From the Department of Laboratory Medicine
Karolinska Institutet, Stockholm, Sweden

Physical Capacity, Physical Activity and Skeletal Muscle in Heart Failure: Studies of Pathophysiology

Michael Melin

Stockholm 2021

All previously published papers were reproduced with permission from the publisher.
Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021

ISBN **978-91-8016-361-3**

Physical Capacity, Physical Activity and Skeletal Muscle in Heart Failure: Studies of Pathophysiology

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Michael Melin

The thesis will be defended in public in lecture hall 4V, ANA 8, Alfred Nobels Allé 23, Stockholm, 2021-11-05 9.00 AM

Principal Supervisor:
Associate Professor Eric Rullman
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Physiology

Co-supervisors:
Professor Thomas Gustafsson
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Physiology

PhD Inger Hagerman
Karolinska Institutet
Department of Medicine, Huddinge

Opponent:
Professor Jan Engvall
Linköping University
Department of Health, Medicine and Caring Sciences
Division of Diagnostics and Specialist Medicine

Examination Board:
Professor Jan Henriksson
Karolinska Institutet
Department of Physiology and Pharmacology

Associate Professor Elin Ekblom Bak
The Swedish School of Sport and Health Sciences, GIH
Department of Sport and Health Sciences

Professor Gustav Smith
Gothenburg University
University of Gothenburg
Department of Molecular and Clinical Medicine

To Aino, Cecilia, Ella and Cleo

POPULÄRVETENSKAPLIG SAMMANFATTNING

I avhandlingen har olika samband mellan det sviktande hjärtat och periferin hos hjärtsviktpatienter studerats för att belysa bakomliggande patofysiologiska mekanismer och möjlig prognostisk valör av biomarkörer. Nya sätt att mäta kvalitativa aspekter på fysisk aktivitet baserade på accelerometer visade sig ha stark bäring på prognosen hos patienter med svår hjärtsvikt, även som tillägg till de bästa prognostiska modellerna som finns att tillgå idag. I studierna har också utnyttjats metoder för att mäta ett stort antal cirkulerande proteiner (targeted proteomics) för att finna biologiska processer, som kopplar samman mått på det sviktande hjärtat med fysisk aktivitet och prestationsförmåga. Metoden kunde bekräfta några väl etablerade samband mellan hjärtats pumpförmåga, cirkulerande så kallade natriuretiska peptider och prognos men identifierande också ett antal proteiner kopplade till inflammation, extracellulär matrix remodellering, celltillväxt och celladhesion samt angiogenes som betydelsefulla för både fysisk prestationsförmåga och hjärtfunktion vid svår hjärtsvikt. Studierna har också visat att ett protein av betydelse för kontraktionsförmågan (RyR1) i skelettmuskeln verkar vara förändrad genom så kallade posttranslationella förändringar hos patienter med hjärtsvikt. Undersökning av en specifik behandlingsmetod vid hjärtsvikt, som har liknats vid passiv träning, påvisade inga signifikanta förändringar av genexpression och talade emot adaptiva förändringar i skelettmuskulaturen som förklaring till ökad gångförmåga. Sammanfattningsvis har studiena påvisat samband som kan belysa möjliga patofysiologiska mekanismer vid hjärtsvikt som delvis kan spegla periferins inflytande. Studierna har identifierat vissa cirkulerande proteiner kopplade till processer som angiogenes och även till prognos. Förändringar i ett protein av möjlig negativ betydelse för kontraktionsförmågan och nya, kvalitativa aspekter på fysisk aktivitet utgör länkar som binder samman det sviktande hjärtat med periferin.

ABSTRACT

The overall aim of the present thesis was to provide a better understanding of the pathophysiology of heart failure (HF), especially to explore possible mechanistic links between the failing heart and the periphery, as well as to explore variables with possible prognostic utilisation.

In **Study I** we asked if the degree of variability in physical activity (PA) could hold prognostic value. We examined 60 patients with HF, using echocardiography, blood sampling, VO_2 peak and accelerometer. Accelerometer-derived variables were analysed for covariance using a PCA, bi-plotted together with mortality and added to the established clinical score, HFSS, in Cox regression models. Skewness and kurtosis, measurements of asymmetry in intensity level of periods of high PA, were analysed. **Conclusion:** skewness had additive value to predict all-cause mortality.

In **Study II** we asked if we could identify links between physical capacity, PA, myocardial function and circulating proteins, comparing patients with HF with controls, and if circulating proteins could hold prognostic information. We examined 66 patients and 28 controls, with echocardiography, blood sampling, VO_2 peak and accelerometer. Circulating proteins were quantified via a multiplex immunoassay. Proteins that differed between groups and that were linked with prognosis were identified using OPLS-DA and univariate analyses. **Conclusion:** 10 circulating proteins covaried with physical capacity, PA and myocardial function, identifying possible links in HF pathophysiology, and 8 of these carried prognostic information.

In **Study III** we asked if circulating proteins could give insights into disease progression and prognosis.16 patients with HF were followed for 2 to 4 years. Depending on changes in LVEF, VO_2 peak and NT-proBNP between inclusion and follow-up, the patients were divided into stable or deteriorated. Data was analysed, at baseline (t-test) as were the changes between baseline and follow-up (ANOVA). **Conclusion:** 10 circulating proteins covaried with disease progression, while 5 different circulating proteins were prognostic.

In **Study IV**, we asked if skeletal muscle in patients with HF undergoes ryanodine receptor 1 (RyR1) posttranslational remodelling. 8 patients with HF and 7 controls were examined using VO_2 peak, echocardiography, NT-proBNP, accelerometer and lateral vastus muscle biopsies. Biopsies were analysed with immunoblots. **Conclusion:** skeletal muscle RyR1 was post-translationally modified, excessively phosphorylated, S-nitrolysated and oxidized in HF.

In **Study V**, we asked if EECP in HF patients showed significant up or down-regulation of gene expression in skeletal muscle. 9 patients had 7 weeks of EECP. Before and after, lateral vastus muscle biopsies and 6MWT were obtained. Quality of life (QoL) was assessed by MLHF questionnaire. Skeletal muscle expression was analysed using microarray transcriptional profiling with subsequent differential expression and network analysis. **Conclusion:** EECP significantly improved 6MWT. QoL remained unchanged. No significantly expressed genes were identified, ruling out skeletal muscle adaptation as the reason behind increase in 6MWT.

SAMMANFATTNING

Det övergripande målet med avhandlingen var att, hos patienter med hjärtsvikt, studera bakomliggande patofysiologiska mekanismer som sammanbinder det sviktande hjärtat med periferin, samt att utforska variabler med möjlig prognostisk användning.

I **studie I** studerade vi om variabiliteten i fysisk aktivitet mätt med accelerometer kan ha prognotisk information. Vi undersökte 60 patienter med hjärtsvikt med ekokardiografi, blodprover, VO_2 peak och accelerometer. Variabler från accelerometern analyserades för samvarians med hjälp av PCA och en biplot gjordes. **Slutsat**s: Skewness, ett mått på variabiliteten i fysisk aktivitet, hade additivt prognostiskt värde utöver Heart Failure Survivial Score och VO_2 peak.

