate the RAAS, further promoting water retention (Silbernagl *et al.*, 2020). While these compensatory mechanisms counteract the heart’s increased workload for an extended period, in the long run, they prove non-beneficial and accelerate cardiac remodeling by increasing preload and afterload.

Atrial natriuretic peptide and BNP are released in response to elevated atrial and ventricular pressures, increasing water excretion to compensate for volume overload (Silbernagl *et al.*, 2020). These hormones are commonly used to diagnose heart failure (McDonagh *et al.*, 2021).

Mechanical and neurohumoral stressors induce myocardial remodeling through various signaling molecules and growth factors, leading to myocardial hypertrophy

and decreased sympathetic sensitivity. Furthermore, this remodeling results in a weak cardiomyocyte action potential and a reduction in resting potential elevating the risk of arrhythmias (Silbernagl *et al.*, 2020).

1.1.7 Heart failure due to hypertension

1.1.7.1 Epidemiology and etiology

This thesis specifically focuses on myocardial disease and heart failure resulting from hypertension, so an emphasis will be put on this topic. Hypertension stands out as a primary cause of heart failure and myocardial infarction in the western world. The global prevalence of hypertension is anticipated to surpass 1.5 billion people by 2025 (Slivnick and Lampert, 2019), (Kearney *et al.*, 2005). The development of hypertension is a complex phenomenon unique to each patient, encompassing factors such as increased sympathetic nervous system activity, long-term high sodium intake, overproduction of sodium-retaining hormones and vasoconstrictors, diabetes, inappropriate renin secretion, vasodilator deficiencies, vessel abnormalities, and alterations in the kallikrein-kinin system (Elliott, 2007).

1.1.7.2 Pathophysiology

Hypertensive cardiomyopathy emerges as a significant end-organ damage triggered by hypertension, manifested as ventricular hypertrophy that, over time, leads to diastolic dysfunction and, in the end phase, systolic dysfunction (Phillips and Diamond, 2001), (Nadruz, 2015). This cardiomyopathy affects 20% of patients with mild hypertension and nearly 100% of patients with severe hypertension, imposing a substantial socio-economic burden on healthcare (Ruilope and Schmieder, 2008). Additionally, the Framingham study, spanning over 14 years and following more than 5000 patients, reveals that 91% of patients with newly developed heart failure had underlying hypertension (Levy *et al.*, 1996).

Furthermore, hypertension is intricately linked to other cardiovascular diseases such as coronary heart disease, stroke, atrial fibrillation, and peripheral vascular diseases (Slivnick and Lampert, 2019). These connections negatively impact the development of heart failure. For instance, hypertension induces endothelial dysfunction, exacerbating the atherosclerotic process in coronary heart disease. Left ventricular hypertrophy in hypertensive cardiomyopathy reduces coronary

reserve and increases myocardial oxygen demand, both contributing to myocardial ischemia that further worsens heart failure (Escobar, 2002).

Although several stages have been consistently observed, understanding how hypertension leads to hypertensive cardiomyopathy and subsequently heart failure remains incomplete (Figure 3). In the early stages, hypertensive patients may develop left ventricular hypertrophy, either concentric or eccentric, without symptoms but with elevated filling pressures. However, only 23-48% of hypertensive patients reach this stage (Devereux *et al.*, 1987). Later symptoms may progress to HFpEF and, in a subset of patients, a reduced ejection fraction may develop. The reasons why only a subset of hypertensive patients develop HFrEF are not fully understood. The clinical study by Salas-Pacheco et al. suggests that the extent of hypertrophy determines the development of systolic dysfunction, and LVEF is an insensitive method to determine systolic dysfunction in hypertensive patients (Salas-Pacheco *et al.*, 2022). Patients with an eccentric hypertrophy phenotype have a higher probability of developing HFrEF presenting with dilated cardiomyopathy (Velagaleti *et al.*, 2014). In their review article, Borlaug and Redfield propose that a second hit, such as an infarction, cardiotoxic substances like drugs or medication, infection, or inflammation, damages the heart and leads it from HFpEF to HFrEF (Borlaug and Redfield, 2011). When patients reach HFrEF, the mortality rate is significantly higher (Frazier *et al.*, 2007). The variability in disease progression may be caused by differences in pressure and volume loads, neurohormonal status, genetic basis, and cardiovascular risk factors such as diabetes, obesity, and smoking (Palmiero *et al.*, 2014).

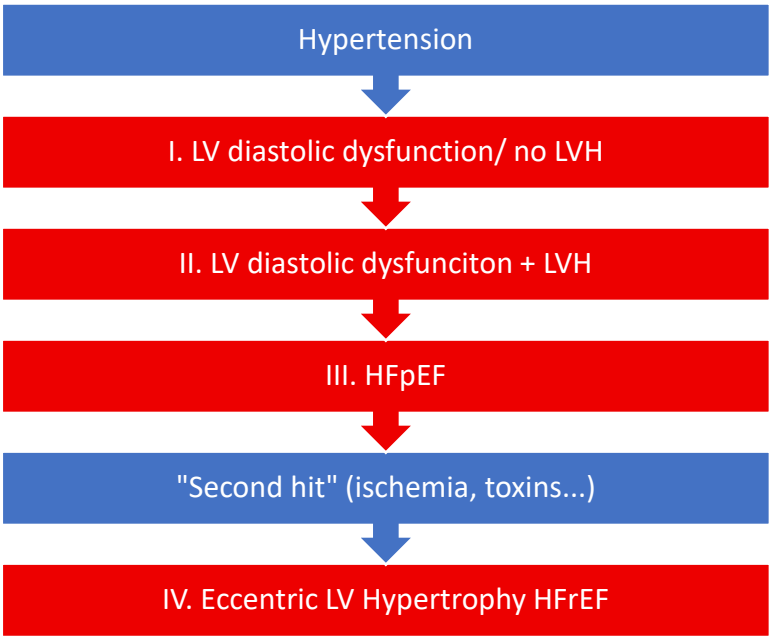

Figure 3: Development of hypertensive cardiomyopathy

Flow chart illustrating the potential development of heart failure due to hypertension. Patients with hypertension may progress through various stages of cardiac remodeling, potentially leading to heart failure, although this progression is not obligatory. The reasons behind why some patients undergo these stages while others do not are still under research. Slivnick and Lampert propose a second hit, such as ischemia, inflammation, or exposure to cardiotoxic substances, which may contribute to hypertensive patients with HFpEF transitioning to HFrEF. LV = Left ventricular, LVH = Left ventricular hypertrophy, HFrEF = heart failure with reduced ejection fraction. This figure was adapted from the Review “Hypertension and Heart Failure” (Slivnick and Lampert, 2019).

1.1.7.3 Treatment

Effectively treating hypertensive cardiomyopathy involves the prevention and treatment of hypertension before the onset of cardiac remodeling (Slivnick and Lampert, 2019). This can be achieved through ACE-I, ARB, dihydropyridine calcium channel blockers (CCB), and thiazide diuretics (Whelton and Carey, 2017). During left ventricular hypertrophy, antihypertensive medications are—at least in part—able to reverse the phenotype (Slivnick and Lampert, 2019). In cases where heart failure manifests, the treatment approach should align with established heart failure guidelines (see 1.1.5). A blood pressure target of 130/80 should be attained (Cushman *et al.*, 2016). Furthermore, prioritizing the prevention of coronary heart disease in individuals with hypertension is of utmost importance. This goal can be effectively pursued by initiating a combined regimen involving antiplatelet agents, lipid-lowering medications, and antihypertensive drugs (Slivnick and Lampert, 2019).

1.2 Cellular and molecular insights

In the following section, I will explain various mechanisms that occur during the development and presentation of heart failure at the cellular and subcellular levels, that are already within our understanding.

1.2.1 Hypertensive cardiac remodeling

Hypertensive heart disease leading to heart failure can be explained by cardiac remodeling—a complex restructuring involving cellular and subcellular alterations in the heart. This encompasses processes such as cardiomyocyte hypertrophy, interstitial inflammation, cardiomyocyte apoptosis, interstitial fibrosis, reorganization of the cellular matrix, changes in receptor expression, and reduced capillarization (Figure 4). These cellular changes lead to ventricular dilation, dysfunctional filling, altered metabolic homeostasis, and reduced contractility (González, Ravassa, *et al.*, 2018), (Martin *et al.*, 2012). Cardiac remodeling already starts in the compensated stage of heart failure where symptoms on the level of the whole organism are not yet present. While β-adrenergic signaling plays a crucial role in the heart's adaptation to external stressors, chronic stimulation of these receptors plays a pivotal role in cardiac remodeling and the development of heart failure.

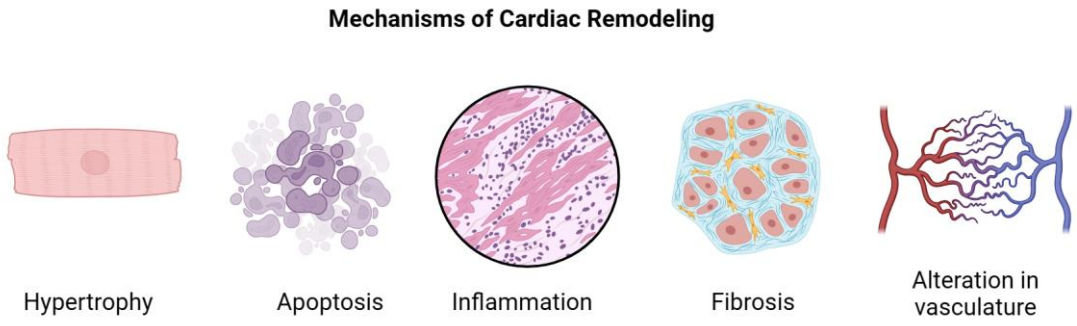

Figure 4: Mechanisms of Cardiac Remodeling

This figure provides an overview of mechanisms involved in cardiac remodeling. This process occurs as a response to a cardiac stressor, encompassing cardiac hypertrophy, apoptosis, inflammation, fibrosis, and alterations in vasculature. The figure has been adapted from the paper "Myocardial Remodeling in Hypertension" (González, Ravassa, *et al.*, 2018) and was created using BioRender.com.

1.2.1.1 Hypertrophy

Pressure overload, a stressor for the heart, induces protein synthesis in cardiomyocytes, which enlarges intracellular sarcomeres, leading to cell growth (Sugden and Clerk, 1998). Neurohumorally, pressure overload triggers a response involving angiotensin II (ATII) (Lijnen and Petrov, 1999) and catecholamines through activation of the sympathetic nervous system (Zimmer, 1997). Additionally, this stressor leads to the release of growth factors and cytokines by non-cardiomyocytes such as fibroblasts, vascular cells, and blood cells. These factors contribute to the development of hypertrophy (Kamo, Akazawa, and Komuro, 2015). The exact mechanisms by which the heart senses strain is not fully understood. Hypotheses include stretch-sensitive ion channels and other membrane-bound and internal stretch sensors (McCain and Parker, 2011).

Cardiomyocyte hypertrophy further induces genetic changes that disrupt various cellular processes including metabolism, contraction, cytoskeleton, and membrane properties (González, Ravassa, *et al.*, 2018). Metabolic alterations associated with cardiomyocyte hypertrophy include reduced fatty acid oxidation, increased glucose utilization, dysfunction of the mitochondrial electron transport chain, and reduced adenosine triphosphate (ATP) production, crucial for both systolic and diastolic function (Tuomainen and Tavi, 2017), (Neubauer, 2007), (Facundo *et al.*, 2017).

Furthermore, at myocyte level increased microtubule density initially leads to improved contractility and left ventricular mass (Tagawa *et al.*, 1996). Later on, derangements in calcium handling, microtubule disarray, and hyperphosphorylation of titin protein lead to increased cardiomyocyte stiffness (Shah *et al.*, 2014), (Zile *et al.*, 2015).

1.2.1.2 Pressure overload-induced Apoptosis

As cells hypertrophy, there is a dysregulation of protein synthesis and processing in the endoplasmic reticulum. Such dysregulation results in an accumulation of unfolded proteins that can cause dysfunction and therefore trigger apoptosis—the process of programmed cell death (Dickhout, Carlisle, and Austin, 2011), (Sun *et al.*, 2008). The increase in apoptosis can lead to left ventricular dysfunction, manifested by reduced systolic function and chamber dilation. This outcome can be

attributed, in part, to the reduction in ventricular mass and the activation of various mechanisms during apoptosis. These mechanisms can influence cardiomyocyte function even before their demise, such as the impairment of ATP production (Narula *et al.*, 2001). In addition to apoptosis, other forms of cell death, including necrosis, i.e. unplanned cell death, and excessive autophagy, i.e. intracellular recycling of cellular components, are induced by cardiac injury and hypertension contributing to myocardial remodeling (Wang, Rothermel and Hill, 2010), (Whelan, Kaplinskiy and Kitsis, 2010).

1.2.1.3 Inflammation

There is accumulating evidence that indicates hypertensive cardiac injury may be caused by inflammation (Kvakan, Luft, and Muller, 2009), (McMaster *et al.*, 2015). Animal models suggest that hypertension-induced inflammation is triggered through neurohumoral factors such as ATII (Kvakan, Luft, and Muller, 2009), aldosterone (Yoshida *et al.*, 2005), mineralocorticoids (Ogata *et al.*, 2004) and the sympathetic nervous system (Levick *et al.*, 2010). These models demonstrate increased capillary wall permeability, elevated secretion of cytokines and chemokines, and infiltration of inflammatory cells into the myocardium. Another pathomechanism involves inflammation due to cardiomyocyte injury resulting from pressure overload in hypertension. When cardiomyocytes are damaged, the release of molecules (e.g., DNA fragments, heat shock proteins) prompts healthy cardiomyocytes to produce inflammatory mediators, leading to leukocyte activation and recruitment (Ghigo *et al.*, 2014). In experimental models and clinical trials, an imbalance in T effector and regulatory lymphocyte subsets, key cell components of the immune system, has been observed during hypertension, contributing to low-grade inflammation and end-organ damage (Idris-Khodja *et al.*, 2014). Moreover, hypertensive patients not only experience local inflammation but also manifest an increase in systemic inflammatory markers such as the C-reactive protein (CRP) (Hage, 2014) and CXCR3, triggering T cell homing. This homing mechanism directs T cells to migrate to the required tissue, in this case, the myocardium (Youn *et al.*, 2013).

All of these inflammatory signals, in combination with changes to the extracellular matrix, drive the activation and differentiation of fibroblasts, which then regulate inflammation and promote fibrosis (Shinde and Frangogiannis, 2017). Systemic inflammation induces endothelial oxidative stress, reducing myocardial nitric oxide

availability, resulting in cardiomyocyte stiffness and hypertrophy (Franssen *et al.*, 2016).

1.2.1.4 Fibrosis

The accumulation of type I and type III collagen fibers in the interstitium and perivascular space is a characteristic feature of cardiac fibrosis (Berk, Fujiwara, and Lehoux, 2007). The pathogenesis of fibrosis involves the differentiation of fibroblasts into myofibroblasts, which synthesize procollagen, various enzymes, cytokines, and growth factors promoting fibrosis (Shinde and Frangogiannis, 2017). This response can be triggered by the stretching of the myocardial wall, the replacement of small areas of necrosis/apoptosis, as well as hormones such as the RAAS, and chemical factors such as reactive oxygen species (González, Schelbert, *et al.*, 2018). Enzymes called membranometalloproteases may play a key factor in increased collagen turnover during fibrotic remodeling (Ahmed *et al.*, 2006). Fibrosis is associated with left ventricular dysfunction as it impairs relaxation and diastolic filling and, in later stages, compromises systolic performance (Brower *et al.*, 2006), (López *et al.*, 2006). Additionally, it may also compromise coronary blood flow by compressing intramural arteries and induce arrhythmias by promoting local re-entry mechanisms through conduction abnormalities and the interaction of cardiomyocytes with myofibroblasts (Schwartzkopff *et al.*, 1993), (Dai *et al.*, 2012), (McLenachan and Dargie, 1990), (Rohr, 2012).

1.2.1.5 Change in Myocardial perfusion and Oxygen supply

Hypertension is linked to alterations in the macrocirculation, such as coronary artery disease (Tackling and Borhade, 2023). Additionally, it induces changes in the coronary microvasculature, leading to arterial stiffness, reduced diastolic myocardial perfusion pressure, and increased susceptibility to ischemia and end-organ damage (Feihl *et al.*, 2008),(François, Liaudet, and Waeber, 2009).

Furthermore, the arterial medial wall thickness-to-lumen ratio increases. In this context, the sympathetic nervous system plays a role in the hypertrophy of smooth muscle in the vasculature; while α1-adrenergic activation leads to growth, β1-adrenergic stimulation leads to atrophy of these cells (O'Callaghan and Williams, 2002). Furthermore, myocardial vascular density decreases, potentially due to dysfunction in so-called pericytes situated in close proximity to the vasculature.

These pericytes play a crucial role in angiogenesis and fibrosis (Avolio and Madeddu, 2016). One significant factor is the interplay between high pulsatile blood pressure in hypertension combined with relatively low vascular resistance (Feihl *et al.*, 2008). Another point to consider is the increased fibrotic tissue in the perivascular area that extends the oxygen diffusion distance, leading to impaired oxygen delivery to the cardiomyocytes (Frohlich, 2001). Lastly, hypertension can damage the endothelium, a critical regulator of microvascular tone, resulting in compromised coronary vasodilation (Konukoglu and Uzun, 2017). Collectively, these changes contribute to a reduction in coronary flow reserve, which is associated with left ventricular systolic and diastolic dysfunction (Galderisi *et al.*, 2002).

1.2.2 Adrenergic signaling in the heart

Hypertension triggers multiple mechanisms that alter the microstructure of the heart, leading to functional impairments. One key neurohumoral pillar in this process is the sympathetic nervous system, specifically adrenergic signaling. As explained above, chronic overstimulation of adrenergic signaling contributes to cardiac remodeling by promoting hypertrophy, inflammation, apoptosis, fibrosis, and influencing angiogenesis (de Lucia *et al.*, 2014). That is why the blockade of β-adrenergic signaling through β-blockers is one of the main pillars of heart failure therapy (McDonagh *et al.*, 2021).

The upcoming section will provide a more in-depth exploration of adrenergic signaling mechanisms, detailing the various receptors, their role in cardiac function, and the alterations observed in adrenergic signaling during heart failure, placing special emphasis on the beta 2 adrenergic receptor (β2AR).

Adrenergic receptors are receptors that react to the hormones released by the sympathetic nervous system, such as adrenalin (epinephrin) from the adrenal glands and noradrenalin (norepinephrine) from the postganglionic nerve endings and adrenal glands. These hormones play a crucial role by interacting with diverse cells in various organs, triggering the renowned "fight or flight" reaction (Romero, 2010).

There are different types of adrenergic receptors expressed in the heart, with α receptors mostly in the vasculature of the heart and β receptors in the myocardium. These are divided into β1, β2, β3, α1A, and α1B adrenergic receptors (Romero, 2010).

Beta adrenergic receptors (βAR) are the primary receptors in the heart, with beta 1 adrenergic receptors (β1AR) being the predominant subtype, constituting about 70-80%, followed by β2ARs at approximately 20-30% in human hearts (Bristow *et al.*, 1986). α1 receptors account for approximately 20% of the adrenergic receptors in the heart (Woodcock *et al.*, 2008). β3 receptors are nearly non-expressed; however, de Lucia suggests they might have a role in cardiac function, and remodeling, as well as in cardiac diseases (de Lucia, Eguchi and Koch, 2018).

1.2.2.1 Molecular mechanisms of GPCR

α and βARs are G-protein-coupled receptors (GPCR) located in the cell membrane. GPCRs are involved in most of the heart's physiological processes, including contraction, growth, apoptosis, hypertrophy, and fibrosis. These receptors consist of a perceiving extracellular N-terminus that interacts with the specific hormone and an intracellular C-terminus that mediates the intracellular response.

A contributing unit in the intracellular response is the G-protein subset of the GPCR receptor, the so-called heterotrimeric guanine nucleotide-binding protein, composed of α, β, and γ subunits. When bound to the activated GPCR, the α subunit releases a guanosine diphosphate (GDP) molecule, takes up a guanosine triphosphate (GTP) molecule, and dissociates from the G-protein. The free α subunit and bound βγ subunits then modulate the downstream effectors. The cell recycles the α subunit by hydrolyzing GTP to GDP, thereby inactivating it, and then the G-protein subunit is bound to the GPCR (Martin *et al.*, 2012) (Figure 5).

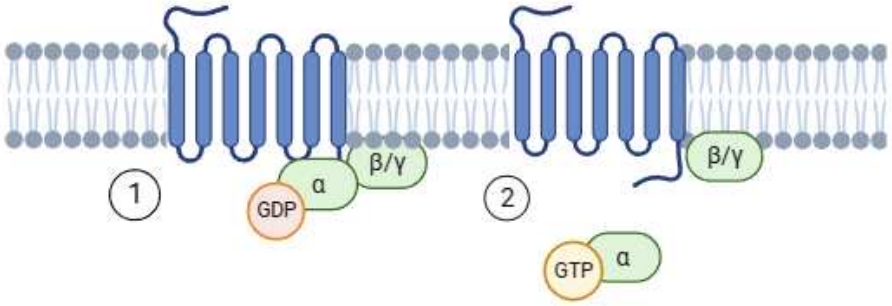

Figure 5: G-protein-coupled receptor (GPCR) with an N- and C-terminus and G-protein in its subunits

This figure illustrates the structure of a GPCR receptor embedded in a cell membrane, featuring its extracellular N-terminus for communication with its effector molecule and the intracellular C-terminus interacting with the G-protein subunits. 1) Depicts the GPCR in the inactivated state, where α and βγ subunits are attached to the receptor, and the α subunit is bound to a GDP molecule. 2) Portrays the GPCR in its activated state, with the α subunit incorporating a GTP molecule and dissociating from the receptor. Created with BioRender.com and adapted from (Martin *et al.*, 2012).

The response to the sympathetic stimulus varies due to different G-protein types, which can be made up of different α, β, and γ subunits. These families of G-proteins are categorized into Gαs, Gαi, Gαq, and Gα12/13 (Martin et al., 2012). The β1AR interacts with Gαs proteins and β2AR binds with both Gαs and Gαi. The beta 3 adrenergic receptors (β3AR) interact with Gαi while α1A and α1B adrenergic receptors interact with Gαq proteins (Martin *et al.*, 2012), (Motiejunaite, Amar and Vidal-Petiot, 2021), (Xiao, Ji and Lakatta, 1995).

Gαs proteins activate adenylyl cyclase (AC), which generates the messenger cyclic AMP (cAMP) from ATP. Gαi proteins on the other hand reduce adenylyl cyclase activity. cAMP stimulates cAMP-dependent protein kinase (PKA). This enzyme can phosphorylate and regulate various downstream targets in the cardiomyocyte, including L-type calcium channels (LCC), ryanodine receptors (RyR), phospholamban (PLB), and troponin I. These regulatory actions result in an increased influx of Calcium^{2+} ions (Ca^{2+}) through LCCs, enhanced Ca^{2+} reuptake facilitated by Sarco/Endoplasmic reticulum calcium ATPase (PLB/SERCA) activity, reduced Ca^{2+} gating of RyRs, and modulation of myofilament Ca^{2+} sensitivity of troponin I. These combined effects lead to heightened contractility and improved cardiac output.

α receptors linked to Gαq activate phospholipase Cβ (PLC). This enzyme cleaves phospholipids, specifically phosphatidylinositol 4,5-bisphosphate (PIP2), generating inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 leads to Ca^{2+} release from the sarcoplasmic or endoplasmic reticulum, and in conjunction with DAG, it activates Protein Kinase C (PKC). In the case of α receptors, this signaling cascade results in vasoconstriction in vascular cells and the regulation of cardiomyocyte contractility in cardiomyocytes (Martin *et al.*, 2012) (Figure 6).

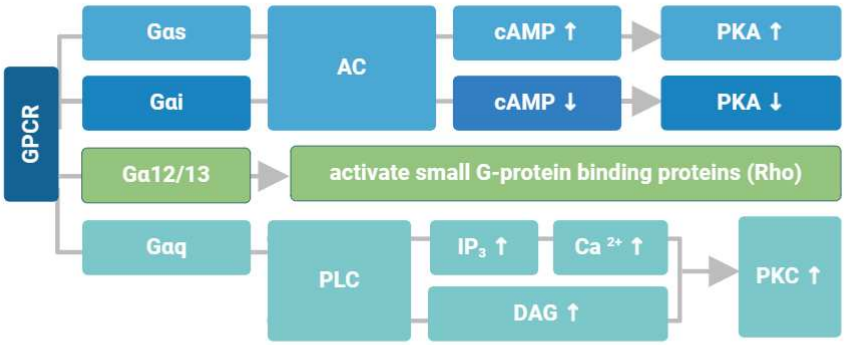

Figure 6: Overview of G-protein subsets and effects

This graphic provides an overview of the G-protein-coupled receptor (GPCR) and the potential G-proteins with their respective effects. Gαs triggers an increase in Protein Kinase A (PKA) through cyclic adenosine monophosphate (cAMP), generated by the activation of adenylyl cyclase (AC). Conversely, Gαi reduces PKA levels by inhibiting AC. Gαq induces a rise in Protein Kinase C (PKC) through a series of steps initiated by phospholipase Cβ (PLC) activation, leading to a cascade effect that elevates calcium, diacylglycerol (DAG), and inositol 1,4,5-trisphosphate (IP_3). Gα12/13 activates small G-protein binding proteins. These variations in protein levels within the cell result in diverse effects impacting metabolism, cell growth, and more. Adapted from the book "Muscle: G-Protein-Coupled Receptors in the Heart" (Martin *et al.*, 2012) Created with BioRender.com.

βAR can be deactivated through G-protein-coupled receptor kinases (GRK), which phosphorylate agonist-activated GPCRs. This phosphorylation recruits β-arrestins, uncoupling the GPCR from the G-proteins and promoting internalization and downregulation of the receptors (de Lucia et al., 2018). Downstream, β-arrestins can terminate signaling by recruiting phosphodiesterases (PDEs) and diacylglycerol kinase (DAGK), contributing to the breakdown of second messengers such as cAMP (Shenoy and Lefkowitz, 2011) (Figure 7).

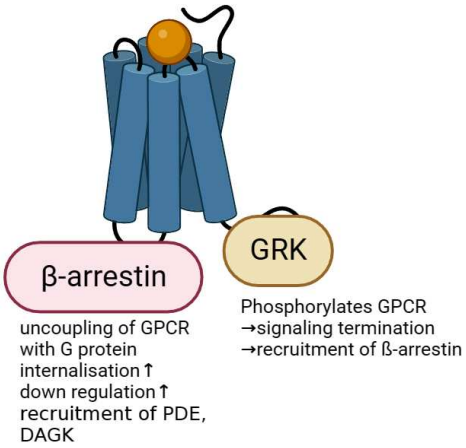

Figure 7: Deactivation of GPCR signals

This figure outlines the steps involved in the deactivation of GPCR signals. G-protein-coupled receptor kinase (GRK) and β-arrestin are key players in this process. GRK phosphorylates the GPCR, terminating its signal, and recruits β-arrestin. Subsequently, these proteins decouple the GPCR from its G-protein, internalize the receptor, and promote gene downregulation. Additionally, they recruit phosphodiesterase (PDE) and diacylglycerol kinase (DAGK) to break down second messengers crucial for GPCR signals. Created with BioRender.com.

Another form of downregulation is heterologous desensitization. It desensitizes a GPCR by the activation of another GPCR, without the need for phosphorylation of the former GPCR by GRKs (Woo *et al.*, 2015).

The outcomes of these signaling cascades depend on various factors, including the cellular localization of the receptors (Gorelik *et al.*, 2013), the presence of scaffolding proteins creating subcellular compartments, the types of signaling molecules present, and the extent and duration of receptor stimulation (Tilley, 2011). Additionally, it is essential to consider the high degree of simultaneous signals occurring within a cell at any given moment. The addition of these complex signals and pathways contributes to the ultimate cellular response (Martin *et al.*, 2012).

1.2.2.2 β1 physiology

The β1 receptors are primarily concentrated in the heart, with an additional presence in the kidneys, eyes, and lungs (Khan, 2006). In cardiac tissue, these receptors play a pivotal role as the primary adrenergic receptors expressed in cardiomyocytes (*cellxgene*, 2024). Their distribution concentrates evenly on the plasma membrane, including the caveolin 3-enriched and other plasma membrane fractions (Rybin *et al.*, 2000), Golgi apparatus (Nash *et al.*, 2019), nuclear envelope (Boivin *et al.*,

2006), and the sarcoplasmic reticulum (Wang *et al.*, 2021). Cardiomyocytes employ the organic cation transporter family of proteins 3 (OCT3 proteins), (Wang *et al.*, 2021) to facilitate the stimulation of intracellular β1 receptors with norepinephrine. β1AR demonstrates a binding affinity to norepinephrine that is tenfold higher than that of β2AR, while having an equivalent affinity to epinephrine (Xu *et al.*, 2021).

Evidence points in the direction that the location of β1ARs affects the respective response upon stimulation. Plasma membrane-activated β2ARs produce a global cAMP signal (Yang *et al.*, 2020). On the other hand, Golgi-localized β1 receptors cAMP signals induce phosphatidylinositol-4-phosphate (PI4P) hydrolysis by phospholipase C (PLC), a response not accessed by cell surface β1 receptors (Nash *et al.*, 2019). This PI4P hydrolysis in the Golgi further triggers the release of diacylglycerol (DAG) and inactive inositol-4,5-bisphosphate (IP_2), facilitating nuclear protein kinase D (PKD) and hypertrophic downstream signaling (Zhang *et al.*, 2013).

Physiologically, their activation leads to chronotropic (increase in heart rate), inotropic (increase in contractility), and lusitropic (enhancement of relaxation) effects, contributing to the overall regulation of cardiac function. The chronotropic effect is elucidated through the activation of PKA in pacemaker cells, resulting in PKA-mediated phosphorylation of membrane ion channels and Ca^{2+} handling proteins. This process enhances the Ca^{2+} cycling rate (Woo and Xiao, 2012). In addition, β1 receptors play a role in activating the RAAS (Blumenfeld *et al.*, 1999), (van Zwieten and de Jonge, 1986).

1.2.2.3 β1 pathophysiology during failure

During heart failure, the balance between β1ARs and β2ARs undergoes a shift from approximately 80:20 to 60:40 due to the downregulation of β1AR (Bristow *et al.*, 1989). The mechanisms implicated in βARs signaling are summarized in Figure 8. This alteration in βAR signaling is also characterized by RyR hyperphosphorylation, decreased PLB phosphorylation, and β1AR desensitization (Hartupee and Mann, 2017). Although the initial desensitization of β1AR is considered an adaptive response to counteract chronic norepinephrine overstimulation, it subsequently contributes to further cardiac remodeling and the progression of heart failure.

Internalization and desensitization of GPCR, evident in both human and animal models, are mediated by G-protein-coupled receptor kinase 2 (GRK2) (Ungerer *et al.*, 1994). Initially, GRK2 upregulation is triggered by sympathetic nervous system hyperactivity, acting as a countermeasure against excessive catecholaminergic drive. However, prolonged elevated levels of GRK2 ultimately result in dysfunctional β-AR signaling and a decline in contractility and inotropic reserve (Penela *et al.*, 2006), (Ribas *et al.*, 2007), (Giuseppe Rengo, Perrone-Filardi, *et al.*, 2012), (Sato *et al.*, 2015), (Zhai *et al.*, 2022). The pivotal role of GRK in the development of heart failure and cardiac remodeling has been consistently demonstrated across various models, encompassing both human (Montó *et al.*, 2012), (Dzimiri *et al.*, 2004), (Leineweber *et al.*, 2005), (Agüero *et al.*, 2012) and animal studies (Choi *et al.*, 1997), (Shah *et al.*, 2001), (Tevaearai *et al.*, 2001), (Rengo *et al.*, 2009), (Raake *et al.*, 2008). Additionally, the upregulation of GRK2 emerges as a common event in hypertension (Gros *et al.*, 1997).

The β1AR downregulation reinforces β2AR Gαi signaling, inhibiting β1AR-mediated contractile responses. Consequently, there is a reduced ability to initiate Ca^{2+} release from the sarcoplasmic reticulum (SR), resulting in a disruption of Excitation-Contraction Coupling (ECC), particularly evident under conditions of intensified workload (W. Zhu *et al.*, 2005). However, this concept has been challenged by Xiao et al., who demonstrated that Gαi selectively inhibits the inotropic effect of β2AR without interfering with β1AR signaling. (Xiao *et al.*, 2003)
β1AR stimulation through Gαs signaling also activates extracellular signal-regulated kinase 1/2 (ERK1/2) via the Gβγ subunit, which can induce hypertrophic remodeling (Vidal *et al.*, 2012).

A study demonstrated that only β1AR, with the assistance of β-arrestin, can activate Ca^{2+}/calmodulin-dependent kinase II (CamKII) with cAMP through a PKA-independent mechanism when chronically stimulated (Zhu *et al.*, 2003). CamKII is known to stimulate apoptosis in cardiomyocytes (Zhu *et al.*, 2007), induce hypertrophy (Ljubojevic-Holzer *et al.*, 2020), and promote arrhythmias (van Oort *et al.*, 2010), (Bengel *et al.*, 2021). It also induces cardiac remodeling (Ling *et al.*, 2009), (Singh and Anderson, 2011) and initiates a fetal gene program in the context of heart failure (Woo and Xiao, 2012). While fetal gene programs are activated in

response to pathological conditions and are meant to initially be adaptive, sustained activation of fetal genes is associated with remodeling.