I **studie II** studerade vi möjliga samband mellan fysiologiska, prognostiska variabler (fysisk kapacitet, fysisk aktivitet och ejektionsfraktion) och cirkulerande protiener för att utröna potentiella patofysiologiska och prognostiska samband. Vi undersökte 66 patienter med hjärtsvikt och 28 kontroller, med ekokardiografi, blodprover, VO_2 peak och acclerometer. Cirkulerande proteiner kvantifierades med immunoassay. **Slutsat**s: Analyser med OPLS-DA, PCA och MI networks visade 10 cirkulerande proteiner som samvarierade med fysisk kapacitet, fysisk aktivitet och ejektionsfraktion, samt 8 av dessa hade prognotisk valör.

I **studie III** följde vi upp 16 av patienter från kohorten i studie II över en period på 2 till 4 år. Utifrån en sammanvägning av förändring i fysiologiska, prognostiska variabler (VO_2 peak, ejektionsfraktion och NT-proBNP) delades patienterna in i stabila eller försämrade. **Slutsats:** 10 cirkulerande proteiner samvarierade med sjukdomsprogression, medan 5 andra cirkulerande proteiner hade prognostisk valör.

I **studie IV** undersökte vi om skelettmuskulaturen hos patienter med hjärtsvikt genomgår posttranslationell remodellering av ryanodine receptor 1 (RyR1). 8 patienter med hjärtsvikt och 7 kontroller undersöktes med VO_2 peak, ekokardiografi, NT-proBNP, accelerometer och biopsier av vastus lateralis. Biopsierna analyserades med immunoblots. **Slutsats:** RyR1 var nitrolyserad, fosforylerad och oxiderad hos patienterna med hjärtsvikt.

I **studie V** undersökte vi om enhanced external counterpulsation (EECP) ökade gångsträckan hos patienter med hjärtsvikt och om denna ökning kunde förklaras av förändringar i skelettmuskulaturen. **Slutsats:** EECP ökade signifikant gångsträckan men inga signifikanta förändringar i genexpressionen i skelettmuskeln kunde detekteras.

LIST OF SCIENTIFIC PAPERS

I. Melin M, Hagerman I, Gonon A, Gustafsson T, Rullman E

Variability in Physical Activity Assessed with Accelerometer is an Independent Predict of Mortality in CHF Patients

PLOS ONE 2016 Apr 7;11(4) https://doi.org/10.1371/journal.pone.0153036

II. Rullman E, Melin M, Mandić M, Gonon A, Fernandez-Gonzalo R, Gustafsson T

Circulatory Factors Associated with Function and Prognosis in Patients with Severe Heart Failure

Clinical Research in Cardiology 2020 Jun;109(6):655-672

III. Melin M, Hagerman I, Mandić M, Lovric A, Gustafsson T, Jansson E, Rullman E

Circulating Proteins in Progression and Pathophysiology of Heart Failure with Reduced Ejection Fraction

In manuscript

IV. Rullman E, Andersson DC, Melin M, Reiken S, Mancini DM, Marks A R, Lund LH, Gustafsson T

Modifications of the Skeletal Muscle Ryanodine Receptor Type 1 and Exercise Intolerance in Heart Failure

J Heart Lung Transplant 2013 September; 32(9): 925-929

V. Melin M, Montelius A, Rydén L, Gonon A, Hagerman I, Rullman E

Effects of enhanced external counterpulsation on skeletal muscle gene expression in patients with severe heart failure

Clin Physiol Funct Imaging 2016 August; 2018 Jan;38(1):118-12

LIST OF ABBREVIATIONS

ACEi	Angiotensin-converting enzyme inhibitor
ANP	Atrial natriuretic peptide
ARB	Angiotensin receptor blocker
ARNI	Angiotensin receptor neprilysin inhibitor
BMI	Body mass index
BNP	Brain natriuretic peptide
CPM	Counts per minutes
CPX	Cardiopulmonary exercise test
CRT	Cardiac resynchronization therapy
CV	Coefficient of variation
DAVID	Database for annotation, visualization, and integrated discovery
EECP	Enhanced external counterpulsation
EF	Ejection fraction
eGFR	Estimated glomerular filtration rate
ESC	European Society of Cardiology
FDR	False discovery rate
FGF-2	Fibroblast growth factor 2
HF	Heart failure
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
HFSS	Heart failure survival score
HR	Heart rate
ICD	Implantable cardioverter defibrillator
IL-6	Interleukin-6
IGF-1	Insulin-like growth factor-1
IPA	Ingenuity pathway analysis
IQR	Interquartile range
KCCQ	Kansas City Cardiomyopathy Questionnaire

LA-area	Left atrium area
LBBB	Left bundle branch block
LIMMA	Linear models for microarray data
LVAD	Left ventricular assist device
LVEF	Left ventricular ejection fraction
MAGGIC	Meta-analysis Global Group in Chronic Heart Failure risk score
MAP	Mean arterial pressure
MI	Mutual information
MLHFQ	Minnesota Living with Heart Failure Questionnaire
MRA	Mineral receptor antagonist
MR-proADM	Mid-regional pro-adrenomedullin
NT-proBNP	N-terminal pro–B-type natriuretic peptide
NYHA	New York Heart Association
OPLSDA	Orthogonal projections to latent structures discriminant analysis
OUES	Oxygen uptake efficiency slope
PA	Physical activity
PCA	Principal component analysis
PON3	Paraoxonase 3
PSV	Peak systolic velocity
QoL	Quality of life
RAAS	Renin angiotensin aldosterone system
RBBB	Right bundle branch block
RMA	Robust Multi-array Average
ROS	Reactive oxygen species
RT-PCR	Real-time quantitative polymerase chain reaction
RyR	Ryanodine receptor
RyR1	Ryanodine receptor 1
RyR2	Ryanodine receptor 2
RyR3	Ryanodine receptor 3
SAM	Significance Analysis of Microarrays

SD	Standard deviation
SHFM	Seattle Heart Failure Mode
SF-36	Short Form-36 Health Survey
SGLT1-inhibitors	Sodium glucose cotransporters1-inhibitors
SGLT2-inhibitors	Sodium glucose cotransporters2-inhibitors
6MWT	Six-minute walk test
SR	Sarcoplasmatic reticulum
ST2	Suppression of tumorigenicity 2
TAPT5	Tartrate-resistant acid phosphatase type 5
TfR1	Transferrin receptor protein 1
TNFα	Tumour necrosis factor alpha
TNFR1	Tumour necrosis factor receptor 1
TNFR2	Tumour necrosis factor receptor 2
VE/VCO_2 slope	Minute ventilation/carbon dioxide release
VO_2 peak	Peak oxygen uptake

CONTENTS

1 INTRODUCTION

Heart failure (HF) is ‘a clinical syndrome characterised by typical symptoms that may be accompanied by signs, caused by a structural and/or functional cardiac abnormality. This results in in a reduced cardiac output and/or elevated intra-cardiac pressures at rest or during stress’ (1,2). The overall prevalence of HF is about 1–2% in the western world with an estimated 64.3 million people affected globally (3). The prevalence is believed to increase due to more people living longer and better survival for myocardial infarction (4). In HF quality of life (QoL) is generally poor (5,6). Common causes of HF are ischemic heart disease and hypertension as well as valve disease (1,2).