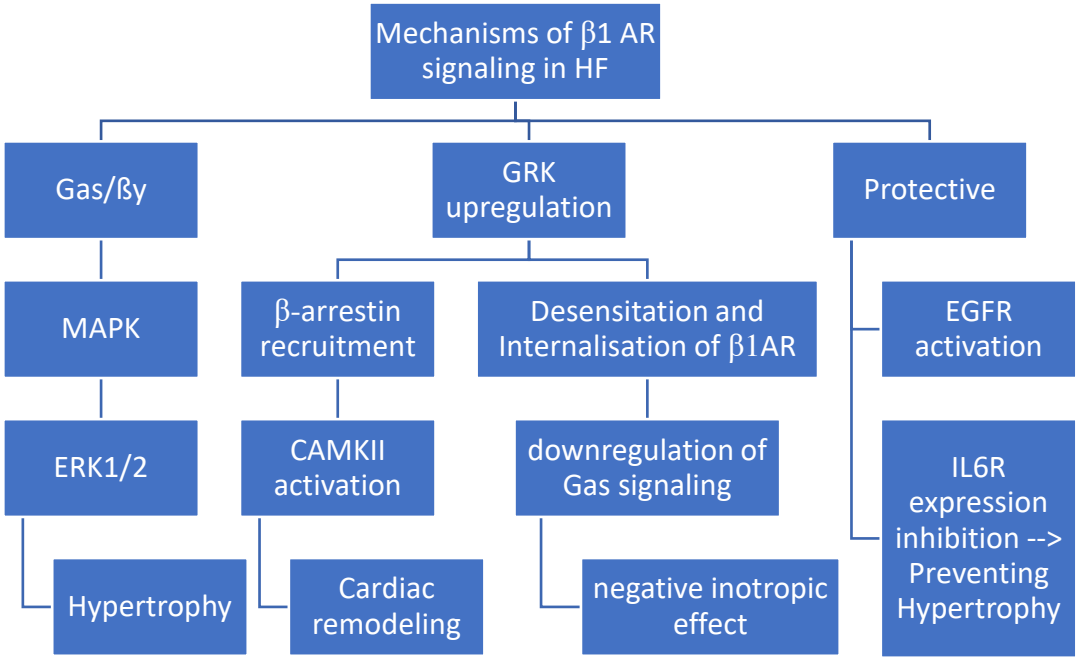

Figure 8: Mechanisms of β1AR signaling in heart failure

The following overview explains the effects of β1AR in cardiomyocytes. During cardiac stress, β1AR can induce hypertrophy through the MAPK-ERK 1/2 pathway. Moreover, under cardiac stress conditions, upregulation of GRK proteins results in the desensitization of β1AR, leading to a reduction in ß1AR-dependent Gas signaling and subsequently, a decrease in contractility. GRK proteins also recruit β-arrestins, activating the β1AR-dependent CAMKII pathway that promotes cardiac remodeling. Conversely, two cardioprotective mechanisms of β1ARs have been identified: EGFR activation and a pathway that inhibits IL6R overexpression, preventing hypertrophy.

Interestingly, treatment with a β1AR-blocker does not affect phospho-CaMKII and CaMKII expression levels in experimental and human heart failure (Dewenter *et al.*, 2017). Consequently, the uncoupling of β1AR-CaMKII signaling during cardiac remodeling and the initiation of the transition to heart failure remains unclear.

Moreover, a study suggests that β1-adrenergic signaling may play a protective role in cardiac remodeling by preventing IL6R overactivation and sodium/calcium exchanger (NCX) dysregulation when stimulated in combination with tachycardia, which is associated with hypertrophy (Matzer *et al.*, 2022). Furthermore, β1ARs have been found to transactivate receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR). This transactivation, mediated by β-arrestins and

involving GRK5 and GRK6, has shown a cardioprotective role (Noma *et al.*, 2007), (Patel, Tilley and Rockman, 2008).

1.2.2.4 β2AR physiology

The β2AR was the first GPCR to be cloned and sequenced in 1986 (Dixon *et al.*, 1986). It is predominantly expressed in the bronchi, vascular smooth muscle, insulin-secreting tissues of the pancreas, gastrointestinal tract, and, to a limited extent, in the heart and coronary arteries (Khan, 2006).

The study by Bristow et al. analyzed whole heart tissue, stating about 20% of βARs are β2AR (Bristow *et al.*, 1986). A study that examined isolated cardiomyocytes reported a minor fraction (5%) of β2AR in healthy mice (Myagmar *et al.*, 2017). This aligns with data from the human heart atlas, which explores mRNA patterns of different proteins in the heart. There, β2AR was primarily expressed in endothelial cells and lymphoid cells, with a small portion in cardiomyocytes and myeloid cells (*cellxgene*, 2023), (Kanemaru *et al.*, 2023). At the organ level, the expression of βAR subtypes varies, and β2 receptors exhibit a higher concentration in the apex of the heart (Gorelik *et al.*, 2013). Notably, the β2-subtype is more than 2.5-fold more abundant in the sinoatrial node than in the atrial myocardium in humans. The relatively elevated β2AR density in the human sinoatrial node aligns with physiological studies implicating this receptor in the regulation of cardiac chronotropism (Rodefeld *et al.*, 1996).

In the cardiomyocyte, research by Marc Bathe-Peters et al. and Rybin et al. reveal that β2 receptors are confined to the T-tubular network and caveolae, in contrast to β1 receptors found on both evenly distributed plasma membranes and T-tubules. This compartmentalization of the cAMP signal likely contributes to signal specificity (Rybin *et al.*, 2000), (Bathe-Peters *et al.*, 2021), (Kuschel *et al.*, 1999). Functional β2AR-Gαs-cAMP signaling occurs almost exclusively on the cell surface sarcolemma of rat ventricular myocytes (Cros and Brette, 2013).

The β2 receptor exhibits distinct signaling pathways depending on the cell state, efficiently coupling with both Gαs and Gαi, displaying stronger cAMP coupling than β1 receptors (Xiao, Ji, and Lakatta, 1995). In the healthy human heart, β2-adrenoceptors favor coupling to Gαs proteins, although coupling to Gαi proteins is

also detected (Kilts *et al.*, 2000). In transgenic mice without heart failure, Gαi signaling also did not play a role (Nikolaev *et al.*, 2006). The inotropic and lusitropic responses of β2AR are associated with PKA-mediated phosphorylation of phospholamban at Ser16 (MacDougall *et al.*, 2012), (Molenaar *et al.*, 2007).

In the context of β2AR signaling downstream, there is considerable discourse. One example is the Mitogen-activated protein kinase (p38 MAPK). This kinase is linked to various outcomes such as hypertrophy, cell death, as well as cardioprotective functions (Zheng *et al.*, 2000). Research indicates activation of p38 MAPK through the Gαs PKA pathway in mouse cardiomyocytes (Zheng *et al.*, 2000) while another line of inquiry suggests Gαi-protein-driven activation (Communal, Colucci and Singh, 2000). Furthermore, β2AR operates through G-protein-independent pathways, such as direct inhibition of the Na^+/H^+ exchange regulation factor (NHERF), leading to sustained activation and modulation of LCC activity (Hall *et al.*, 1998). Subsequently, β2AR plays a crucial physiological role in the regulation of βAR signaling and contributes significantly to cardiac function (Figure 9).

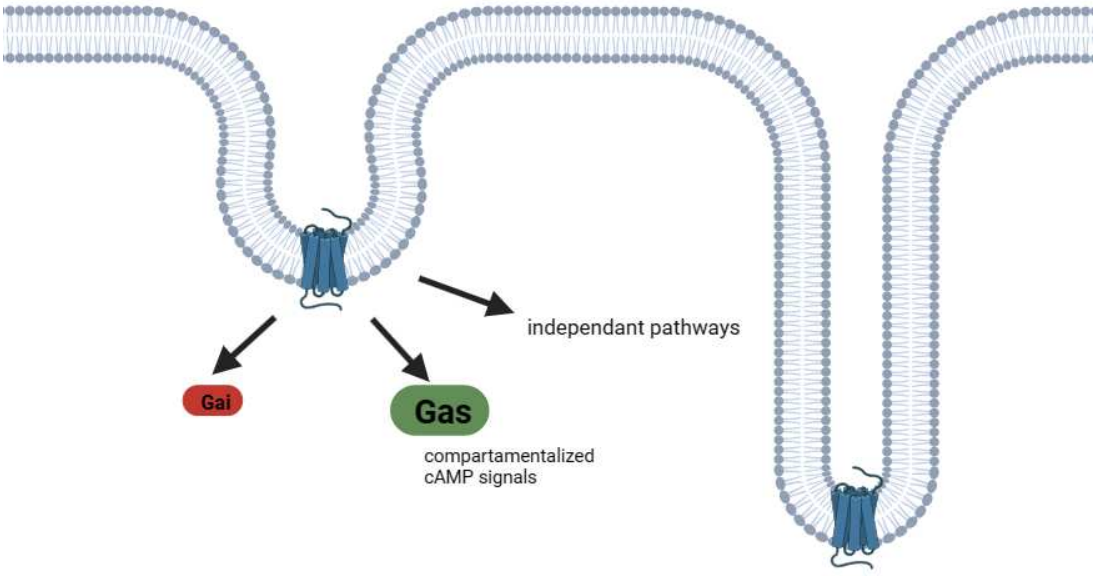

Figure 9: Physiological signaling of β2AR:

The figure illustrates β2AR in cardiomyocytes under physiological conditions. The receptors are predominantly located in invaginations of the cell membrane, such as caveolae and tubules, with a Gαs predominance, resulting in compartmentalized cAMP signals. Signaling pathways mediated by Gαi or G-protein-independent pathways play a secondary role. Figure created with BioRender.com.

1.2.2.5 β2AR pathophysiology in heart failure

During heart failure, while β1ARs are chronically downregulated, the expression levels of β2AR remain relatively unchanged, giving the β2AR more influence on cardiac function (Bristow *et al.*, 1986).

The following studies examining β2AR receptor localization in healthy and heart failure rats and humans revealed significant differences. Whereas Gorelik et al. found there was a physiologically higher gradient of β2ARs in the apex of the heart in rat models compared to the base of the heart, Brodde et al. found that in humans with end-stage congestive heart failure β2ARs expression was more prominent in the atria than in the ventricles (Gorelik *et al.*, 2013), (Brodde *et al.*, 1986). In healthy cells, β2ARs resided within the surface invagination of cell membranes (caveolae) and T-tubules, producing spatially confined cAMP signals. In failing cardiomyocytes, β2ARs redistributed to other cell surface areas, generating a diffuse cAMP signal, similar to β1AR signaling (Gorelik *et al.*, 2013), (Nikolaev *et al.*, 2010), (Nikolaev *et al.*, 2006).

Molecular components of the β2AR signaling also undergo alterations, such as increased GRK-mediated receptor phosphorylation, reduced Gαs, and increased Gαi-coupling with higher Gαi-expression levels (Ceolotto *et al.*, 2008). This minimizes β1AR-Gαs signaling, leading to a negative inotropic response (Woo and Xiao, 2012), (Böhm, Eschenhagen, *et al.*, 1994), (Böhm, Castellano, *et al.*, 1994).

βARs can also form heterodimers, altering ligand binding profiles and enhancing signaling efficiency in regulating myocyte cAMP production and contractility (W.-Z. Zhu *et al.*, 2005). The β1AR and β2AR heterodimer inhibits the internalization of βAR, leading to the complete loss of the Gi-ERK1/2 MAPK pathway, which may play a role in the development of cardiac hypertrophy, as shown in HEK293 cells (Lavoie *et al.*, 2002), (Zou *et al.*, 1999). It is plausible to hypothesize that the downregulation of β1 receptors may result in decreased heterodimerization of β2AR, thus enhancing Gαi signaling and contributing to cardiac remodeling.

1.2.2.5.1 Cardioprotective effects of β2AR signaling

β2ARs are thought to exert cardioprotective effects during heart failure. *In vitro* models demonstrate that β2 stimulation prevents apoptosis in response to various stimuli, such as hypoxia or chronic norepinephrine stimulation, mediated through Gαi-Gβγ induced activation of the PI3K-Akt pathway, a pro-survival kinase (W.-Z. Zhu *et al.*, 2005). These findings were confirmed in various animal models (Zhu *et al.*, 2001), (DeGeorge *et al.*, 2008). Communal et al. propose a β2AR- Gαi mediated anti-apoptotic pathway via MAPK p38 (Communal, Colucci, and Singh, 2000), while Hussain and colleagues propose that the Gαi pathway plays a role in regulating the basal contractility of failing hearts (Hussain *et al.*, 2013).

Evidence supports the idea that β2-adrenoceptor-Gαi activation is not only beneficial but also life-saving in acute heart failure associated with Takotsubo syndrome (Paur et al., 2012), (Shao et al., 2013). This is likely attributed to the acute nature of catecholamine overstimulation and disease progression, inhibiting the β1AR cardiotoxic effects, in contrast to the slow, progressive chronic nature of congestive heart failure.

Another cardioprotective role is the positive feedback loop between the growth factor ErbB2 and β2AR. Inhibition of ErbB2 kinase increases susceptibility to heart injury induced by chronic β-AR stimulation, highlighting the crucial role of ErbB2 kinase in cardioprotection during β-adrenergic stress (Sysa-Shah *et al.*, 2016).

A novel cardioprotective mechanism entails β2-AR preventing hypertrophy by inhibiting PLCe signaling at the Golgi apparatus. This mechanism requires the internalization of β2AR, activation of Gαi and Gβγ subunit signaling at endosomes, and ERK activation. The pathway inhibits both angiotensin II and Golgi-b1-AR-mediated stimulation of phosphoinositide hydrolysis at the Golgi apparatus, ultimately leading to decreased PKD and HDAC5 phosphorylation and protection against cardiac hypertrophy (Wei and Smrcka, 2023).

The β2AR-Gαs pathway has shown a positive inotropic effect without activating the CAMKII pathway when chronically stimulated, in contrast to β1AR. This is because the specific β-arrestin CamKII-EPac1 does not bind to the C-terminus of β2AR

(Mangmool, Shukla, and Rockman, 2010), (Woo *et al.*, 2015). A rat study model with myocardial infarction showed β2AR overexpression had an association with pro-angiogenic pathways through activation of the VEGF/PKB/eNOS pathway, which led to a higher capillary density (G. Rengo *et al.*, 2012).

In vivo studies have shown enhanced cardiac function with no pathology at high expressions of β2AR (60-fold). However, grossly overexpressing β2 in mice (100 and 350-fold) led to heart failure, hypertrophy, fibrosis, increased apoptosis, and ventricular failure of myocytes (Milano *et al.*, 1994), (Liggett *et al.*, 2000), (Dorn *et al.*, 1999), (Zou *et al.*, 1999). Rodent model studies showed that β2AR stimulation was beneficial against cardiac remodeling (Xydas *et al.*, 2006).

A study using a dog model of heart failure found no expression differences in β2AR protein during heart failure disease development, but overexpression of β2AR genes led to improved contraction and function (Gong *et al.*, 2017).

The discovery of these cardioprotective effects has prompted efforts to gain a deeper understanding of β2 adrenergic signaling, opening up the possibility of exploring it as a novel target for therapies in the realm of heart failure.

1.2.2.5.2 β2AR signaling and the development of heart failure

The β2AR has not only cardioprotective functions but is also involved in mechanisms that exacerbate heart failure. One hypothesis is that β2ARs activation only produces desirable signaling in the failing heart if applied before the structure of the cardiomyocytes changes (Gorelik *et al.*, 2013). The cardioprotective and cardiotoxic effects of β2AR signaling are comprehensively summarized in Table 1.

Gαi signaling was also attributed to weakening cardiomyocyte contraction through negative regulation of Ca^{2+} dynamics, leading to HF exacerbation (El-Armouche *et al.*, 2003), (Fajardo *et al.*, 2013). Both β1 and β2ARs are found to be involved in pathways that promote hypertrophy (Zhao *et al.*, 2011). β2ARs stimulation through Gαi signaling also activates ERK1/2 via the Gβγ subunit, which can induce hypertrophic remodeling (Zou *et al.*, 1999).

As explained in the β1ARs passage, GRK2 plays an important role in β1ARs desensitization and internalization. Additionally, GRK2 exhibits cardiotoxic effects such as the promotion of adrenal sympathetic activity (Lymperopoulos, Rengo, and Koch, 2012), and an increased incidence of myocyte death in ischemic models (Sato *et al.*, 2018). Recent findings suggest that β2-adrenoceptor Gαi-biased signaling is the link between GRK2 up-regulation and the progression to decompensated heart failure (Zhu *et al.*, 2012).

Additionally, β2ARs expressed in other cells in the heart, other than cardiomyocytes, may play a role in heart failure and cardiac remodeling. For example, immune cell β2ARs are important for proinflammatory macrophage infiltration to the heart. Macrophage infiltration leads to cardiac injury, cardiomyocyte death, fibrosis, and hypertrophy (Tanner, Maitz, and Grisanti, 2021). Furthermore, both βARs seem to play a role in the development of fibrosis in cardiac remodeling (Brouri *et al.*, 2004).