1.1 Variability in physical activity: role in prognosis

HF is in most cases a chronic, progressive disease that despite advances in medical treatment, surgical procedures and device therapy has a grim prognosis, and ultimately leads to death - generally there is a 50% five-year mortality (7), and in severe HF one-year mortality could be as high as 50% (8). Despite improvement in prognosis in the twentieth century, prognosis has not improved significantly in later years (9). There is a great heterogeneity in the rate of disease progression (10). In some patients the deterioration is rapid despite adequate or aggressive treatment, but in others treatment can (temporarily) hinder the disease progression. Different prognostic variables and tools are used for identification of patients most at risk of dying. It is difficult to accurately identify which patients have the worst prognosis, especially in patients in a clinical grey-zone of moderately to severely impaired aerobic exercise capacity (11), as well as to identify which patients will deteriorate rapidly, despite the prognostic tools available. For the clinician it is important to determine prognosis to enhance management, improve patient outcomes and decide which patient is most in need of more aggressive treatment such as heart transplantation or left ventricular assist device (LVAD). Many clinicians ask for more refined prognostic tools.

Measuring left ventricular ejection fraction (LVEF) is a cornerstone in the diagnosis of HF, and for determining prognosis (12,13). However, there is a weak correlation between LVEF at rest and peak oxygen uptake (VO_2 peak), which holds the strongest prognostic value in HF (14–17). To aid the clinician in evaluating prognosis, several scoring models are available. The HF survival score (HFSS) prediction model , Meta-Analysis Global Group in Chronic (MAGGIC) HF Risk Score and the Seattle Heart Failure Model (SHFM) are validated multivariate risk scores, that are used in the evaluation for heart transplantation or LVAD (18–20). The HFSS prediction model combines VO_2 peak with selected clinical variables.

Impaired aerobic exercise capacity is a hallmark of HF (21–23). Cardiopulmonary exercise test (CPX), or VO_2 peak, is considered gold standard in determining prognosis in HF (24,25). Other parameters derived from CPX also carry prognostic information, e.g. the minute ventilation/carbon dioxide production (VE/VCO_2 slope) and oxygen uptake efficiency slope (26–28). 6-minute walk test (6MWT) is sometimes used to assess aerobic exercise capacity, because it is less time consuming than CPX and does not require elaborate equipment or technically trained personnel. While neither precision nor accuracy of 6MWT is as good as VO_2 peak, 6MWT also carries prognostic information in HF (29). Patients with HF have generally somewhat lower levels of physical activity (PA) compared to healthy individuals

(30,31). PA is correlated with aerobic exercise capacity in HF (32). Studies with accelerometers in patients with HF, have shown that low daily PA correlates to a poor New York Heart Association Functional Class (NYHA) (33) and high mortality (34). Da Silva et al. (35) evaluated PA patterns from accelerometers and potential association with CPX variables and showed a strong correlation between PA and aerobic exercise capacity in HF. Considering the almost linear associations between PA and exercise capacity, we questioned if measuring mere volumes of PA could hold any additive information above VO_2 peak, or if other aspects of PA should be evaluated. Interestingly, patients with severe HF have a distinct walking pattern with frequent stops (36). This walking pattern has the potential of containing additive information that could be of value. Therefore, in **Study I** we asked if variability in PA could be identified and characterised through analysis of accelerometer data and if the degree of variability could hold additive prognostic value on mortality above established survival scores.

1.2 Links between failing heart and periphery: role of circulating proteins

The pathophysiology of HF is complex and involves activation of renin-angiotensin-aldosterone system (RAAS), vasopressin and the sympathetic system (37). The effects of the neurohormonal activation are fluid retention, leading to increased filling pressures and direct increased contractility aiming to restore cardiac output (38). The adaptive mechanisms with neuroendocrine activation contributes to the damaging processes that affect several other organs such as renal, respiratory and skeletal muscle, resulting in a vicious circle with an increased load on the heart and more impaired function (remodelling) (39,40). The activation of the sympathetic system leads to peripheral vasoconstriction to maintain circulation of central organs and the brain (38).

The role of circulating proteins is increasingly acknowledged in HF management for making the diagnosis and determining prognosis. One of the most used in clinical context is N-terminal pro–B-type natriuretic peptide (NT-proBNP), which is a strong prognostic marker to identify patients with high risk of mortality (41,42). However, the prognostic value of NT-proBNP is weaker in HF with mid-reduced ejection fraction (HFMrEF) and HF with preserved ejection fraction (HFpEF) compared to HF with reduced ejection fraction (HFrEF) (43). Many other circulating proteins carry prognostic information, like galectin-3, insulin-like growth factor-1 (IGF-1), interleukin-6 (IL-6), mid-regional pro-adrenomedullin (MR-proADM), suppression of tumorigenicity 2 (ST2), tumour necrosis factor-alpha (TNFα), however these are not commonly used in clinical practice (44–49). If a circulating protein holds prognostic value, it is reasonable to assume it could play a role in the pathophysiology and disease progression of HF but there are gaps in knowledge that need to be filled. Therefore, in **Study II** we wanted to explore possible associations between circulating proteins and established prognostic variables, such as physical capacity, PA, myocardial function, to identify pathophysiological links that connect circulating proteins with the failing heart to the periphery, as well as to explore variables with possible prognostic ability.

1.3 Disease progression and prognosis: role of circulating proteins

Heart failure is a progressive disease that deteriorates over time but there is a large heterogeneity in how fast a patient deteriorates (50). Despite recent advances in HF therapy, many patients experience an increase in symptom burden over time as the HF progresses (51). However, symptoms of HF are often under-recognized and as a consequence thereof the HF is left undertreated (52). There is also a discrepancy between symptoms and objective measures of functional capacity (53). An observation shared by many clinicians is that risk stratification of HF patients is difficult. Despite predictable patterns of progression that parallel worsening of the HF syndrome and survival risk score models available, it is difficult to accurately identify the individual patient most at risk of dying. Some patients with a seemingly good prognosis deteriorate fast, while other patients with a bad prognosis according to prognostic tools live on for years (50,54,55). By analysing changes at baseline and follow-up in three prognostic, physiological variables (VO_2 peak, LVEF, NT-proBNP), and studying association with circulating proteins, our hypothesis was that it would be possible to explore mechanistic links related to disease progression by identifying patients that deteriorated in HF. Therefore, in **Study III** we asked if circulating proteins measured at follow-up could give insights into disease progression and thereby on pathophysiology. We also asked if circulating proteins at baseline could carry prognostic information on disease progression.

1.4 Reduced exercise capacity in heart failure: role of skeletal muscle

The mechanisms behind reduced exercise capacity in HF have historically been attributed to the malfunction of the heart as a pump, with systolic dysfunction and reduced cardiac output. However, even though the pathophysiology of HF starts with an abnormality of the heart, strong evidence shows that central hemodynamic factors, correlates poorly with exercise capacity (14,56,57). Ventricular dysfunction or peak cardiac output is not the only limiting factor in exercise capacity in HF (58), in contrast to subjects without HF where cardiac output is the major limiting factor (59). Supporting this are studies that have showed gains in aerobic exercise capacity and VO_2 peak in HF, without demonstrated improvements in cardiac output, stroke volume or LVEF (60). The skeletal muscle undergoes a variety of alternations in HF, including muscle atrophy, alterations in fibre type, reduced mitochondrial enzymes, and decreased mitochondrial volume density (61–63). The muscle hypothesis is derived from the fact the exercise-limiting symptoms in HF, skeletal muscle fatigue and dyspnoea, could be explained by abnormalities in peripheral blood flow and in the skeletal muscle, in addition to disturbances in central haemodynamic blood flow (23). The exact mechanisms behind skeletal muscle fatigue and the contribution to exercise intolerance as well as symptoms are not fully understood. Fatigability has been shown in small muscle groups in which blood flow is unlikely to be limited by cardiac reserve, which supports the fact that intrinsic muscle factors mediate fatigability, and dysfunctional Ca^{2+} handling has been suggested as a possible mechanism (64). Skeletal muscle contraction is a complex process that involves excitation-contraction coupling. The muscular action potential activates the voltage-gated L-type Ca^{2+} channels dihydropyridine receptors (DHPRs). The action potential spreads across the cell surface and into the muscle fibre's network of T-tubules, depolarizing the inner portion of the muscle fibre. The depolarization activates DHPRs in the terminal cisternae, which are near

the calcium-release channel, ryanodine receptor (RyR), in the adjacent sarcoplasmic reticulum (SR) and physically interact with RyR to activate them. By this, RyR opens and releases calcium from the SR into the cytoplasm. The calcium released into the cytosol binds to the troponin C present on the actin-containing thin filaments of the myofibrils, to allow cross-bridge cycling, and thereby enabling production of force. RyR1 is primarily found in skeletal muscle (65,66), RyR2 in myocardium (67,68), and RyR3 in the brain (69). Animal HF models have shown that maladaptive changes of the RyR1, including excessive phosphorylation, S-nitrolysation, oxidation and depletion of the stabilizing protein FK506 binding protein 12 (FKBP12 or calstabin1), play an important role in the disturbed calcium handling. However, no studies of skeletal muscle in human HF have been performed. In animal models, elevated levels of catecholamines, which is seen in HF, leads to hyperphosphorylation of RyR1 in skeletal muscle (70,71). We wanted to get a better understanding of the maladaptive calcium metabolism in HF. Therefore, in **Study IV**, we asked if skeletal muscle in human HF undergoes RyR1 posttranslational remodelling as shown in animal models and if this is associated with reduced exercise capacity.

1.5 Effects of External Enhanced Counterpulsation: role of skeletal muscle

There exists robust evidence for the treatment of HF with reduced ejection fraction (HFrEF) with several treatments that improve survival. Treatment could be divided into non-pharmacological, pharmacological, implantation of devices and surgical. Examples of medicines that increase survival are angiotensin-converting enzyme inhibitor (ACEi), angiotensin receptor blocker (ARB), angiotensin receptor neprilysin inhibitor (ARNI), betablockers, mineral receptor antagonist (MRA), sinus node inhibitor, and recently the addition of sodium glucosecotransporters2 (SGLT2)-inhibitors (1,72–85). Diuretics are often needed but should be kept at minimal dose possible (1,2,86). All symptomatic patients with HFrEF, unless there are contraindications, should be on pharmacological treatment of ACEi, or ARNI, betablockers, MRA and SGLT2-inhibitors (2). Devices are used as the next step in the treatment algorithm, such as implantable cardioverter defibrillator (ICD) and treatment of electrical dyssynchrony with cardiac resynchronization therapy (CRT) (increased width ECG QRS ≥150 ms (class I indication according to ESC guidelines 2021) or 130-149 ms (class IIa indication) particularly seen in left bundle branch block (LBBB)) in patients with LVEF ≤ 35% despite optimal medical treatment (2,87). Heart transplantation and LVAD are the final treatment options available when everything else is done (2).

Despite several novel treatment options, prognosis for patients with HF has not improved much in recent years (9). Physical activity and exercise programmes are recommended in the current guidelines and improves QoL, wellbeing and physical function. There is however little support that exercise programmes decrease mortality or hospitalisations (31,88,89). External Enhanced Counterpulsation (EECP), a treatment used for refractory angina, has been put forward as a potential new treatment in HF. EECP, where cuffs around the legs inflate and deflate and thereby increase blood flow to the heart, is sometimes described as a form of passive training. Studies have shown improvement in physical capacity with increased walking distance (90,91). However, the mechanisms behind this improved physical capacity remains elusive as there seems not to be any significant central hemodynamical effect to explain the improvement. Therefore, in **Study V**, we asked if treatment with EECP

in HF affects gene expression in skeletal muscle. This would indicate that the effect of EECP om physical capacity is mediated by peripheral changes.

2 RESEARCH AIMS

The overall aim of the present thesis was to explore possible mechanistic links between the failing human heart and the periphery, as well as to explore variables with possible impact on pathophysiology and prognosis.

Specifically, the objectives were:

1) To explore if a high degree of variability in physical activity could be identified and characterised through an analysis of accelerometer data as well as predict all-cause mortality in heart failure in addition to established prognostic factors.
2) To explore associations between circulating proteins and established prognostic models including myocardial function, physical capacity and physical activity in heart failure in relation to mortality.

3) To explore if changes in circulating proteins in heart failure differed between patients that remained stable in relation to those with deteriorating clinical status over time and to explore possible links behind disease progression.

4) To investigate if skeletal muscle in heart failure displayed posttranslational RyR1 remodelling.

5) To explore if external enhanced counterpulsation, used in treatment of heart failure, had an effect on skeletal muscle and evaluate possible effect on physical capacity as well as quality of life.

3 MATERIAL AND METHODS

3.1 Methods at a glance

Table 1 summarises the design, number of participants, study population and main statistics and bioinformatics used in **Studies I-V**:

Study	I	II	III	IV	V
Design	Cross-sectional	Case control	Cross-sectional, longitudinal	Cross-sectional, case control	Prospective, interventional
Numbers of participants	60	HF: 66 C: 28	16	HF: 8 C: 7	9
Age, years	70	HF: 70 C: 70	Baseline: 69 Follow-up: 72	HF: 65 C: 71	62
EF, %	24	HF: 25 C: 58	Baseline: 26 Follow-up:27	HF: 24 C: 61	Before: 19 After: 24
NYHA	III	III	III	III	III-IV
VO_2 peak, mL/(kg x min)	10	HF: 13 C: 24	Baseline: 16 Follow up:14	HF: 16 C: 29	-
6MWT (m)	-	-	-	-	Before: 329 After: 377

Variables are means, medians, frequencies or categories.

C Controls, HF Heart Failure.

3.2 Study populations

In **Studies I, II** and **III**, the same population of patients with severe HF, was investigated. The patients had HF with reduced ejection fraction (HFrEF), with LVEF ≤ 35%, and were in NYHA-class III. The patients were predominantly male, clinically stable and had not been hospitalized within the last 8 weeks prior to study enrolment. In **Study II** an age-matched control group was also included, consisting of 28 patients that were referred to the out-patient clinic because of dyspnoea, but where HF was ruled based om LVEF >50% and NT-proBNP levels <300 ng/L. In **Study IV**, a cohort of eight patients with HF was investigated. The

patients all had severe HF, NYHA III, EF ≤35% and NT-proBNP >300 ng/L. A control group of seven age-matched healthy controls was included. The absence of HF was defined as LVEF >50% and NT-proBNP <300 ng/L. The patients and controls were predominantly males. In **study V** a second cohort of nine patients with severe HF, NYHA III-IV, LVEF ≤35% was investigated. The patients were clinically stable and were not readmitted during the study. The patients were all male.

3.3 New York Heart Association classification

The severity of symptoms is often described by the New York Heart Association (NYHA) classification. Patients with HF grade their symptoms in relation to physical activity (PA) by means of a self-reported form. Patients in NYHA class I have no limitation of PA, NYHA II experience symptoms upon strong PA, NYHA III experience symptoms upon light PA, whereas patients in NYHA class IV have symptoms at rest and cannot carry out any PA without discomfort (92). The NYHA classification is related to prognosis (93–95).