A topic of significant debate is the reason behind the superiority of the non-selective β-blocker Carvedilol over its β1-selective counterparts in heart failure mortality (Rain and Rada, 2015). The central question is: Is this superiority attributed to its blockade of β2AR signals, which promote heart failure? Various hypotheses attempted to elucidate why Carvedilol may exhibit enhanced efficacy in the treatment of heart failure. In their review, Talan et al. proposed that therapies involving a β1AR blocker + β2AR agonist, β1AR blocker + ACE inhibitors, and Carvedilol, all coincide with vasodilation, potentially contributing to superior outcomes compared to β1AR-selective blockers (Talan *et al.*, 2011). Another theory involves β-arrestin-dependent signaling that activates ERK through β2AR (Wisler *et al.*, 2007), and the EGFR via β-arrestin-biased signaling at β1AR (Kim *et al.*, 2008) conferring cardioprotection in animal models (Noma *et al.*, 2007).

Table 1: Overview of β2AR signaling effects

The table provides an overview of β2AR signaling effects categorized as either cardiotoxic or cardioprotective, along with corresponding references. Numbers added in the "Cardiotoxic" or “Cardioprotective” column correspond to the number in the references provided.

Effects of β2AR signaling			
Cardioprotective	**Ref.**	**Cardiotoxic**	**Ref.**
Antiapoptotic →Gαi-Gβγ -PI3K-Akt (1)	(1) (Zhu *et al.*, 2001), (DeGeorge *et al.*, 2008)	Gαi → GRK upregulation → βAR desensitization and internalization →promotion of adrenal catecholamine production (1) →Apoptosis (2)	(1)(Lymperopoulos, Rengo and Koch, 2012) (2) (Sato *et al.*, 2018)
→Gαi-MAPKp38 (2)	(2) (Communal, Colucci, and Singh, 2000)		
Counteracts hypertrophy: →Internalisation of β2AR →Gαi-Gβγ activation at endosome →ERK activation →Inhibition of ATII, and Inhibition of β1AR mediated PIP hydrolysis at the Golgi apparatus	(Wei and Smrcka, 2023)	Hypertrophic: Gαi-Gβγ →ERK1/2	(Zou *et al.*, 1999)
		Fibrosis	(Brouri *et al.*, 2004)
β2AR Gαs positive inotropic pathway does not activate the CAMKII apoptotic pathway	(Mangmool, Shukla and Rockman, 2010), (Woo *et al.*, 2015)	Neg. Inotropic: Gαi inhibition of the Gαs-PKA-cAMP pathway	(El-Armouche *et al.*, 2003), (Fajardo *et al.*, 2013).
Angiogenic: VEGF/PKB/eNOs pathway	(G. Rengo *et al.*, 2012)	Promotes inflammation: β2AR in Immune cells → Macrophage infiltration	(Tanner, Maitz and Grisanti, 2021)
Positive correlation of β2AR and ErbB2	(Sysa-Shah *et al.*, 2016)		

1.2.2.6 Possible novel treatment strategies with β2AR as a target

The pursuit of novel treatment strategies in the realm of β2AR signaling and downstream targets has yielded promising avenues for addressing heart failure.

One innovative approach involves a combination of the fully escalated heart failure medication which involves β-blockers, an ACE-I, an ARB, digoxin, and an aldosterone receptor combined with β2-adrenoceptor-agonist clenbuterol, presenting a protective drug design strategy for mechanical left ventricular assistance in advanced heart failure stages (Hall *et al.*, 2007).

Exploring further combination therapies, the combination of a β1ARs antagonist with β2ARs agonists like fenoterol is proposed (Talan *et al.*, 2011). In their review, Woo and colleagues suggest a Gαs-biased β2AR agonist combined with a β1AR antagonist, leveraging β2AR-specific Gαs signaling to achieve a positive inotropic effect without the cardiotoxicity associated with the β1AR Gαs pathway and mitigating the negative Gαi-mediated effects (Woo *et al.*, 2015).

Normalizing β-adrenergic signaling encompasses multiple therapeutic targets, with GRK2 inhibition emerging as a proposed strategy for heart failure treatment (Lymperopoulos, Rengo, and Koch, 2012). This approach could potentially reduce Gαi signaling, minimizing βARs desensitization and internalization. Studies combining GRK2 inhibition with a β-blocker have demonstrated additive or synergistic effects in various heart failure models (Sato *et al.*, 2015). Another approach is to target the β-arrestin signaling (Patel, Tilley, and Rockman, 2008). Additionally, ErbB2 kinase emerges as a potential novel therapeutic target within the downstream β2AR signaling cascade (Sysa-Shah *et al.*, 2016).

These innovative strategies provide valuable insights into the evolving landscape of therapeutic interventions centered around β2AR signaling and downstream targets in the context of heart failure.

1.2.2.7 The unknown in β2AR signaling

In conclusion, the uncertainties surrounding β2AR signaling contribute to ongoing debates within the scientific community. Questions persist regarding the delicate balance between the antiapoptotic and negative inotropic effects of Gαi-mediated β2AR signaling, as highlighted by W. Zhu et al. (W. Zhu *et al.*, 2005).

The functional existence of β2-adrenoceptor-Gαi signaling in the chronically failing human heart has been a matter of debate. Proponents such as Kilts and Gong argue for the presence of increased Gαi proteins in human heart failure cells, suggesting a potential role in the pathophysiology of the condition (Kilts *et al.*, 2000), (Kilts *et al.*, 2003), (Gong *et al.*, 2002). Contrastingly, Molenaar challenges the idea of an important negative inotropic role of Gαi coupling in human heart failure (Molenaar *et al.*, 2007). Another human study countering the idea of β2AR receptors playing a role in heart failure found no evidence of β2AR activity correlating with heart failure risk (Baudier *et al.*, 2023).

Navigating the intricate nature of β2AR, a multifaceted receptor with both promoting and counteracting effects on cardiac remodeling and heart failure, underscores its complexity in engaging diverse pathways and manifesting distinct effects in various cardiac cells. The ongoing discourse raises fundamental questions about whether β2AR signaling acts as a regulator, balancing between the promotion and counteraction of cardiac remodeling and heart failure.

Given these complexities, some suggest that targeting downstream molecules may offer a more intuitive approach to achieving specific therapeutic effects (Giuseppe Rengo, Femminella, *et al.*, 2012). While the scientific community generally agrees on the overarching goal of restoring βAR signaling to its former state, diverse opinions persist on the most effective strategies to attain this objective.

Despite extensive research, the precise role of β2AR in human heart failure remains elusive, emphasizing the need for continued exploration. The complexities surrounding β2AR signaling underscore the demand for further research to unravel its exact role in cardiac function and pathology.

1.3 Objectives and description of the thesis project

β2ARs play a pivotal role in regulating cardiac function and contribute to the development of heart failure. While regulatory pathways are being discovered, much remains unknown, leading to debates on how these pathways interact within cells and their roles in human heart cells. Therefore, it is crucial to gather more data on β2ARs and their implications for humans to assess the translatability of insights from animal models to human cardiomyocyte function. Therefore, the main aims of this thesis are to investigate:

1. Alterations in β2AR expression patterns in cardiomyocytes isolated from control and hypertensive Dahl salt-sensitive rats during early- and late-stage cardiac remodeling.
2. Alteration of β2AR expression patterns during βAR stimulation from control and hypertensive Dahl salt-sensitive rats
3. Changes in β2AR expression levels during heart failure in the human myocardium.
4. Comparative alterations of β2ARs in relation to changes in β1ARs expression in Dahl salt-sensitive rats

2. Material and Methods

2.1 Overview

In this experiment, we employed a hypertensive rat model, dividing it into two groups of healthy and hypertensive rats based on the salt concentration in their diet. Upon reaching a predetermined age, we euthanized the rats and isolated the cells using the Langendorff method. Furthermore, heart tissue samples were collected for Western Blot analysis and RNA microarray, with a focus on β2-adrenergic receptors. The isolated cardiomyocytes were stained for β2AR, and the cells were analyzed using fluorescence microscopy. This data was compared with the β1AR data collected using Immunocytochemistry (ICC) (Figure 10).

For human samples, we utilized a bank of heart tissues obtained from transplant donors. Western Blot analyses were conducted to quantify β2AR expression.

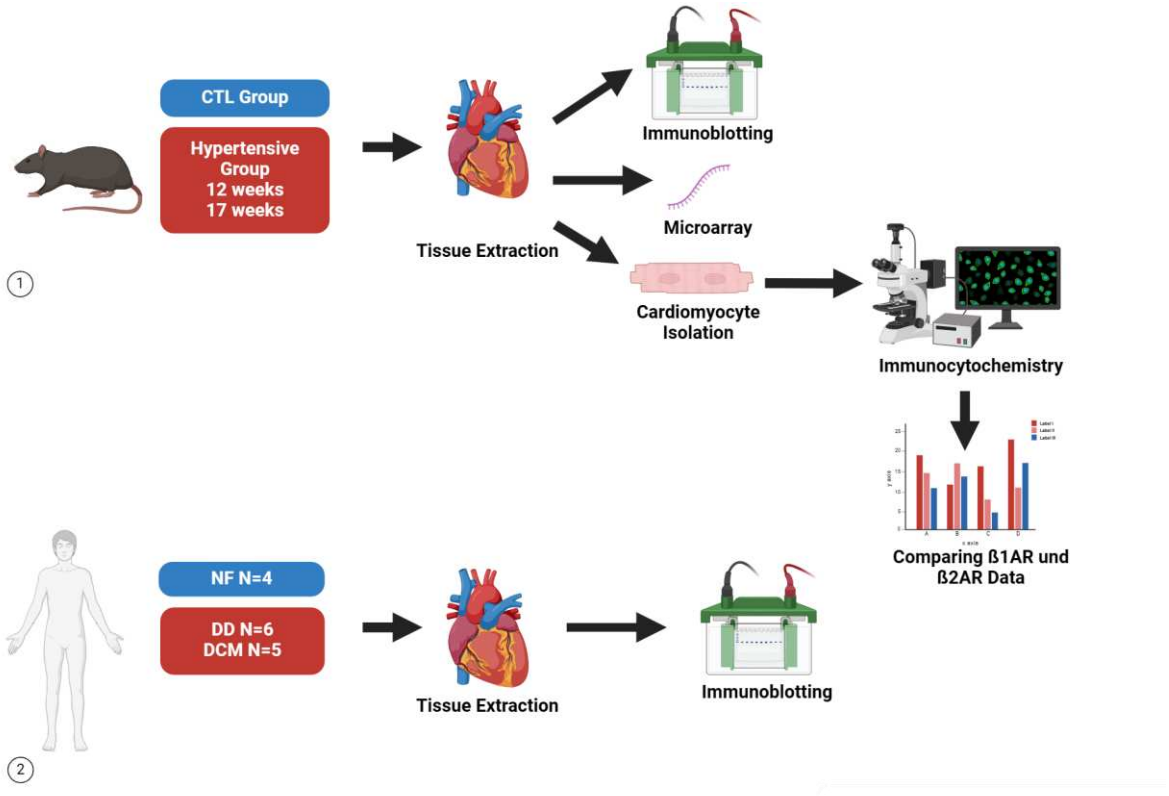

Figure 10: Overview of the experiments

The figure provides an overview of the methods employed in this thesis. Data collection included (1) Dahl-salt-sensitive rats, with a hypertensive group euthanized at 12, 17, and 19 weeks and a corresponding normotensive control group (CTL). β2ARs were analyzed in rat hearts using immunoblotting, microarray, and Immunocytochemistry (ICC) of isolated cardiomyocytes. Isolated cardiomyocytes were also treated with a β-agonist to analyze the rapid desensitization behavior of

β2AR in both groups. The ICC data was then compared to β1AR ICC data collected by Matzer, Ingrid Msc. (Matzer, 2021). Additionally, (2) human data was obtained from the Human Organ Donor Bank, consisting of 5 non-failing (NF) hearts, 6 hearts with diastolic dysfunction (DD), and 5 hearts with dilated cardiomyopathy (DCM). Immunoblotting of left ventricular tissue was utilized for quantifying β2AR in the human hearts. Created with BioRender.com.

2.2 Dahl salt-sensitive rat model

To replicate the human scenario of hypertension-induced cardiomyopathy, we utilized the male Dahl salt-sensitive rat model from Charles River Laboratories in the USA. These rats are selectively bred for their development of hypertension due to salt sensitivity, exhibiting low renin, a salt-sensitive form of hypertension, which is uncommon in salt-resistant wild-type rats (Charles River Laboratories, 2009). It is reported that these rats develop heart failure at 15-20 weeks due to induced hypertension (Schwarzer, 2016). This model was selected specifically for its close resemblance to the phenotype observed in humans with hypertension, including alterations in left ventricular geometry and heart function (Doi *et al.*, 2000). Ethical approval for the use of these rats was obtained from the Austrian Ministry (BMBWF: 2020-0.192.430) and the rats were treated according to European ethical regulations.

From weeks 0-7 both groups received a low-salt-diet (LSD, 0.3% NaCl; Research Diets; D10001R). The test group received a high-salt-diet (HSD, 8% NaCl; Research Diets; D05032408Y) starting at week seven, while the control group continued with the LSD.

Starting at week 7, live parameters including blood pressure and body weight were collected every two weeks. Systolic and diastolic blood pressure were measured, using the CODA® tail cuff system (Kent Scientific Corporation, Torrington, CT, USA). Recordings were performed in a steady state and were the average of three consecutive measurements. Transthoracic echocardiography measurements, including ejection fraction (EF) to assess systolic heart failure and E/e' to evaluate diastolic heart failure, were performed shortly before the experimental timepoints with Vevo 3100 (VisualSonics, Toronto, ON, Canada) under light anesthesia (Isoflurane, 1-1.5%, and 1.5–2 l/min oxygen flow). The EF was measured in the parasternal long axis. To obtain the E/e’ ratio, the mitral annular velocity (e’) was

obtained using the tissue doppler in a parasternal short axis, while the early mitral inflow velocity (E) was acquired using the flow doppler in a modified parasternal long axis.

Rats received deep anesthesia with Isoflurane before euthanization by guillotine. After euthanizing the rats, heart mass was obtained and normalized to body mass to indicate hypertrophic cardiac growth. Heart samples were collected at an early stage (12 weeks) and late stage (17 weeks) timepoints, either for subsequent microarray or utilized for the isolation of ventricular cardiomyocytes to assess differentiation in receptor expression and hypertrophy. Rats used for immunoblotting were used from an older cohort which was euthanized at 19 weeks (Figure 11).

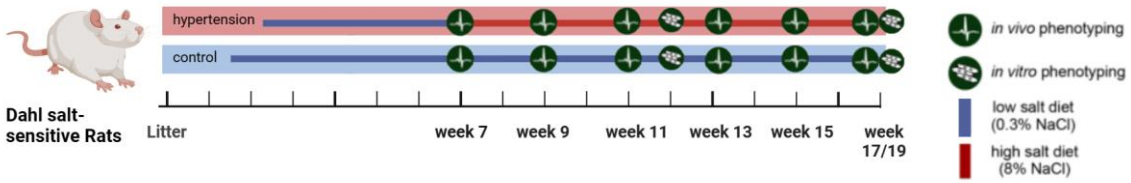

Figure 11: Experimental timeline and parameters for the high-salt-diet induced hypertension study

The male Dahl salt-sensitive rat cohorts were divided into two groups: a control group receiving a low-salt-diet and the hypertension group receiving a high-salt-diet from week seven. *In vivo* phenotyping, encompassing blood pressure and body weight, was conducted biweekly starting from week seven. Heart tissue collection was performed at both an early timepoint of 12 weeks and a late timepoint of 17 or 19 weeks. Adapted from the figure by Holzer, Senka (Holzer, Senka, 2019), created using Biorender.

The *in vivo* data and results presented in this thesis are integral to a broader project, involving Ms. Ingrid Matzer's Master's thesis “Role of β1-adrenergic signaling in early- and late-stage hypertensive cardiac remodeling” (Matzer, 2021), Dr. Julia Voglhuber's Ph.D. thesis “the perinuclear region in cardiac remodeling” which is under revision and the paper “β-Adrenergic Receptor Stimulation Maintains NCX-CaMKII Axis and Prevents Overactivation of IL6R-Signaling in Cardiomyocytes upon Increased Workload” (Matzer *et al.*, 2022). Dr. Julia Voglhuber and Ms Ingrid Matzer collected the *in vivo* data analyzed in this thesis. The *in vivo* data used for analysis pertain to the rats utilized in Microarray, immunoblotting, and ICC experiments, as well as the hypertrophy data that I contributed to collecting.

2.3 Human tissue collection

All experiments with human heart probes were approved by the Ethical Committee of the Medical University of Graz (Nr. 28-508 ex 15/16), and they were performed in accordance with principles outlined in the *Declaration of Helsinki*. Informed consent was obtained from patients with terminal heart failure. However, it was not feasible to collect informed consent from all patients who did not have terminal heart failure. Therefore, the ethical committee waived the requirement for informed consent for these patients, considering that their underlying medical condition prevented them from providing it. Upon transport in ice-cold Custodiol cardioplegic solution supplemented with 2,3-butanedione monoxime (BDM 10mM), Sigma cat.no B0753, before transportation to the laboratory as a precaution against hypoxic damage.