3.4 Echocardiography

LVEF is widely used as a phenotypic parameter to classify HF. In modern terminology, HF is classified into three groups: HFrEF with LVEF ≤ 40%, HF with mildly reduced ejection fraction (HFmrEF) with LVEF 41-49% and HF with preserved ejection fraction (HFpEF) with LVEF ≥ 50% (2).

Echocardiography uses sound waves that are reflected by the different structures of the heart as well as the blood within to produce images (96). Echocardiographic assessment gives information about the heart´s dimensions, and through the doppler phenomena information about valvular function as well as systolic and diastolic function.

Systolic function is a proxy for contractility but not a direct measurement. Using echocardiography, systolic function can be assessed by different methods, e.g. fractional shortening, fractional area change, LVEF, stroke volume and systolic myocardial velocity. In the context of HF and all five studies we assessed LVEF as a marker for systolic function and defined systolic HF as LVEF ≤ 35%, which was also the inclusion criteria in all studies. The most common ways of calculating LVEF is by using Teicholtz method (97) or Simpson's method (98). Of these, Simpson´s method is currently recommended in clinical guidelines (99) and the method used in all studies. In Simpson's method, LV area is measured at end diastole and end systole in both four-chamber view as well as two-chamber view. (98). TAPSE is a marker for right ventricular function, where measurements of the longitudinal displacement of the tricuspid annulus is performed (100,101). LVEDD and LA-area was measured for dimensions.

Diastolic function is measurement of the heart´s ability for relaxation. Diastolic function is criteria-based and graded. Diastolic function can be assessed by combining various measurements, e.g. mitral in flow E/A-wave, transmitral flow velocity, left atrial volume index as well as tricuspid regurgitation velocity (102,103). In **Study II** assessment of tissue doppler index E/É and É was used for estimation of left ventricular filling pressure and diastolic dysfunction. PA-pressure was calculated by tricuspid valve velocity (according to the formula $4v^2$ = TV pressure gradient) + estimated CVP (104–106). Assessing diastolic

function in sinus rhythm is easy, however assessing diastolic function in patients with atrial fibrillation or who have pacemakers is difficult, because of the missing atrial contraction.

In all five studies, echocardiographic measurements were carried out in the same manner and in accordance with clinical guidelines (Vivid 7, General Electric Healthcare, Little Chalfont, United Kingdom). The echocardiograms were analysed by an echocardiographer blinded to the specific clinical history of the patient.

3.5 Functional capacity

There are several ways of measuring functional capacity. NYHA-classification is an easy self-administered questionnaire assessing activity and exercise limitation. NYHA-classification is routinely used in the clinic to stratify the severity of HF in patients. NYHA I indicate that the patient has no symptoms or limitation of physical exercise. NYHA II means slight limitation of physical exercise, whereas NYHA III means marked limitation of physical exercise. NYHA III is often divided into NYHA IIIa and IIIb, the latter means that the patient can walk 200 meters at the most without stopping for rest. NYHA IV indicates that the patient has symptoms of HF at rest and is unable to perform any physical activity without discomfort. Notwithstanding its very crude estimations, NYHA-classification has a surprisingly good prognostic impact and is easy to use (107). In all studies NYHA-classification was used to stratify the severity of HF.

The six-minute walk test (6MWT) is a submaximal exercise test that entails measurement of distance walked over a span of 6 minutes. 6MWT is widely used and has advantages over VO_2 peak; simple, inexpensive, less patient discomfort. The reproducibility of 6MWT has been shown to be good (108). In **Study V** 6MWT was used as assessment of functional capacity due to feasibility.

During a cardiopulmonary exercise test (CPX), peak oxygen uptake (VO_2 peak) was measured, as it holds strong prognostic information. In fact, VO_2 peak is considered gold standard in estimating functional capacity in HF (17). VO_2 peak is a diagnostic procedure to measure oxygen consumption during a maximal exercise test. VO_2 peak is affected by pulmonary and cardiovascular factors including the ability to extract oxygen in peripheral tissues and haemoglobin concentration (59), as well noncardiac factors like age, gender and muscle mass. Further insights into cardiopulmonary exercise testing have resulted in other variables than mere VO_2 peak being used in the risk stratification in HF, like VE/VCO_2 slope and the presence of exercise oscillatory ventilation, both among the strongest predictors of poor outcome in HF (55,109,110). VE/VCO_2 slope has the advantage of carrying prognostic information even when the exercise test is submaximal, in contrast to VO_2 peak (111).

In **Studies I, II, III** and **IV** VO_2 peak was performed for measures of functional capacity, for risk stratifying HF as well as for providing prognostic information. In **Study V** we examined the effect of EECP on functional capacity in patients with HF. Functional capacity was assessed by 6MWT. In hindsight, VO_2 peak would have provided more information, but was not part of the study protocol.

VO_2 peak was performed in **Studies I, II, III** and **IV**. The test consisted of maximum symptom-limited exercise either on a cycle ergometer (increments of 10 W every 60 s) or on

a treadmill (1 m/s with a stepwise increase in the angle of 0.5 degree/min). Continuous assessment of gas-exchange data (Vmax, Sensor Medics, Anaheim, CA, USA) was performed. The exercise was terminated due to volitional exhaustion and/or the patient's inability to maintain the correct speed despite strong verbal encouragement. In **Study V** a 50-m course was marked in the hospital corridor and patients were instructed to walk from end to end at their own pace with the objective of walking as far as possible within six minutes (112).

3.6 Borg Rating of Perceived Exertion (RPE) Scale®

The Borg RPE scale is a rating scale used to estimate perceived physical exertion and goes from 6 to 20 (104). The Borg RPE scale was used in **Studies I, II, III, IV** and **V**.

3.7 Daily physical activity

Measurements of daily PA describes various measures of time spent active or inactive in different exercise intensities during daily living. These measurements include self-assessment questionnaires, pedometers and accelerometers. Accelerometric assessment of daily PA is considered gold standard (113). Accelerometers measure acceleration and integrate the data collected into intensity of activity. Accelerometers are used to assess the total activity and time spent at varying intensities of activity. Usually intensity is integrated into one-minute intervals. The temporal resolution in accelerometers is better than measurements of PA derived from devices like implantable cardioverter defibrillator (ICD) and cardiac resynchronization therapy (CRT) (114,115), where data is often compressed into a period of a day. A pedometer only measures discrete movement and is sensitive to the correct use. Unlike pedometers, accelerometers are not as sensitive to correct positioning on the body (116).

In **Studies I, II** and **III** PA was assessed using accelerometers and the various intensity of activity was evaluated. The patients were instructed to wear the accelerometer for 7 consecutive days. The definition of non-wear time was 60 consecutive minutes of 0 cpm, with allowance for 1-2 minutes of 0-99 cpm. Intensity of activity was assessed using the following accelerometric metrics: 1) total number of minutes the monitor was worn; 2) sedentary time (vertical axis cpm <100); 3) light activity time (vertical axis cpm between 100 and 1951); moderate vigorous physical activity time (vertical axis cpm greater than 1952). 1952 cpm corresponds to walking at 4 km/h. In **Study II** episodes of continuous PA were identified by applying a rolling mean to the raw data and 1-, 3- and 12-hour periods with the highest mean activity over the recorded 7-day period. In these three time periods an average activity was measured, and variables used for pattern recognition in accelerometers were calculated: IQR, skewness and kurtosis. High skewness indicates high levels of activity during a particular time, whereas kurtosis is a measure of 'peakedness' and also illustrates the number of outliers.

In **Studies I, II** and **III** accelerometers were used by the patients and the controls in the same manner. Daily activity was assessed in all participants by accelerometers (GT3X; Actigraph, Pensacola, FL, USA), which were mailed to all patients within 6 weeks of the CPX measurements. The patients were instructed to attach the accelerometer to their waist belt upon rising in the morning and to remove it only for showering, bathing, and sleeping. The

monitors were set to begin collecting data 1 day before the delivery date, as estimated by the postal service, and to continue recording data until they were downloaded. The patients were asked to return the monitor by mail using a prepaid return envelope after having worn it for 7 consecutive days. Raw data collected by the accelerometer were integrated into 60-s epochs using ActiLife software with the normal filter option and expressed as cpm. Wear time was estimated using the algorithm described by Troiano et al (117).

3.8 Heart Failure Survival Score

There are several prediction models used in determining prognosis in HF. Perhaps the most established prediction model in HF to date is HFSS (118), that uses seven parameters including variables taken from echocardiography, blood sampling, VO_2 peak, ECG and medical history (19). Another prognostic score model, Seattle Heart Failure Model (SHFM), uses 20 variables combining clinical, laboratory and therapeutic data (18) while Meta-Analysis Global Group in Chronic Heart Failure (MAGGIC) risk score is composed of 13 clinical variables (119).

We used HFSS since it is one of the most studied prediction models in HF and, unlike SHFM and MAGGIC, includes VO_2 peak as a measure of physical capacity.

HFSS was calculated by summarizing the beta-coefficients of VO_2 peak, LVEF, resting heart rate, serum sodium, ischemic aetiology (categorical) and intraventricular conduction delay (categorical) for each patient in accordance with Aaronson et al. (19). HFSS was used in **Studies I** and **V**.

3.9 Quality of life

There are several validated instruments to measure quality of life (QoL) in patients with HF. One of the most frequently used generic measures is the SF-36 (19,120). Other instruments include the Kansas City Cardiomyopathy Questionnaire (KCCQ) (121) and Minnesota Living with Heart Failure Questionnaire (MLHFQ) (122). Of these, MLHFQ is the most commonly used in studies of the QoL in hospital settings and has been shown to have good reliability and validity. In comparison to SF-36, MLHFQ is considered better for patients with more advanced HF and more sensitive to changes over time (123).

In **Study V** QoL was assessed using the disease specific MLHFQ (122). MLHFQ is a 21-question self-assessment questionnaire addresses the physical, social, emotional, dietary and economic limitations and the side-effects of treatment typical for HF. MLHFQ assesses HRQoL, from 0 (none) to 5 (very much). It provides a total score (range 0–105, from best to worst HRQoL). The questionnaire assesses both physical (8 items, range 0–40) and emotional (5 items, range 0–25) aspects.

3.10 External Enhanced Counterpulsation

External Enhanced Counterpulsation (EECP) is used to relieve pain and to increase physical capacity in patients with refractory angina pectoris (124). It gives subjective and objective reduction of myocardial ischemia and is considered an established treatment (124–126). In HF, EECP treatment is still considered to be experimental.

In **Study V**, the EECP equipment (Vasomedical, Westbury, New York) comprised an air compressor, a console, a treatment table, and two sets of three cuffs. Treatment was given for 1 hour per day, 5 days per week, for a total of 35 hours. Before the start of each treatment session, the cuffs are positioned around the legs (127). The cuffs are thereafter sequentially inflated and deflated in synchrony with the patient's electrocardiogram. A finger plethysmograph monitors diastolic and systolic pressure waveforms. From the systolic and diastolic curves two ratios are computed. A ratio >1 is considered as optimal treatment, corresponding to diastolic values greater than the systolic pressure (128). In early diastole, a pressure of 260 mmHg is applied sequentially in order to propel blood back to the heart. This augments the diastolic pressure which in turn increases coronary perfusion pressure, and also leads to a reduction of afterload and increased venous return with a subsequent increase in cardiac output (129,130). At end diastole, air is instantaneously released from all cuffs, reducing vascular impedance and decreasing peripheral vascular resistance.

3.11 Muscle biopsies

Skeletal muscle biopsies of the vastus lateralis muscle were performed at rest, using the Bergström needle technique (131). The biopsies were frozen in liquid nitrogen and stored at -80 °C until processing. In **Study IV** analysis of skeletal muscle RyR1 complex was performed with quantification of phosphorylation, S-nitrosylation, oxidation and calstabin1 association to the RyR1. In **Study V** gene expression analysis of the muscle biopsies was performed, as was analysis of specific mRNA.

3.12 Blood samples

Blood samples were collected from a vein with the subject in a fasting state in the morning in EDTA-coated tubes. The blood samples were put on ice and then centrifuged; plasma was aliquoted and stored at −80 °C until analysis.

3.13 Protein measurements

3.13.1 Multiplex immunoassay

Immunoassays is a technique to measure the presence or concentration of proteins, through binding of antibody to antigen.

The Proseek Multiplex (Olink Bioscience, Uppsala, Sweden), is a 92-plex immunoassay based on a proximity ligation extension assay. Proximity extension assays use target-specific antibody pairs that are linked to DNA strands that, upon simultaneous binding to the target analyte, create a real-time polymerase chain reaction amplicon by the action of a DNA polymerase. In **Studies II** and **III** we used the Proseek Multiplex immunoassay, because of its major advantage over conventional multiplex immunoassays in that only correctly matched antibody pairs give rise to a signal

3.13.2 Immunoprecipitation

Immunoprecipitation is a technique of precipitating a protein antigen out of solution. It is a technique that uses an antibody that specifically binds to a specific protein. The advantage is

that the process can isolate and concentrate a specific protein from samples containing many different proteins.

3.13.3 Immunoblot

Immunoblotting is a technique to detect specific proteins. After denaturation, the sample undergoes gel electrophoresis, where the protein is separated through an electric current. An antibody is added and recognizes and binds to a specific target protein. A second antibody is then added, binding to the primary antibody. Via immunofluorescence, the second antibody is visualized.

In **Study IV** skeletal muscle samples were isotonically lysed. RyR1 was immunoprecipitated by incubating homogenate with anti-RyR antibody (Affinity Bioreagents, Boulder, CO, USA) followed by immunoblotting with anti-calstabin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-RyR1-pSer2843 (local production in the laboratory), and anti-SNO (Sigma, St. Louis, MO, USA) antibodies. To assess oxidation-mediated formation of carbonyls on RyR2, the immunoprecipitate was treated with 2, 4-dinitrophenyl hydrazine (DNP), and derivatized carbonyls were detected using an OxyBlot Protein Oxidation Detection Kit (Chemicon, Temecula, CA, USA). Quantifications were normalized to total RyR1.

3.14 RNA measurements

3.14.1 RNA – extraction

Fifteen mg of skeletal muscle tissue was homogenized in 0.5-ml TRIzol, and the RNA was extracted using TRIzol (Sigma, Sigma-Aldrich, Saint Louis, Missouri, USA), in accordance with the manufacturer's instructions (132). The quality of the extracted total RNA was analysed by spectrophotometry and chromatography (Agilent Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA).

3.14.2 Microarray

Microarray is a technique to analyse gene-expression by DNA hybridization. RNA is extracted from tissues or cells, reversed-transcribed and labelled with a dye (usually fluorescent), and hybridized on the prefabricated array. See 3.14.1 for the procedure of RNA extraction. The amount of fluorescence reflects the amount of mRNA in the sample.