Cardiomyocyte Isolation via the Langendorff perfusion method was attempted.
Tissue samples were extracted from the left ventricle, anonymized, and stored in cooled nitrogen at -40°C. These samples were later utilized in immunoblotting for β2AR. Patient data, including LVEF, weight, age, and gender, were anonymized and recorded in a file. The patients were then classified into three groups: not failing (NF), diastolic dysfunction (DD), and dilated cardiomyopathy (DCM). In our study, Diastolic Dysfunction is observed with HFpEF.

DCM is characterized by left ventricular dilation and contractile dysfunction, resulting in a reduced LVEF. Echocardiography reveals ventricular hypokinesis, and dilation can occur in both the left and right ventricles (Weintraub, Semsarian, and Macdonald, 2017).

2.4 Ventricular cardiomyocyte rat isolation via Langendorff perfusion

Langendorff Perfusion is an isolation method wherein various solutions are slowly infused through the coronary arteries of the heart. Our Langendorff perfusion setup maintained constant flow through a Peristaltic Pump, and pressure overload was counteracted with a bubble trap. (Figure 12).

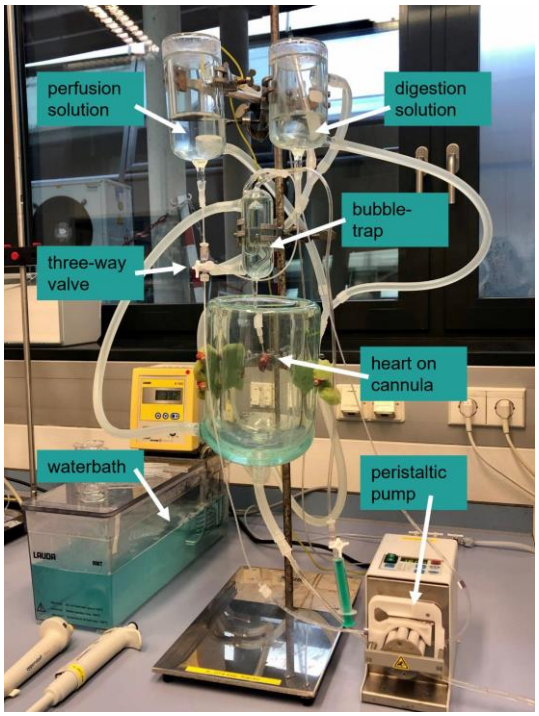

Figure 12: Langendorff perfusion setup

In the following figure, the Langendorf perfusion setup is depicted, consisting of a water bath regulating temperature, a peristaltic pump controlling solution velocity into the vessels, a three-way valve directing solution flow, and a bubble trap preventing pressure overload. The solutions then infiltrate the heart, attached to the cannula via the aorta. After the digestion process is complete, the heart is removed from the Langendorf perfusion setup for subsequent steps in the isolation protocol. Source: (Matzer, 2021) used with kind permission from Matzer Ingrid Msc.

The isolation of ventricular cardiomyocytes from rat hearts involved an adapted Langendorff-based perfusion protocol (Ljubojevic *et al.*, 2014). Dahl salt-sensitive HSD- and LSD-fed Rats at 12 and 17 weeks received an intraperitoneal injection of Heparin (500 IE; Gilvasan; ET34622) 15 minutes before anesthesia with isoflurane (Baxter; 1087151) and subsequent decapitation via guillotine. Following the opening of the thoracic cavity, the heart was excised, and the aorta was promptly cannulated.

The Langendorff setup was employed, and after retrograde perfusion with cannulation solution, the heart underwent perfusion with Ca^{2+}-free solution (perfusion solution) for 1 minute and 30 seconds. The primary function of the isolation process involves the cannulation solution, designed to wash out toxic metabolites that accumulate during hypoxia and stress associated with euthanization. This solution aims to mimic an environment in which cardiomyocytes feel comfortable. Butanedione monoxime (BDM) is added to inhibit permanent contracture, thereby reducing ischemia-reperfusion injury (Li *et al.*, 1985).

The hearts were then digested with myocyte digestion solution for approximately 10 minutes until clear signs of digestion were observed. The digestion solution is intended to break down the tissue surrounding the cardiomyocytes, including fibroblasts, connective tissue, and vascular cells, using enzymes such as Liberase and Trypsin. The digestion process should be stopped to prevent the cardiomyocytes from being digested as well, and this is achieved with the rat myocyte stopping solution 1. Digested ventricles, excluding the atria, were cut from the cannula, immersed in myocyte stop solution 1, and sieved through a 300 µm mesh. The obtained myocytes settled through gravity and underwent exposure to a series of solutions with increasing Ca^{2+} concentrations, to obtain functioning contracting cardiomyocytes. The last Ca^{2+} solution is BDM-free to allow proper contraction.

Finally, the isolated ventricular cardiomyocytes were plated on Laminin-coated glass-bottom cell culture dishes (Greiner bio-one; 627861) and allowed to attach for 1 hour at room temperature. These cells were then used for hypertrophy measurements and ICC.

2.4.1 Solutions for rat ventricular cardiomyocyte isolation

All solutions were prepared using ddH20 (Millipore) unless stated otherwise.

Table 2: Rat perfusion solution, pH =7.4

Reagent	Company	Cat. No.	Conc. [mM]
NaCl	Roth	3957.1	135
KCl	Roth	P017.1	4.7
KH2PO4	Roth	P018.1	0.6
HNa2PO4 dibasic	Sigma	S0876	0.6
MgSO4 *7H2O	Roth	P037.1	1.2
HEPES	Roth	6763.1	10
Taurin	Sigma	T0625	30

Table 3: Rat perfusion buffer

Reagent	Company	Cat. No.	Conc. [mM]
Perfusion solution	See table 2	-	-
Butanedione monoxime	Sigma	B0753	10
Glucose	Merck	50-99-7	10

Table 4: Rat cannulation solution

Reagent	Company	Cat. No.	Conc. [mM]
Perfusion solution	See table 2	-	-
CaCl2	Sigma Aldrich	21115	1

Table 5: Rat calcium solutions

Reagent	Company	Cat. No.	Conc. [mM]
CaCl2	Sigma Aldrich	21115	100
CaCl2	Sigma Aldrich	21115	10

Table 6: Rat myocyte digestion solution

Reagent	Company	Cat. No.	Quantity
Perfusion solution	See table 2	-	40mL
Liberase TM Research Grade	Roche	0540 1127001	1200 – 1300µL
Trypsin	GIBCO	15090-046	222µL
10mM CaCl2	Sigma Aldrich	21115	150µL

Table 7: Rat myocyte stopping solution 1

Reagent	Company	Cat. No.	Quantity
Perfusion solution	See table 2	-	22.5mL
BCS	Sigma Aldrich	12133C	2.5mL
10mM CaCl2	Sigma Aldrich	21115	31.26µL

Table 8: Rat myocyte stopping solution 2

Reagent	Company	Cat. No.	Quantity
Perfusion solution	See table 2	-	48mL
BCS	Sigma Aldrich	12133C	2.5mL
10mM CaCl2	Sigma Aldrich	21115	63.16µL

Table 9: Rat calcium series.

Calcium solutions were diluted from the myocyte stopping solution 2.

No.	CaCl2 [mM]
1	0.125
2	0.25
3	0.5
4	1
5	1.5

Table 10: Rat normal tyrode solution (NT)

Reagent	Company	Cat. No.	Conc. [mM]
NaCl	Roth	3957.1	140
KCl	Roth	P017.1	4
MgCl2*6H2O	Roth	HN03.2	1
HEPES	Roth	6763.1	10
CaCl2	Sigma Aldrich	21115	1.5
Glucose	Merck	50-99-7	5

One of my responsibilities was to optimize the human heart Langendorff isolation protocol. There were two timepoints during my laboratory work when the parameters were aligned, coinciding with the extraction of organs from deceased donors at the LKH Medical University Clinic in Graz, where the heart was not suitable for transplantation. Unfortunately, at the first timepoint, I could not yield enough viable cells. In the second timepoint, although I obtained some cells, their survival rate was not sufficient to proceed with ICC, and consequently, this aspect was excluded from my results and discussion.

2.5 Hypertrophy measurements

To assess cardiomyocyte specific hypertrophy, cellular dimensions of isolated cardiomyocytes from LSD- and HSD-fed Dahl salt-sensitive rats at 12 and 17 weeks were measured from Differential Interference Contrast images. All live cell imaging was performed at room temperature (22-24°C). Attached cardiomyocytes were captured at the largest sectional plane available and imaged using confocal fluorescence microscopes (Zeiss LSM 510 Meta or LSM 700 (Carl Zeiss, Germany), Nikon A1 (Nikon Europe B.V., The Netherlands) or Olympus Fluoview 1000 (Olympus, USA). Cell width and cell length measurements were conducted using ImageJ software (National Institutes of Health, USA).

2.6 Immunocytochemistry (ICC)

The ICC protocol was obtained from the paper: “Early Remodeling of Perinuclear Ca^{2+} Stores and Nucleoplasmic Ca^{2+} Signaling During the Development of Hypertrophy and Heart Failure” (Ljubojevic *et al.*, 2014).

2.6.1 Drug treatments and fixing isolated Cardiomyocytes

The cardiomyocytes collected at the late timepoint (17 weeks) were seeded onto cell culture dishes. Subsequently, they were incubated for one hour at 37°C in two groups: one with 3 mL containing 100 nM Isoprenaline hydrochloride (ISO), a beta-agonist, and another with 3 mL of Normal Tyrode (NT), devoid of any drug treatment.

After that cells were fixed for 10 minutes with 1 mL of 4% paraformaldehyde (Morphisto; 1176200500), yielding a 1% paraformaldehyde fixation solution. Cells were then rinsed three times with phosphate-buffered saline (PBS, Sigma Aldrich; P4417-100TAB) and stored at 4°C in the dark until further usage.

2.6.2 ICC staining

Permeabilization was carried out using 0.6% Triton-X100 (Sigma Aldrich; T8787-100ML)/PBS for 10 minutes. Triton-X100 facilitates antibody penetration into intracellular structures, while PBS, a phosphate buffered saline solution, maintains physiological pH and environment. Following a single wash in PBS, cells underwent incubation with the primary antibody (ADRB2, Thermofisher BS-0947R polyclonal LOT: AG 10096795) at a concentration of 1:100 in 5% BSA (Sigma Aldrich; B6917)/3% goat serum (Abcam; ab7481)/0.01% Triton/PBS at room temperature for a minimum of 3 hours (or at 4°C overnight), with gentle shaking. Bovine Serum Albumin (BSA) was employed as a blocking agent to prevent nonspecific binding, stabilize antibodies, and serve as a diluent.

Following two washes in 0.01% Triton-X100/PBS, the cells were exposed to the secondary antibody (anti-rabbit Alexa647 (Cell signaling; 4414) 1:1000 in 5% BSA (Sigma Aldrich; B6917)/3% goat serum (Abcam; ab7481)/0.01% Triton/PBS for 2 hours at room temperature in the dark. Subsequently, cells underwent two washes with 0.01% TritonX-100/PBS and two additional washes with PBS before being stained with DAPI (Carl Roth; 6335.1; 1:200 in 5% BSA/PBS) for 10 minutes at room temperature in the dark. DAPI is a DNA fluorescent stain used to visualize cell

nuclei, emitting blue fluorescence. Following this, cells were washed twice with PBS and kept in PBS for imaging.

Images were recorded on a confocal fluorescence microscope (Zeiss LSM 510 Meta) with a Plan Neofluar 40x/1.3 oil-immersion objective. Image J software (National Institutes of Health, USA) was used to measure the intensity of the recorded fluorescence signal. Ventricular cardiomyocytes with ADRB2 staining were analyzed for their whole cell and fluorescence signal subtracting the nucleus (Figure 13). To ensure equal staining of all treatment groups, cardiomyocytes of each group were stained together. Background corrected data was used for statistical analysis.

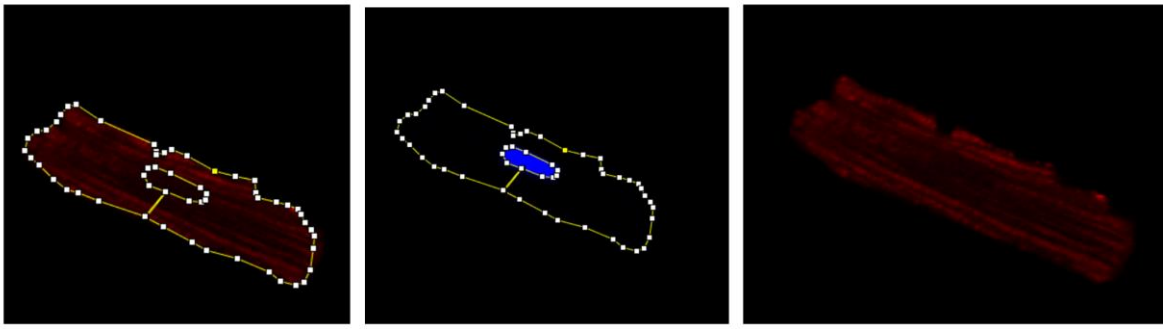

Figure 13: Cardiomyocyte ADRB2 fluorescence analysis using Image J software
This figure illustrates the fluorescence analysis conducted using ImageJ software. The cell image was captured on a confocal fluorescence microscope with two lasers, allowing differentiation between the blue DAPI staining representing the nucleus and the red staining representing the ADRB2 antibody. In the software, the entire cell area was selected, excluding the nucleus.

2.7 Immunoblotting

The Western Blot protocol was used in the master thesis “Role of β1-adrenergic signalling in early- and late-stage hypertensive cardiac remodeling” (Matzer, 2021). Human left ventricular heart tissue was used for one Western Blot comprising of individuals with not failing hearts, diastolic dysfunction, and dilated cardiomyopathy. Dahl salt-sensitive LSD- and HSD-fed rats were used for the other immunoblot. These comprised rats from the first cohort which were euthanized at 12 weeks and 19 weeks.

2.7.1 Tissue homogenization

Homogenization of tissue is crucial for obtaining reliable results in subsequent protein quantification. Tissue samples were homogenized using a freshly prepared ice-cold homogenization buffer and an electric pestle (VWR). After obtaining the supernatant through two brief centrifugation steps at 8000 rpm at 4°C, the homogenate underwent final centrifugation at maximum speed for 10 minutes at 4°C to isolate the protein fraction in the supernatant. The samples were then either immediately utilized for a BCA assay or stored at -80°C for subsequent use.

Table 11: Homogenisation buffer

Reagent	Company	Cat. No.	Conc.
IGEPAL CA-630	Sigma Aldrich	I8896-50ML	1%
Glycerol	Carl Roth	3783.1	10%
NaCl	Carl Roth	3957.1	137 mM
Tris-HCl, pH=7.4	Carl Roth	8789.2	20 mM
NaF	Sigma Aldrich	S7920-100G	20 mM
Sodium orthovanadate	Sigma Aldrich	S6508	1 mM
Sodium pyrophosphate	Sigma Aldrich	S6422-100G	1 mM
β-Glycerophosphate	Fluka BioChemika	50020	50 mM
EDTA (Ethylenediamin-etetraaceticacid), pH=8	Sigma Aldrich	EDS-500G	10 mM
EGTA (Ethylene glycol-bis-2-aminoethyl-ether), pH=7	Sigma Aldrich	E4378-100G	1 mM
Aprotinin	Carl Roth	6367.1	4 µg/mL
Leupeptin	Sigma Aldrich	L2023	4 µg/mL
Pepstatin A	Sigma Aldrich	P4265-5MG	4 µg/mL
PMSF (Phenylmethyl-sulfonylfluorid)	Carl Roth	6367.1	1 mM

2.7.2 Pierce BCA assay

To determine the total protein concentration of the samples intended for Western Blot analysis, a BCA Assay (ThermoFisher Scientific; 23225) was conducted following the manufacturer's protocol. A standard curve was generated using nine dilutions of BSA (Pierce; 23209) ranging from 0.0 to 2.0 mg/mL. Subsequently, samples were diluted 1:15 in ddH2O, and 10 µL of each sample or standard was added in duplicate to a clear flat-bottom 96-well plate.

The BCA protein assay reagent A and BCA protein assay reagent B were mixed in a ratio of 50+1, and 200 µL of the mixture was added to each well. After incubation for 30 minutes at 26°C, absorbance was measured at 562 nm using a SpectraMax® Plus 374 photometer (Avantor by VWR). Individual protein concentrations were calculated by interpolating the absorption values of unknowns into the BSA standard curve.

2.7.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

For Each sample, 30µg were prepared by combining them with 4XT sample buffer (Bio-Rad, 1610791), XT reducing agent (Bio-Rad; 161-0792), and homogenization buffer, resulting in a final volume of 25 µl per sample.