In **Study V** we used Affymetrix for analysis of DNA microarrays. Gene-expression was analysed on the Affymetrix HuGene 1.0 ST1 gene chip at the Bioinformatics and expression core facility. Quality control and normalization were carried out using the AffyPLM and Oligo packages from Bioconductor on the R platform. As a means to control the quality of the individual arrays, all arrays were examined using hierarchical clustering, Normalized Unscaled Standard Error (NUSE), a variance-based metric to identify outliers and normalised using robust multiarray average (RMA).

3.14.3 Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-PCR), is a technique that measures the amplification of nucleic acids during the PCR. In conventional PCR, mRNA is amplified and

measured at the end of the amplification. The RT-PCR products are identified and quantified via fluorescence through binding to specific DNA or RNA. The advantage of RT-PCR is that it allows for sensitive, specific and reproducible quantitation of DNA and RNA.

In **Study V** one microgram of total RNA was reverse transcribed to cDNA by Superscript reverse transcriptase (Life Technologies) using random hexamer primers (Roche Diagnostics) in a total volume of 20 µl. Detection of mRNA was performed on an ABI-PRISM7700 Sequence Detector (Perkin-Elmer Applied Biosystems). Primer and probe were ordered as assay on demand (IGF-1: Hs00153126_m1; FGF-2: Hs00960934_m1; GAPDH: 4352934E; Perkin-Elmer Applied Biosystems). Target gene expression was calculated by 2-ΔCT using GADH as reference gene.

3.15 Main statistics and bioinformatics

3.15.1 T-test

Continuous variables were tested using t-test in **Studies II**, **III**, **IV** and **V**. T-test is used for group and paired comparisons. Statistical significance was set at $p < 0.05$ (two-tailed).

3.15.2 ANOVA

In **Study III** repeated two-way ANOVAs were carried out to analyse the effect of group (stable and deteriorated) and time (baseline and follow-up) and of interaction between group and time. A p-value of 0.05 was considered significant.

3.15.3 Wilcoxon matched-pairs signed-ranks test

Wilcoxon's test is a nonparametric statistic test and was used in **Study V** for paired comparisons.

3.15.4 Chi-square test

In **Study II** and **IV** chi-square test was used to test frequencies. Chi-square test is nonparametric test for categorical data. Chi-square test compares how a model compares to observed data, comparing the size of discrepancies between the expected and actual results.

3.15.5 Fischer's test

In **Study II** Fischer's test was used. Fischer´s test is a nonparametric test for categorical data. It can be used when the chi-square test cannot, such as with small sample sizes. Generally, Fisher's exact test is preferable to the chi-squared test because it is an exact test. The chi-squared test should be particularly avoided if there are few observations (e.g. less than 10) for individual cells. Since Fisher's exact test may be computationally infeasible for large sample sizes and the accuracy of the chi-square test increases with a larger number of samples, the chi-square test is a suitable replacement in this case. Another advantage of the chi-square test is that it is more suitable for contingency tables whose dimensionality exceeds 2×2.

3.15.6 C-index

C-index, or concordance statistic, was used in **Study I**. C-index is equal to the area under the Receiver Operating Characteristic (ROC) curve. It measures the probability that a patient that

has been affected by an event is at greater risk than a patient that has not. C-index ranges from 0.5 to 1.

3.15.7 Cox proportional regression

In **Studies I** and **II** Cox proportional regression was used. Cox proportional regression measures the effect of several variables upon the time a specified event takes to happen.

3.15.8 Multiple hypothesis compensation

In **Study I,** Bonferroni correction was used to manage the problem of multiple hypothesis tests.

In **Study V**, false discovery rate (FDR) was used. FDR is used when conducting multiple comparisons, and is defined as the expected proportion of false positives among the declared significant results (133,134). Microarray studies are not suitable for the P-value scale, and FDR has an advantage with greater power, however with the risk of increased Type I errors (false negatives).

3.15.9 Principal Component Analysis

In **Studies I** and **II** Principal Component Analysis (PCA) was used together with biplots to characterise variance of the individual variables and to identify collinearities. All variables were scaled to unit variance and mean-centred. PCA is a way to reduce multidimensionality of large sets of data. A large set of variables is transformed into a smaller variable that maintains most of the information. A biplots is a graph, a form of two-variable scatterplot, with variables visualized as vectors, linear axes or nonlinear trajectories. A biplot can incorporate both continuous and categorical variables.

3.15.10 Orthogonal Projections to Latent Structures Discriminant Analysis

In **Study II** Orthogonal Projections to Latent Structures Discriminant Analysis **(**OPLS-DA) was used. OPLS-DA is similar to PCA but is used for classification

rather than correlation. OPLS-DA is a classification model constructed using the rOPLS-library in R. Each value was represented by a loading value compared with what was predicted (patient or control). In short, the OPLS model finds the multidimensional direction in the X space that explains the maximal variance in the Y space. OPLS regression is particularly suitable when the matrix of predictors has more variables than observations and when there is multicollinearity among X values.

3.15.11 Differential gene expression analysis

In **Study V** Significance Analysis of Microarray (SAM) was used. SAM is a non-parametric, permutation-based statistical test for identifying significant genes in a microarrray (135). It calculates the empirical False-Discovery Rate (FDR). FDR was set at p-value <0.1, corresponding to a FDR of less than 10%.

In **Study V** Linear Models for Microarray data **(**LIMMA) was used for analysing microarrays and RNA data.

3.15.12 Functional analysis of differential gene expression

In **Study V** Ingenuity Pathways Analysis **(**IPA) (IPA, http://www.qiagen.com) is a web-based bioinformatics application allowing researchers to upload data analysis results from microarray and next generation sequencing for functional analysis and integration (136).

In **Study V** Database for Annotation, Visualization and Integrated Discovery (DAVID) was used. DAVID is a set of functional annotation tools for researchers to comprehend biological meaning behind large sets of genes.

3.16 Ethical considerations

Ethical approvals were obtained for all studies. The studies were conducted in accordance with the Declaration of Helsinki. All data was handled using good research ethics.

Patients with severe HF have poor quality of life and prognosis. Therefore, recruitment to participate in research projects can be a delicate matter. In this dissertation project, biological material was collected from both patients and healthy volunteers. For many participants, it should be noted that the disadvantages of participation (inconvenience of collecting biological material) are probably less important than the advantages (contributing to a good cause). Skeletal muscle biopsies are invasive, but the risk of permanent damage is low and the pain during the procedure is brief. For patients suffering from HF, it can be stated that the disadvantages of participating in a study are likely outweighed by the benefits of study participation for the individual. Research can benefit individuals directly and/or indirectly in terms of their health, perhaps not today, but hopefully in the future. When it comes to recruiting patients, it is important to consider the dependency that patients feel towards the healthcare system and caregivers. To minimise the risk of patients participating against their true will, it is important to emphasize that participation is voluntary and without consequences. It is important to minimize the fear of, for example, worse treatment and care. It is important to emphasize that it is possible to discontinue participation at any time and that discontinuation can occur without justification. The studies we conducted cannot be considered interchangeable, and similar studies in experimental animals would not be clinically relevant. As the clinical problems addressed were of importance to many patients, any potential gain in knowledge was of great interest.

4 RESULTS

4.1 Study I – 'Variability in Physical Activity Assessed with Accelerometer is an Independent Predictor of Mortality in CHF Patients'

Protocol:

Sixty patients with HF were enrolled in the study over a period of three years, and the patients underwent echocardiography, blood sampling and VO_2 peak. Individual HFSS score was calculated from these measurements. Daily physical activity was assessed by an accelerometer worn for a period of seven days. 23 patients died during follow-up. The deaths were categorized as cardiovascular.