The Western Blot Apparatus was assembled, placing gels in the running chambers. The top chamber was filled with running buffer (XT Mops, Biorad 161-0788), while the lower chamber was filled 1/3 with diluted running buffer (100 ml MOPS + 1900 ml ddH2O). Subsequently, 24 µl of each sample was pipetted onto 4-12% Bis-Tris gradient gels (Bio-Rad: 345-0124). Standards, including Precision Plus Protein, All Blue, and Dual Color Standards (Bio-Rad: 161-0374), were loaded on the first and last wells. Electrophoresis started at 70 Volts for 10 minutes, followed by an increase to 120 Volts for approximately 2.5 hours.

For wet protein transfer a membrane, a transfer cassette with 2 sponges, 2 filter papers, and a transfer chamber were employed with a Transfer Buffer composed of 250 ml Transfer buffer, 500 ml Methanol, and 1750 ml ddH2O. The transfer took place at 400 mA and 4°C for 2 hours.

Following the transfer, membranes were incubated for 3-5 minutes in Ponceau S (Sigma Aldrich; P7170), washed with water until bands were visible without a strong background, and imaged using a ChemiDoc Touch Imaging System (BioRad) to assess protein transfer efficiency.

Subsequently, membranes underwent a 1-hour blocking step in 5% milk powder (MP; Carl Roth; T145.1)/TBST at room temperature. A primary antibody for detecting the β2AR (ADRB2, Thermofisher BS-0947R polyclonal LOT: AG 10096795) at a 1:200 dilution was applied. After three washes in TBST, a secondary antibody (anti-rabbit HRP conjugated; Ge LifeScience; NA934V) at 1:5000 was incubated for 1 hour at room temperature. For detection, membranes were exposed to Clarity Western ECL substrate (Bio-Rad; 1705061) for 2-3 minutes and imaged in signal accumulation mode using the ChemiDoc Touch Imaging System.

Following membrane stripping, anti-GAPDH (Cell Signaling; 5174S) was applied at a 1:2000 dilution overnight at 4°C in 0.5% MP/TBST. Subsequently, the secondary antibody was applied, and proteins were detected using the ChemiDoc Touch Imaging system as described above.

2.7.4 Membrane stripping

Stripping refers to the chemical removal of bound protein from the membrane. Immunostaining for ADRB2 resulted in nonspecific bands near GAPDH (band at 37 kD). The membrane was washed with TBST. Consequently, the stripping buffer (Thermo Fisher; 21059) at room temperature for 7 minutes was applied after immunostaining for ADRB2 to eliminate bound antibodies from this region. The membrane was then washed again. Following the stripping process, the membrane was blocked for 20 minutes in 5% MP/TBST, underwent three washes with TBST, and was subsequently reincubated with the secondary antibody. Stripping efficiency was evaluated using the ChemiDoc touch. After successful stripping (indicating no detectable band), the membrane was further incubated with the primary antibody against GAPDH.

2.7.5 Analysis

Densitometric quantification of bands was carried out using ImageLab software (Bio-Rad Laboratories Inc.) with the "lanes and bands" tool, including manual adjustments. ADRB2 quantification was normalized to GAPDH.

2.8 Microarray

To study β2AR gene expression behavior a microarray was utilized. RNA extraction from the left ventricular tissue of Dahl salt-sensitive LSD and HSD-fed rats at 12 weeks and 17 weeks was performed using the microRNeasy® Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's guidelines. Subsequently, The RNA concentration was evaluated using NanoDropTM 2000 (Thermo Fisher Scientific, Waltham, USA) before forwarding the samples to ATLAS Biolabs (Berlin, Germany) for transcriptomic profiling. Affymetrix WT Expression Profiling microarray Clariom D (Thermo Fisher Scientific, Waltham, USA) was utilized for this purpose. The obtained CEL files were imported into the Transcriptome Analysis Console, version 4.0 (Applied Biosystems, USA), where individual data points were extracted. The values presented in the results are the mean signal intensities transformed to log2.

The entire datasets were accessible to me and I conducted the statistical analysis of the genes of interest.

2.9 Statistical Analysis

Data were presented as mean ± standard error of the mean (SEM). Statistical analyses for *in vivo* and *in vitro* data were conducted using GraphPad Prism 8 (GraphPad Software, CA, USA). Normality testing was performed for all data groups using the Shapiro-Wilk and Kolmogorov-Smirnov tests, depending on the group size.

Phenotypication:

Comparisons of repeated measurements (systolic and diastolic blood pressure and body weight) were conducted using two-way ANOVA with post-hoc Sidak's multiple comparisons test. Heart weight to body weight ratio, Left Ventricular mass, Echocardiography, and Hypertrophy data were analyzed, with normality tested beforehand. Unpaired t-tests were employed for data with Gaussian distribution,

while Mann-Whitney U tests were applied for not normally distributed data. Echocardiography data was analyzed using one-way ANOVA with post-hoc Holm-Sidak's multiple comparisons test or Kruskal-Wallis with Dunn's post-hoc test. A significance level of $p \leq 0.05$ was considered significant.

Microarray:

Normality was tested using the Shapiro-Wilk test. As all groups passed the normality test, data were compared using one-way ANOVA with post-hoc Holm-Sidak's multiple comparisons test. A two-tailed $p \leq 0.05$ was considered significant.

ICC:

Normality was tested. Unpaired t-tests were utilized for data with a Gaussian distribution, and Mann-Whitney U tests were applied for not normally distributed data. A two-tailed $p \leq 0.05$ was considered significant.

Western Blot:

β2AR protein quantification was normalized to GAPH. The normality of data groups was tested with the Shapiro-Wilk test. For rat immunoblotting, as all data passed the test, comparisons were made using one-way ANOVA with posthoc Holm-Sidak's multiple comparisons test. In the human experiment, Kruskal-Wallis with Dunn's post-hoc test was performed instead of ANOVA, as the non-failing heart group did not pass the normality test. A two-tailed $p \leq 0.05$ was considered significant.

3. Results

3.1 Dahl salt-sensitive rats

3.1.1 *In vivo* and *in vitro* phenotyping of hypertensive Dahl salt-sensitive rats

Baseline measurements for *in vivo* parameters, including blood pressure (BP) and body weight (BW), were conducted at seven weeks of age and continued at a biweekly plan. Subsequently, at seven weeks the regular chow diet was replaced either by a low-salt-diet (LSD, 0.3% NaCl) or a high-salt-diet (HSD, 8% NaCl) (Figure 14 A).

Upon the initiation of HSD feeding, both systolic and diastolic blood pressure exhibited significant increases within the first two weeks in HSD rats (Figure 14 B-C). Two-way ANOVA revealed significant differences in systolic and diastolic BP between HSD and LSD rats at each timepoint starting at week nine ($p<0.0001$ for each comparison, except for diastolic BP at week nine with $p=0.0004$). BW consistently increased with age in all groups (Figure 14 D), with the mean weight rising from 228.7 ± 20.14 g at week seven to 375,5 ± 16,21 g at week 17.

Upon euthanizing at 12 weeks of age, the heart-to-body-weight ratio, a determinant of cardiac hypertrophy, did not show a significant increase in HSD animals (Figure 14 E). However, during late-stage remodeling, the increase in the heart-to-body-weight ratio in HSD rats became statistically significant compared to LSD rats ($p=0.0004$). (Figure 14 F). Left ventricular mass was significantly higher in the HSD group at both early and late harvesting timepoints ($p<0.0001$) (Figure 14 G-H).

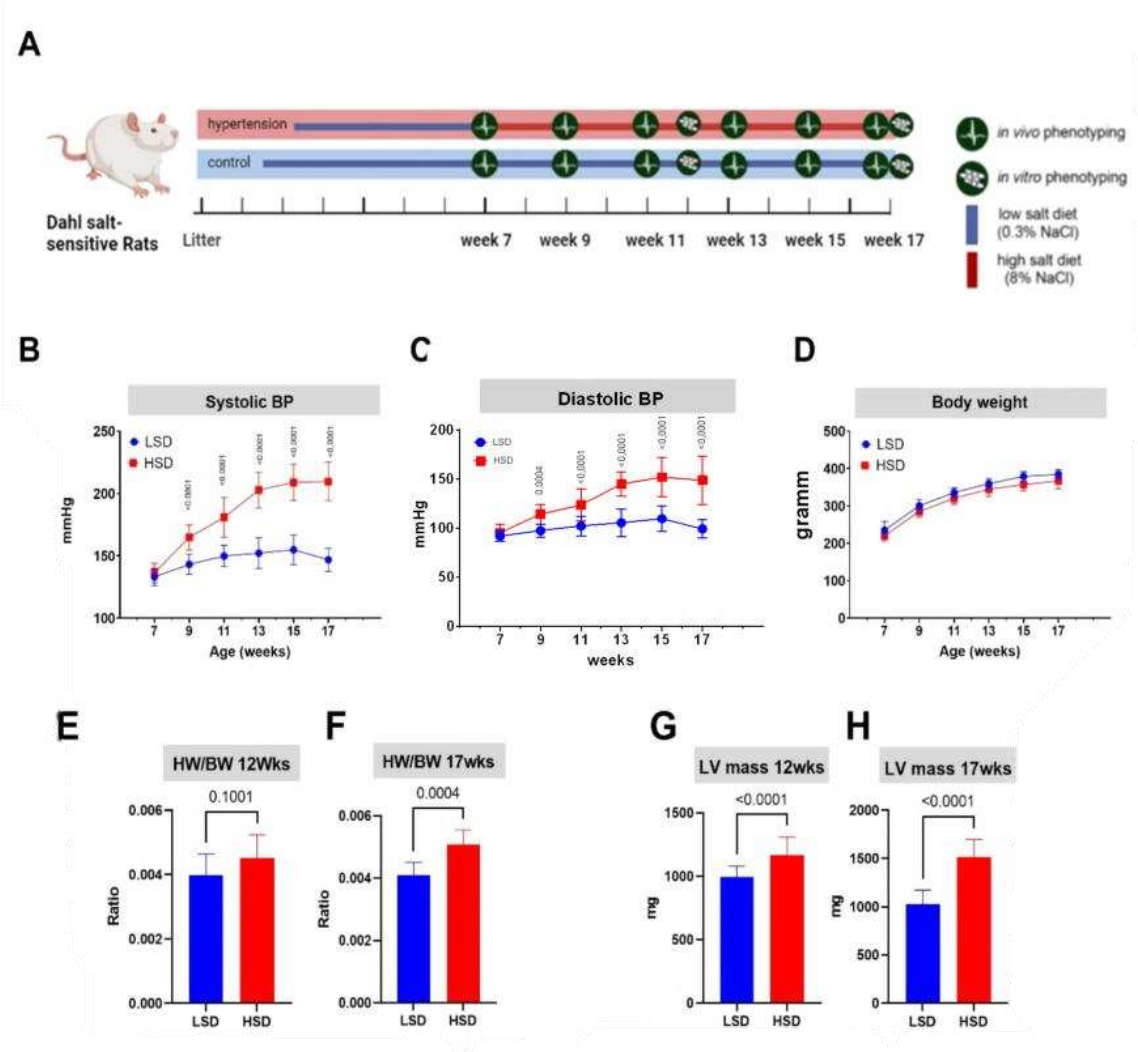

Figure 14: Phenotypical characterization and *in vivo* measurements of Dahl salt-sensitive rats on low-salt-diet (LSD) and high-salt-diet (HSD).

The Dahl salt-sensitive rat group with hypertension is depicted as red, control group is blue. (A) Overview of the rat treatment plan; (B) Systolic blood pressure; (C) Diastolic blood pressure; (D) Body weight; (E) Heart weight/bodyweight ratio at early timepoint 12 Weeks (F) Heart weight/bodyweight ratio at late timepoint 17 weeks; (G) Left ventricular mass at early timepoint 12 Weeks (H) Left ventricular mass at late timepoint 17 weeks; B-D: N=17 HSD rats and N=19 LSD rats at 12 weeks, N=7 HSD rats and N=9 LSD rats at 17 weeks. E; G, N=10 HSD, and LSD rats at 12 weeks. F; H, N=7 HSD and N=9 LSD rats at 17 weeks. (B-D) were calculated by two-way ANOVA with post-hoc Sidak's multiple comparisons test**.** (E-H) were calculated using an unpaired t-test or Mann-Whitney U test.

In vitro, rat cardiomyocytes were assessed for hypertrophy. At both early and late timepoints, HSD rats exhibited statistically significantly larger cells in terms of both length and width compared to the LSD counterparts ($p<0.0001$, except for cell width at 12 weeks, where $p=0.0394$) (Figure 15).

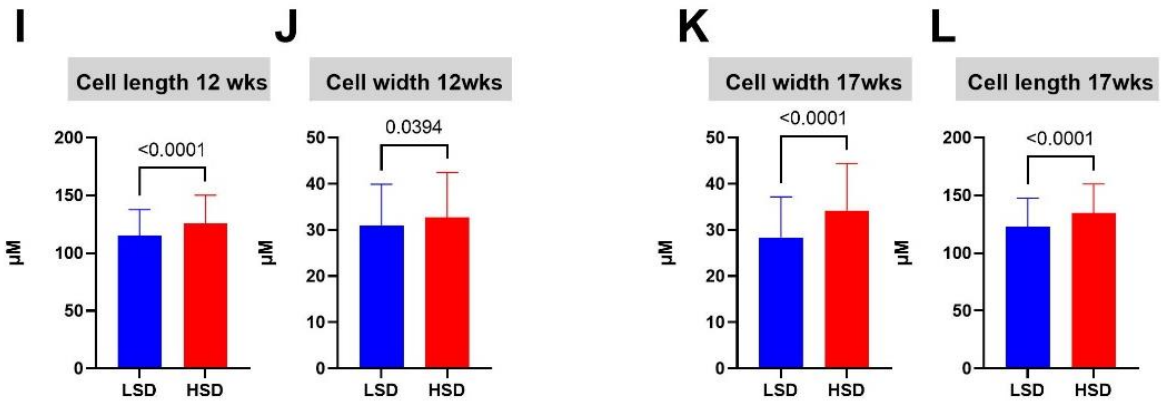

Figure 15: *In vitro* cell hypertrophy of Dahl salt-sensitive rat cardiomyocytes divided into low-salt-diet (LSD) and high-salt-diet (HSD) groups

The Dahl salt-sensitive rat group with HSD is depicted as red, control group with LSD is blue. Cell length in µm at early (I) and late (L) timepoint, Cell width in µm at early (J) and late (K) timepoint; I-J: N=6 HSD rats with N=243 cells, N=6 LSD rats with N=199 cells at 12 weeks; K-L: N=4 HSD rats N=121 cells, N=5 LSD rats N=159 cells at 17 weeks. P-values (I-L) were calculated using an unpaired t-test or Mann-Whitney U test.

Together, these results indicate that the Dahl salt-sensitive rat model was effective in inducing the development of hypertension and left ventricular cardiac hypertrophy.

3.1.2 Echocardiography development of diastolic dysfunction with preserved ejection fraction in Dahl salt-sensitive rats

Echocardiography was performed by Julia Voglhuber PhD, thesis not yet published, at 12 and 17 weeks before euthanizing. The Echocardiography showed no statistically significant difference in EF. It is noteworthy that both rat groups did not manifest systolic dysfunction, as evidenced by EF values around 80% (Figure 16 M-N), which is considered normal for the model (van Ham *et al.*, 2022). It was observed that the HSD group at 12 weeks had a statistically higher EF compared to the LSD group (p=0.005).

Furthermore, a statistically significant difference in E/e', was not noted between LSD and HSD rats at both timepoints. (Figure 16 O-P). Our rats at 12 weeks had a mean value of 18.15 (± 3.38) E/e' in the LSD group and 21.37 (±3.5) in the HSD group. At 17 weeks, the LSD group had a mean of 17.50 (±2.23), and the HSD group had a mean of 20.46 (±4.06). In the review from van Ham et al an E/e' ratio of a maximum of 20 was considered normal in the Dahl-salt-sensitive rat model (van Ham *et al.*, 2022), (Zhang *et al.*, 2020).

In summary, this echocardiography data cannot fully indicate that the HSD group developed diastolic dysfunction. However, the HSD group exhibits a preserved EF.

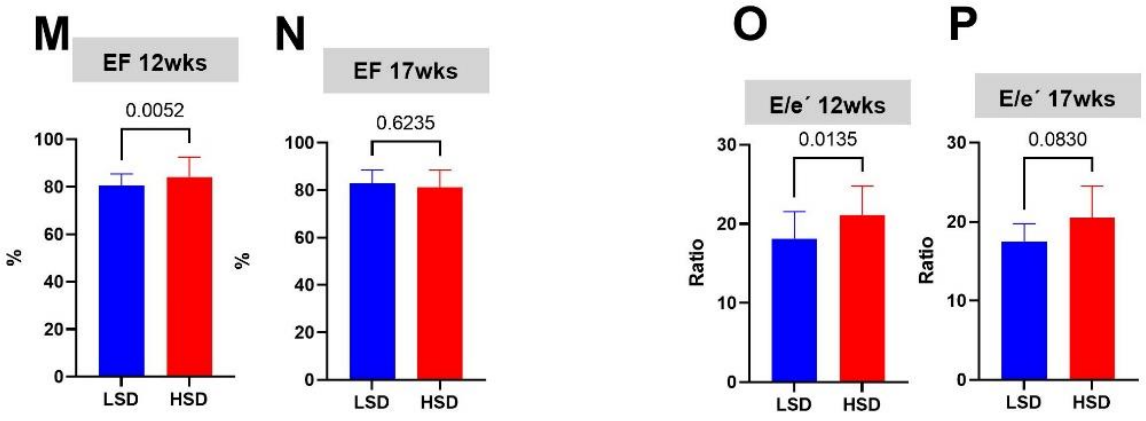

Figure 16: Echocardiography measurements of Dahl salt-sensitive rats on low-salt-diet (LSD) and high-salt-diet (HSD):

The Dahl salt-sensitive rat group with HSD is depicted as red, control group with LSD is blue. Ejection fraction (EF) at early 12 weeks (M) and late 17 weeks (N) timepoint; E/e' at early (O) and late (P) timepoint, M-P, N=18 HSD rats, and N=20 LSD rats at 12 weeks. P-values were calculated using an unpaired t-test or Mann-Whitney U test.

3.1.3 β2AR expression decreases in the whole cell in hypertensive Dahl salt-sensitive rats

The ICC data exclusively depicts cardiomyocytes from the late-stage cardiac remodeling timepoint (17 weeks). The whole cell β2AR fluorescence signal exhibited significantly lower expression of β2AR in cardiomyocytes from the HSD group compared to the LSD group ($p<0.001$). This observation held for both isoproterenol (ISO) treated and untreated cells (Figure 17, Figure 18)

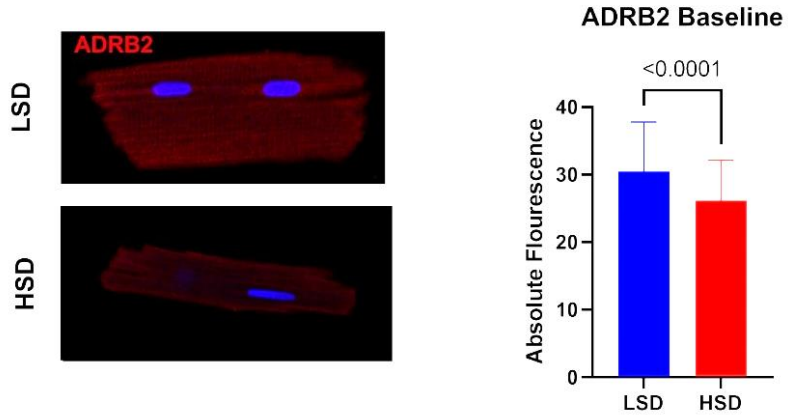

Figure 17: Expression of β2AR in isolated cardiomyocytes from the low-salt-diet (LSD) and high-salt-diet (HSD) Dahl salt-sensitive rats.

The Dahl salt-sensitive rat group with HSD is depicted as red, control group with LSD is blue. On the left: Sample images of LSD and HSD rat cardiomyocytes stained with DAPI (blue) and ADRB2 (red) antibody from the confocal microscope; N=4 rats per group with N=123 cells of HSD rats, and N=160 cells of LSD rats. P-values were calculated using an unpaired t-test or Mann-Whitney U test.

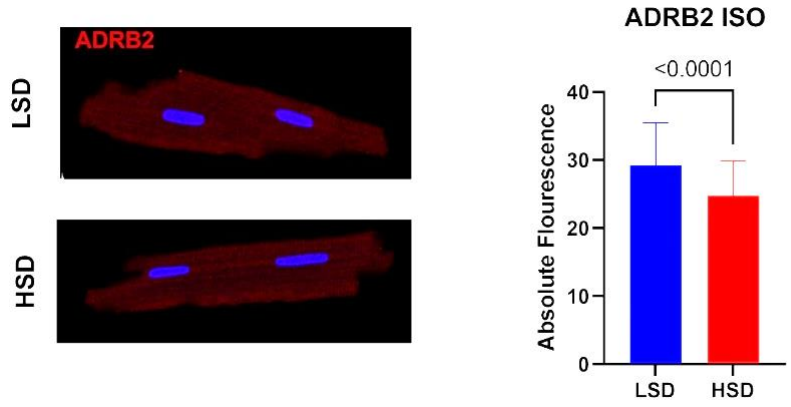

Figure 18: Expression of β2AR in isolated cardiomyocytes from the low-salt-diet (LSD) and high-salt-diet (HSD) Dahl salt-sensitive rats upon β-adrenergic stimulation with 100 nM isoprenaline (ISO) for 1h.

The Dahl salt-sensitive rat group with HSD is depicted as red, control group with LSD is blue. On the left: Sample images of LSD and HSD rat cardiomyocytes stained with DAPI (blue) and ADRB2 (red) antibody from the confocal microscope; N=4 rats per group with N=119 cells of HSD rats, and N=165 cells of LSD rats. P-values were calculated using an unpaired t-test or Mann-Whitney U test.

3.1.4 βAR stimulation decreases β2AR expression in ICC of the LSD Dahl salt-sensitive rat group

β-adrenergic stimulation resulted in the reduction of β2AR expression in cardiomyocytes. This effect was evident in the LSD group at 17 weeks (p=0.008). A similar trend was observed in the HSD group at 17 weeks, although it did not reach statistical significance (p=0.0594) (Figure 19).

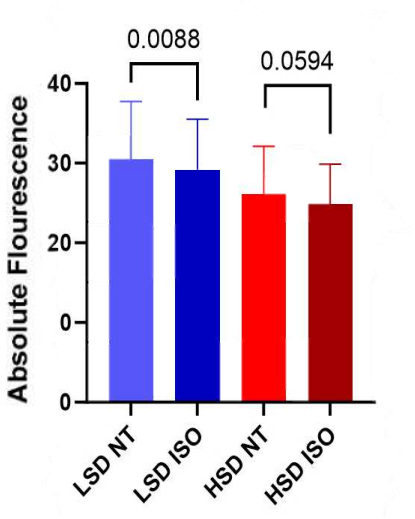

Figure 19: Expression of β2AR in isolated cardiomyocytes from the low-salt-diet (LSD) and high-salt-diet (HSD) Dahl salt-sensitive rats upon β-adrenergic stimulation with 100 nM isoprenaline (ISO) for 1h compared to normal tyrode (NT).

The Dahl salt-sensitive rat group with HSD is depicted as red, control group with LSD is blue. ISO-treated cells are the darker shade of each color. ISO-treated cells encompassed N=4 rats per group with N=119 cells of HSD rats, and N=165 cells of LSD rats. Normal Tyrode (NT) not stimulated cells encompassed: N=4 rats per group with N=123 cells of HSD rats, N=160 cells of LSD rats. P-values were calculated using an unpaired t-test or Mann-Whitney U test.

3.1.5 β2AR expression in relation to β1AR expression in Dahl salt-sensitive rats

β2AR behavior in relation to β1AR was analyzed, as it is frequently cited in the literature. The mean fluorescence of β2AR antibodies decreased from LSD 30.46 (±7.28) to HSD 26.15 (±5.95), signifying a reduction in fluorescence by 14.15% at 17 weeks. The study comprised 4 rats per group, with N=123 cells for HSD and N=160 cells for LSD.

Ingrid Matzer's raw data for comparison with β1AR was used with kind permission, including 3 LSD rats with N=127 cells and 3 HSD rats with N=89 cells. Among the LSD rats, the mean fluorescence was 46.24 (±16.96), which decreased to 41.41 (±10.17), resulting in a 10.45% reduction in fluorescence at 17 weeks (Matzer, 2021). In our experiments on Dahl salt-sensitive rats with HFpEF, the extent of downregulation was similar for both βAR subtypes.

3.1.6 β2AR expression in the left ventricular tissue at early- and late-stage cardiac remodeling

The results discussed above revealed a diminished expression of β2AR in cardiomyocytes from HSD compared to LSD rats during late-stage cardiac remodeling. To verify this observation, an immunoblot with an ADRB2 antibody to quantify β2AR was done with left ventricular tissue, using samples from the first Dahl salt-sensitive rat cohort where the late timepoint of euthanization was set at 19 weeks instead of the 17 weeks of the other cohorts. Unexpectedly, the analysis showed no significant differences in β2AR expression among any of the groups. However, upon closer examination of the data points, a trend of downregulation is discernible, suggesting that with a larger group size, these differences might attain statistical significance (Figure 20).

Consequently, the present study did not allow for the validation of ICC data through immunoblotting.

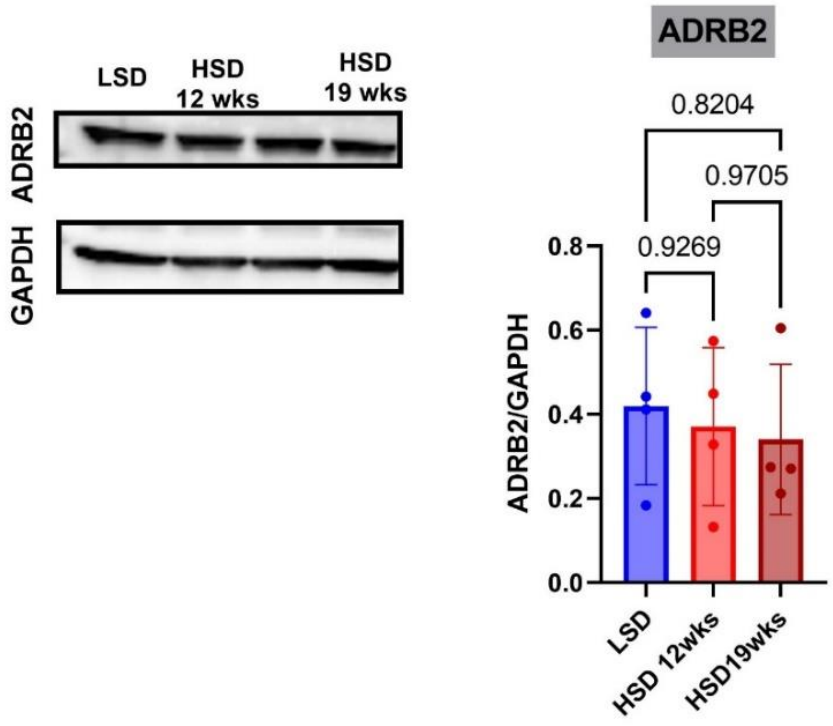

Figure 20: β2AR immunoassay of the left ventricle of Dahl salt-sensitive rats in early (12 weeks) and late (19 weeks)-stage remodeling.

Immunoblot with ADRB2 antibody to detect β2AR in left ventricular tissue lysates. GAPDH was used for normalization. Corresponding quantification of β2AR in low-salt diet (LSD) rats (blue), high-salt-diet (HSD) 12 week old rats (red light), and HSD 19-week-old rats (red dark). Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. N=4 rats/group.

3.1.7 β2AR gene expression is downregulated in the late cardiac remodeling stage

The ICC results indicating the downregulation of β2AR were corroborated by the microarray analysis of Dahl salt-sensitive rat heart tissue. The early timepoint refers to euthanasia at 12 weeks, while the late timepoint corresponds to euthanasia at 17 weeks.

The analysis revealed a significant reduction in β2AR gene expression from the LSD group to the HSD group at the late timepoint (p=0.002) and a significant reduction in β2AR gene expression when comparing the early and late timepoint HSD groups (p=0.002). However, no significant differences in expression were observed between the LSD and early timepoint HSD group (Figure 21).

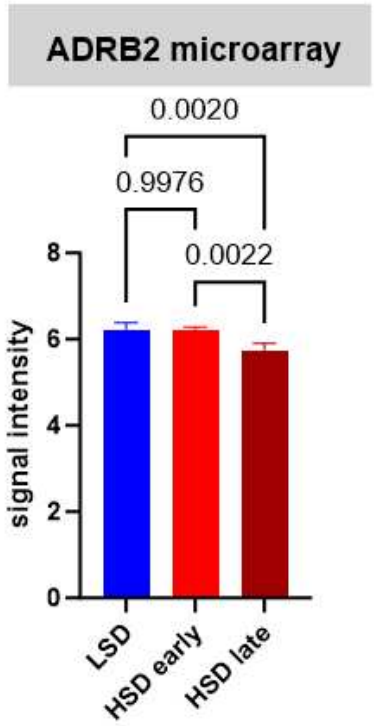

Figure 21: Expression of β2AR mRNA in left ventricular tissue from low-salt-diet (LSD) and high-salt diet (HSD) Dahl salt-sensitive rats in early (12 weeks) and late (17 weeks) timepoints. The Dahl salt-sensitive rat group with HSD is depicted as light red (12 weeks) and dark red (17 weeks), control group with LSD is blue. N=6 rats HSD 17 weeks, N=4 rats HSD 12 weeks, N=4 rats LSD. Differences between groups were calculated using one-way ANOVA with Tukey's post hoc test.

3.2 Human data

3.2.1 Human cohort characteristics

For the human data, a Western Blot analysis was conducted using tissue obtained from organ donors. Among these donors, 4 had NF hearts, 6 exhibited DD, and 5 were diagnosed with DCM.

The non-failing group had a mean age of 53 years (± 14.9), with 40% being female. The mean EF was 61.2% (± 6.1). The Diastolic dysfunction group had a mean age of 60 years (±7.9), 33.3% were female, with an EF mean of 62.2% (±5.4), thereby belonging to the HFpEF group. According to the ESC guidelines, any EF over 50% is generally regarded as normal (McDonagh *et al.*, 2021).

The group with dilated cardiomyopathy had a mean age of 46.6 (± 10.6), with 40% being female. This group exhibited a reduced EF with a mean of 26% (± 10.6), thereby classifying them as HFrEF. Body Mass Index (BMI) was comparable across all groups, averaging around 27 kg/m² which is classified as overweight (Table 12).

Human Cohort Characteristics

Group	NF	DD	DCM
Age	53,6 (± 14,9)	60 (±7,9)	46,6 (±10,6)
Gender (% female)	40%	33,3%	40%
BMI	27,6 (± 5,2)	27,8 (±5)	27,1 (±6)
EF (%)	61,2% (± 6,1)	62,2% (±5,4)	26% (±10,6)

Table 12: Human cohort characteristics for the immunoblot experiment

Parameters from human donors, with N=4 not failing (NF), N=6 diastolic dysfunction (DD), N=5 dilated cardiomyopathy (DCM). Mean value with standard deviations.

3.2.2 β2AR is decreased in humans during diastolic dysfunction

The expression of β2AR in humans showed a significant decrease during DD (p=0.0255) and a trend towards significance in DCM (p=0.0534) compared to the non-failing group. The comparison between the DD and DCM groups revealed no statistical significance (Figure 22).

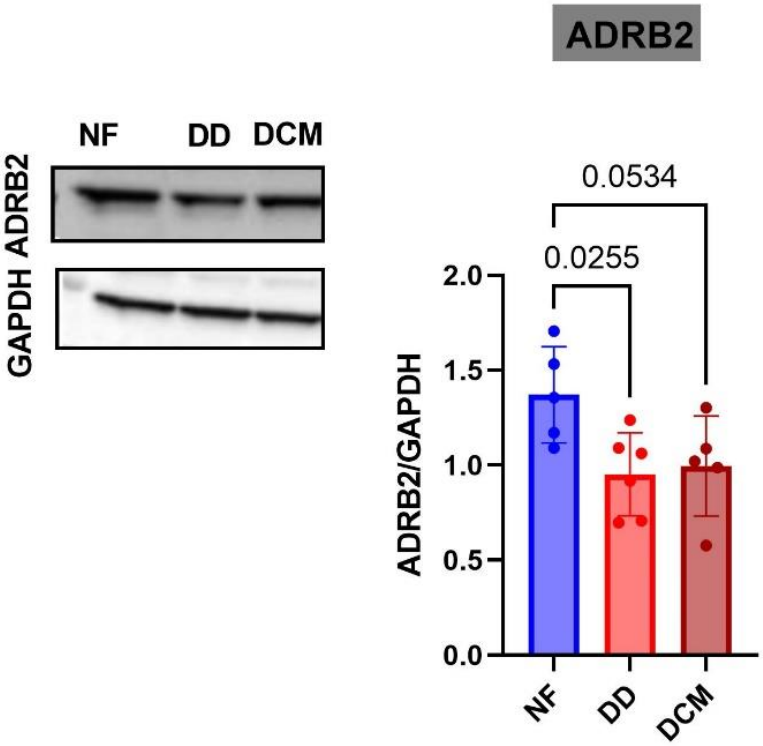

Figure 22: β2AR immunoblot of the left ventricle in humans with not failing (NF) hearts, hearts with diastolic dysfunction (DD), or dilated cardiomyopathy (DCM).

Immunoblot with ADRB2 antibody to detect β2AR in left ventricular tissue lysates. GAPDH was used for normalization. Corresponding quantification of β2AR in NF (not failing) blue N=5, DD (diastolic dysfunction) light red, N=6, DCM (dilated cardiomyopathy) dark red N=5 human hearts. Statistical analysis was performed using Kruskal-Wallis with Dunn's post-hoc test.

4. Discussion

4.1 HSD-fed Dahl salt-sensitive rats develop a hypertensive phenotype with hypertrophy, preserved ejection fraction, and diastolic dysfunction

Research indicates that Dahl salt-sensitive rats serve as a valid animal model for investigating the onset of hypertension-induced cardiac remodeling. These rats demonstrate hypertrophy and diastolic dysfunction comparable to human cardiovascular disease (van Ham *et al.*, 2022).

In our investigation, Dahl salt-sensitive rats fed a high-salt-diet exhibited a hypertensive phenotype, showing relevant systolic and diastolic hypertension within two weeks and left ventricular hypertrophy. Gravimetric and cellular analyses confirmed hypertrophy, with cellular myocyte hypertrophy apparent at an early stage and gravimetric changes becoming evident later. A similar study by Hasan et al. with Dahl salt-sensitive rats at 15 weeks also observed cellular myocyte hypertrophy (Hasan *et al.*, 2018). Notably, significant heart weight to body weight differences were found at nine weeks (Kimura *et al.*, 2019), and studies reported gravimetric differences at weeks 12-13 (Klotz *et al.*, 2006), (Gallet *et al.*, 2016).

The delay in finding relevant heart weight-to-body weight differences in our study compared to others raises discussion points. Matzer et al. suggested differences in gravimetric methods as a potential factor, with two techniques employed: one for organ collection only, directly measuring wet weight after removal of excess blood, and another for Langendorff perfusion, measuring the weight of the heart in a beaker with perfusion solution, wherein the weight of beaker and solution were later subtracted (Matzer, 2021).

Previous studies using this animal model linked it to HFpEF when exposed to a HSD (Abdellatif *et al.*, 2021), (Nakajima *et al.*, 2019), (van Ham *et al.*, 2022). The study from Doi et al. also developed a Dahl-salt-sensitive model with different heart failure phenotypes depending on the HSD starting timepoint. Rat groups starting at seven weeks developed isolated diastolic heart failure while groups starting at eight weeks developed both systolic and diastolic dysfunction (Doi *et al.*, 2000).

In the data provided in this thesis, definitive conclusions regarding the development of HFpEF in our model are not possible. Echocardiographic measurements revealed a preserved EF in the Dahl salt-sensitive rat model. However, assessing E/e' proved to be more challenging. Van Ham et al. suggest that in the Dahl salt-sensitive rat model, an E/e' value exceeding 20 indicates elevated filling pressures, a criterion met by our HSD group. Despite this, Van Ham et al. acknowledge the limited data on E/e' parameters in Dahl salt-sensitive rats, suggesting that normal parameters might not be firmly established (van Ham *et al.*, 2022).

Echocardiography inherently introduces significant subjective bias, particularly dependent on the analyzer. Absolute values should only be used when plentiful data is provided such as in measurements in humans. In humans, an E/e' ratio higher than 15 is a sign of diastolic dysfunction (Hagendorff *et al.*, 2015). The ESC describes an E/e' ratio >9 to have a higher risk of cardiovascular mortality (McDonagh *et al.*, 2021). Our study minimized this bias by conducting all measurements through a single analyzer, Dr. Julia Voglhuber-Höller. However, in animal models where data points are limited, references from the control group are more appropriate to provide valid results compared to absolute values.

This thesis only utilized a subset of the data, primarily from cohorts used for the β2AR experiments, and failed to demonstrate significant changes in E/e' parameters compared to the control group, precluding a definitive HFpEF diagnosis. However, Dr. Julia Voglhuber-Höller's thesis (unpublished), which encompasses a larger sample size and multiple rat cohorts, observed a significant increase in E/e' compared to the control group, allowing for a more reliable diagnosis of HFpEF. Thus, with this additional data, we can conclude that the Dahl salt-sensitive rat model indeed developed HFpEF.

The observed hypertrophy may be the reason why the high-salt-diet group showed a higher ejection fraction at the early 12 week timepoint, possibly adapting to the pressure overload from hypertension. Both groups had EF around 80% which is considered normal (van Ham *et al.*, 2022).

Another important feature to diagnose heart failure in humans is the clinical symptoms, such as shortness of breath, peripheral edema, and weakness. These symptoms could be translated to the rat model as decreased activity levels and weakness compared to the control group (Zhang *et al.*, 2020). These parameters may have given more insight if our rat model developed heart failure with preserved ejection fraction.

With the additional data from Dr. Julia Voglhuber-Höller's thesis, we can conclusively state that our Dahl salt-sensitive rat model has effectively developed hypertension accompanied by left ventricular hypertrophy and diastolic dysfunction, thus characterizing the model as representing HFpEF.

4.2 β2AR expression decreases in rats that develop hypertension in late-stage remodeling

The work of Bristow et al. with human failing and non-failing heart tissue analyzed β-adrenergic subpopulations using radioligand binding techniques. In their study, they observed that the β1:β2 adrenergic receptor profiles change in the failing heart predominately due to β1AR downregulation. (Bristow *et al.*, 1986). However, this finding contrasts with the study using radioligand bindings indicating β2AR receptor downregulation plays a significant role during heart failure in humans (Takahashi *et al.*, 1992).

In a study on rats, Zhao et al. demonstrated a more pronounced downregulation of β2AR compared to β1AR upon norepinephrine stimulation (Zhao, Hagler, and Muntz, 1996). In the case of Dahl salt-sensitive rats, Puleo et al. found elevated plasma norepinephrine levels, likely applicable to animals in our study, and would thus favor β2AR downregulation (Puleo *et al.*, 2020).

We observed a significant downregulation of β2AR in HSD-fed Dahl salt-sensitive rat cardiomyocytes at the 17 week late timepoint, in both ICC experiments: baseline and stimulated with ISO. This finding was replicated in our microarray experiments with HSD-fed Dahl-salt-sensitive rat heart tissue. Considering that hypertension is characterized by higher levels of norepinephrine and epinephrine, it is plausible to expect a certain amount of β2AR downregulation. The pathophysiology may be

explained by an increase in GRK during cardiac remodeling, leading to greater GPCR desensitization and internalization, as β2AR is a GPCR (see section: Molecular mechanisms of GPCR; page: 16).

The observed downregulation was not recapitulated in the immunoblot analysis, as previously discussed. Possible contributing factors include the limited sample size and the nature of immunoblotting, which detects protein fractions from denatured proteins. Furthermore, heart tissue from one of the first Dahl salt-sensitive rat cohorts was used, which were euthanized at 19 weeks, so two weeks later than the other cohorts. This may lead to another factor that should be put into consideration when analyzing the results. Notably, immunoblotting encompasses the entire ventricular tissue, comprising a variety of cell types expressing β2AR, such as vasculature, fibroblasts, or immune cells. This cellular diversity may introduce variations when compared to ICC, where only cardiomyocytes were examined. The microarray analysis also utilized the whole ventricular tissue and demonstrated a downregulation exclusively in the late stage of remodeling, thereby adding complexity to the previous argument. Additional experiments to quantify β2AR in immunoblotting should be repeated with a higher sample size, and ideally, in isolated ventricular cardiomyocytes.

Combining the results, our experiments indicate that hypertensive cardiac remodeling coincides with β2AR receptor downregulation at a late timepoint both in genetic expression as well as receptor concentration in the cell membrane.

4.3 β2AR receptor is downregulated when stimulated with ISO in LSD rats

The capacity for acute adjustments in cardiac output during heightened cardiac demand is a well-established effect of β-adrenergic stimulation. In instances of diminished responsiveness to β-adrenergic signaling, as noted in heart failure (Bristow *et al.*, 1982), individuals experience a decline in contractile function during physiological stress or exercise.

In experimental settings, the conventional approach to emulate β-adrenergic tone involves administering the synthetic catecholamine ISO (Matzer, 2021).

Consequently, our study focused on investigating the impact of acute ISO treatment on the expression of β2AR in cardiomyocytes undergoing cardiac remodeling.

Treatment with ISO leads to rapid desensitization of β2AR (Moffett *et al.*, 2001). This downregulation in healthy cells is probably a compensatory mechanism to prevent cellular exhaustion. Rapid agonist-promoted desensitization is achieved through phosphorylation of the β2AR by a specific GRK, also known as βARK and PKA (Moffett *et al.*, 2001). The study suggests that long-term stimulation, as seen in a hypertensive state, may lead to persistent phosphorylation of the receptor and contribute to long-term desensitization. Here, the enzyme PKA seems to play a more significant role in desensitization when cells are treated long-term with a β-agonist, as it exhibits significantly lower activity in this state (McGraw *et al.*, 1998). Receptor internalization also appears to play a role but at a much slower rate compared to beta ARK and PKA (Roth *et al.*, 1991).

In our experiments, we observed rapid fluorescence signal downregulation in the LSD group when treated with ISO, which may be explained by phosphorylation of the receptor through PKA and GRK. The downregulation was not significant in the HSD group, indicating a potential change or dysfunction in rapid receptor desensitization and internalization. As discussed earlier, the hypertensive state with higher catecholamine levels may already lead to β2ARs being downregulated making them less responsive to rapid changes in catecholamine levels (see section: 4.2 β2AR expression decreases in rats that develop hypertension in late-stage remodeling, page: 62).

Conclusively, ventricular cardiomyocytes lose their responsiveness to fast changes of ISO in the late stage of cardiac remodeling regarding β2AR regulation.

4.4 β2AR is downregulated in humans with diastolic dysfunction

The identification of β2AR downregulation in the human samples with diastolic dysfunction classifying as a HFpEF is a noteworthy discovery, facilitating the translation of insights derived from animal models to clinically relevant human scenarios. A statistically significant β2AR downregulation was not evident in dilated cardiomyopathy, though a trend towards downregulation was observed.

It is worth noting that this human experiment with immunoblotting, with a total sample size of N=16, might show a significant difference compared to the rat immunoblotting results, which had a smaller group size of N=12. The increased sample size in the human study could potentially contribute to the observed differences and variations in statistical significance.

While ICC experiments offer the advantage of detecting both acute and long-term effects, immunoblotting is primarily suited for analyzing long-term effects. Important to say is that human heart tissue was used during the immunoblots, not specific cells. Given the inherent limitations associated with the immunoblotting experiments, the next step would be replicating the findings through ICC experiments of cardiomyocytes and microarray analyses in human heart cells and tissue. Immunoblots with bigger sample sizes could additionally give more statistical strength to our findings. Additional investigation would validate the observed patterns and strengthen the translational relevance of the research with rat models, bridging the gap between preclinical models and clinical implications.

4.5 Comparing β2AR with β1AR expression behavior

In our experiments on isolated Dahl salt-sensitive rat cardiomyocytes with HFpEF at a late timepoint of 17 weeks, the downregulation pattern was similar for both receptors, with β2AR having a downregulation of 14.15% and β1AR being 10.45% downregulated, contradicting Bristow's results in human heart failure which state that β2AR concentration does not change significantly during heart failure. The method used in this study involved radioligand bindings from tissue samples obtained from organ donors with dilated cardiomyopathy exhibiting a HFrEF and pulmonary hypertension (Bristow *et al.*, 1986). These differences in results may be attributed to challenges in translating animal models to the human context, variations in heart failure types (HFrEF vs. HFpEF), as well as differences in samples (heart tissue vs. cardiomyocytes).

The argument that different etiologies might have different receptor profiles is underlined through the study of Brodde's et al. It found that ischemic cardiomyopathy and mitral valve disease in humans led to a decrease of both β1AR

as well as β2AR while idiopathic dilated cardiomyopathy led to isolated β1AR downregulation (Brodde *et al.*, 1989), (Michel, Maisel, and Brodde, 1990).

In literature, the stage of remodeling also plays a role. Takahashi et al. observed a higher downregulation of β2AR in later stages of heart failure (NYHA II-IV) in their *in vivo* study with humans, while β1 downregulation started occurring in mild heart failure. They studied this through the evaluation of adrenoreceptor-mediated contractility measured by echocardiography, adrenergic receptor assays, and radioligand bindings. In the essays, the relations were β1:β2 NYHA I 62:37 proportion, while at NYHA IV, it was 56:44, with both receptors being significantly downregulated. The groups were very diverse with NYHA IV patients having dilated cardiomyopathy, amyloidosis, sarcoidosis, or ischemic cardiomyopathy. NYHA I patients had a preserved EF while the severer cases had a mean EF of 48% (Takahashi *et al.*, 1992).

The timepoint of downregulation is also an intriguing parameter and can coincide with NYHA stages. In the Dahl salt-sensitive rat heart tissue microarray and immunoblots, we differentiated between an early timepoint of 12 weeks and a late timepoint of 17 weeks for the microarray and 19 weeks for the immunoblot. We observed that there was a tendency for downregulation in the immunoblots especially at a late timepoint, though this was not statistically relevant. The microarray on the other hand could show a statistically relevant downregulation on a late timepoint of 17 weeks. At the early timepoint, there were no relevant findings on the β2AR downregulation. β1AR downregulation on the other hand is already found at early timepoint (Matzer, 2021). It would be intriguing to collect ICC data during the early stages of cardiac remodeling, specifically at the 12 week timepoint. This would aim to reproduce the microarray results indicating that β2AR downregulation predominantly occurs in the later stages of cardiac remodeling.

Additionally, my results from human immunoblotting coincide with the fact that patients with HFpEF exhibit β2AR downregulation. This was not reproduced in patients with dilated cardiomyopathy, which were characterized by an HFrEF, indicating that different heart failure subtypes may have a different β-adrenergic profile.

Unlike β1AR, which exhibits staining in distinct regions like perinuclear areas (Matzer, 2021), β2AR showed an overall fluorescence, suggesting their presence on the cell membrane. This finding aligns with existing literature asserting that β2ARs are predominantly expressed on the cell membrane in t-tubules and caveolae (Gorelik *et al.*, 2013), (MacDougall *et al.*, 2012). We did not observe a distinct change in expression behavior in terms of localization on the cell surface during cardiac remodeling, only a reduction in overall fluorescence. This limitation arises from our methods, which cannot differentiate where the β2AR is being expressed on the cell surface. As mentioned above, the literature suggests that in heart failure β2AR ceases to be confined to the structural indentations of the cell membrane and is expressed throughout the cell surface (see section: 1.2.2.5 β2AR pathophysiology in heart failure, page: 25). This phenomenon was identified through the observation of β2AR-mediated cAMP signals (Nikolaev *et al.*, 2010). Another drawback of ICC is that the quality of the quantitative analysis depends on the subjective assessment of the analyzer, which we addressed in this work by strictly predefined analysis criteria (read: 2.6.2 ICC staining; page: 42).

Given the diversity of heart failure types, it is crucial to continue studying β1:β2 downregulation with different types of heart failure and diverse animal models. This will lead to more differentiated data and results, providing a better understanding of the mechanisms behind βAR signaling during heart failure.

5. Conclusion

In the present study, we describe changes and alterations occurring in early (12 weeks) - and late-stage (17 and 19 weeks) cardiac remodeling focusing on β2AR expression and emphasizing the late-stage timepoint. These observations were conducted utilizing a Dahl salt-sensitive rat model and experiments involving human cardiac tissue.

1. A significant downregulation of β2AR was observed especially at late timepoints in the microarray of heart tissue and ICC analyses of cardiomyocytes of the Dahl salt-sensitive rat model. Conversely, rat heart tissue immunoblots failed to reproduce this observation.
2. Impaired rapid-ISO induced β2AR downregulation was observed in HSD Dahl-salt-sensitive rat cardiomyocytes.
3. β2AR downregulation was observed in human heart tissue immunoblot for diastolic dysfunction, but not for dilated cardiomyopathy.
4. Rates of β1AR and β2AR downregulation in Dahl salt-sensitive rat cardiomyocytes in the ICC at late timepoints were found to be comparable.

6. Future perspectives

The complexity of adrenergic receptor profiles seems greater than previously conceived, displaying variations due to heart failure cause, stage, and classification (HFpEF, HFrEF).

To further solidify and expand our results, a more extensive investigation of the early timepoint and a replication of the Immunoblot experiments with an increased sample size is needed. Another experiment of great value would be to redo the immunoblots only with isolated cardiomyocytes, endothelial cells, lymphoid cells, and myeloid cells. This would help to understand the receptors' role in the specific cell. Reproducing the remaining experiments, microarray, and ICC on human tissue would guarantee the translational value of our results. Microarray analysis on healthy human heart tissue has already been done by "the human heart atlas" and brings novel insight into this topic, such as the distribution of β2AR in different cell types of the heart (*cellxgene*, 2023).

Given the ongoing debate surrounding Gαi signaling of the β2AR in human heart failure, a compelling avenue for investigation involves conducting a microarray analysis on Gαi and Gαs proteins. This analysis would shed light on potential differences in early- and late-stage remodeling. Examining downstream molecules, such as cAMP, ERK1/2, PI3K, Akt, and MAPKp38, while blocking the β1AR would provide further insights into the role of β2AR in the heart and during heart failure. This approach may unveil additional information regarding the predominant downstream signaling pathways in various scenarios.

7. References

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