The protocol included:

1) The degree of variability was assessed from the accelerometer data. Various variables were used to describe physical activity pattern, including skewness and kurtosis.
2) Accelerometer-derived variables were analysed for covariance using PCA including bi-plots for mortality.
3) Accelerometer-derived variables were analysed with regard to all-cause mortality and added to a baseline model utilizing Heart Failure Survival Score (HFSS).
4) The predictive value was assessed by c-index.

Results:

1) Analysis of accelerometer derived variables showed that a high degree of variability in periods of high intensity level could be identified and characterised.
2) The PCA analysis identified a high degree of covariance amongst the different accelerometer-derived variables: 69 % of variance was explained by principal components 1 and 2. The PCA analysis further showed that all patients who died earlier than 36 months correlated positively with 1, 3 and 12 h skewness.
3) All accelerometer-derived variables were analysed with regard to all-cause mortality and added to a baseline model utilizing HFSS scores. The variables with the most significant contribution to mortality were 1, 3 and 12 h skewness.
4) The predictive value of adding peak 3h skewness to established prognostic factors (HFSS) was tested and showed that the addition of peak 3h skewness to HFSS had additive value to predict all-cause mortality (likelihood ratio $p<0.02$). The c-index increased to 0.74 (CI, 0.69–0.78).

4.2 Study II – 'Circulatory Factors Associated with Function and Prognosis in Patients with Severe Heart Failure'

Protocol:

Sixty-six patients with HF and 28 controls were enrolled in the study over a period of three years. The patients were examined for circulating proteins as determined in blood samples as well as with echocardiography, VO_2 peak and NT-proBNP. Daily activity was assessed by an accelerometer worn for a period of seven days. Of the 66 monitored patients, 42 died during follow-up (median time, 1.8 years). All mortality events were categorized as cardiovascular.

The protocol included:

1) Circulating proteins were quantified via a multiplex immunoassay. The array contained 92 circulating proteins. By using OPLS-DA circulating proteins that differed significantly between patients and controls were identified.
2) By using PCA and MI networks, links between circulating proteins and established prognostic models including myocardial function, physical capacity and PA were identified.
3) Lastly, association between circulating proteins and all-cause mortality were analysed by using univariate Cox regression crude analysis and by multiple Cox regression controlled for the established prognostic markers: age, eGFR, VO_2 peak and LVEF. FDR was set to <5%.

Results:

1) Thirty-nine circulating proteins that differed significantly between patients and controls were identified.
2) Ten circulating proteins differentially expressed in patients with HF versus controls covaried with physical capacity, daily PA, and myocardial function. These ten circulating proteins were Galectin-4, GDF15, IGFBP7, NT-proBNP, PON3, ST2, TfR1, TRAP5, TNFR1, TNFR. According to MI networks analysis, these ten circulating proteins were involved in inflammation, extracellular matrix remodelling, cell adhesion and migration and angiogenesis. These circulating proteins provide a possible link between peripheral function and systolic function and could be a part of the pathophysiology of HF.
3) Eight of these ten circulating proteins correlated with mortality. The eight circulating proteins were Galectin-4, GDF15, IGFBP7, NT-proBNP, ST2, TfR1,TNFR1, TNFR2. Six factors remained associated with all-cause mortality, after controlling for the established prognostic markers age, eGFR, VO_2 peak, and LVEF. These circulating proteins were Galectin-4, GDF15, IGFBP7, ST2, TfR1,TNFR1.

4.3 Study III – 'Circulating Proteins in Progression and Pathophysiology of Heart Failure with Reduced Ejection Fraction'

Protocol:

Sixteen patients with HF were followed over a period of two to four years. The patients were examined for circulating proteins as determined in blood samples as well as with echocardiography, VO_2 peak and NT-proBNP at inclusion and follow-up.

The protocol included:

1) Circulating proteins were quantified via a multiplex immunoassay. The array contained 92 circulating proteins selected with a cardiovascular profile. Depending on changes in LVEF, VO_2 peak and NT-proBNP between inclusion and follow-up, the patients were divided into stable (n=7) or deteriorated (n=9).
2) Disease progression was analysed by comparing the changes between baseline and follow-up for the groups stable and deteriorated using two-way ANOVA.
3) The prognostic information was analysed by comparing the groups stable and deteriorated by studying the baseline values of the circulating proteins using t-test.

Results:

1) Analyses of the changes between baseline and follow-up showed that ten circulating proteins were significantly different between the two groups stable and deteriorated: FABP4, JAMA, MMP-9, PDGF subunit-A, PECAM-1, PLC, SELP, TIMP4, TNFRSF14, and uPAR. These circulating proteins were associated with disease progression.
2) Analyses at baseline showed that five circulating proteins were significantly different between the groups stable and deteriorated: CD93, CHIT1, IGFBP 7, MB and ST2. These circulating proteins carried prognostic information on disease progression.

4.4 Study IV – 'Modifications of the Skeletal Muscle Ryanodine Receptor Type 1 and Exercise Intolerance in Heart Failure'

Protocol:

Eight patients with HF and seven controls were examined with echocardiography, VO_2 peak, NT-proBNP, and muscle biopsies from vastus lateralis.

The protocol included:

1) Daily physical activity was assessed by an accelerometer worn for a period of seven days.
2) Skeletal muscle biopsy specimens were analysed for post-translational changes in the RyR1 complex with immunoblots and quantification of
    a) phosphorylation.
    b) S-nitrosylation, the oxidation marker DNP.
    c) calstabin1 association to the RyR1.
3) All values are mean ± SD, and statistical group comparison was made with t-test, $p < 0.05$.

Results:

1) Data from accelerometers showed that the patients and controls were equally sedentary in terms of percent active time. During the seven days, the number of episodes of sustained activity lasting longer than five minutes were fewer in HF patients than in controls (1.6±2, 16±17).
2) RyR1 from patients with HF as compared to controls
    a) was excessively Protein Kinase A phosphorylated (quantification of immunoblot in arbitrary units, 3.6±0.3, 1.9±0.2 $p < 0.001$).
    b) was excessively S-nitrosylated (4.2±0.5, 2.1±0.4, $p < 0.001$).
    c) was excessively oxidized (4.0±0.2, 2.0±0.4 , $p < 0.001$).
    d) was depleted of calstabin1 (0.9±0.3, 3.0±0.2, $p < 0.001$).

4.5 Study V – 'Effects of enhanced external counterpulsation (EECP) on skeletal muscle gene expression in patients with severe heart failure'

Protocol:

Nine patients with HF were subjected to 35 hours of EECP five times a week for seven weeks and examined before and after treatment with echocardiography, VO_2 peak, NT-proBNP, and muscle biopsies from vastus lateralis.

The protocol included:

1) Gene expression in skeletal muscle was analysed.
2) Functional capacity was assessed with 6MWT.
3) Quality of Life (QoL) was assessed with Minnesota Living with HF Questionnaire.
4) Symptoms and HF severity were assessed with NYHA classification, echocardiography and plasma NT-proBNP.
5) Global gene expression was analysed by microarray. The results were evaluated by SAM for differentially expressed genes as well as by network analysis. Conformation was done by RT-PCR. Thereafter, comparison of the gene expression profile with previously published datasets was performed.

Results:

1) Skeletal muscle expression did not detect any significantly differentially expressed genes after EECP treatment.
2) Maximum walking distance increased by an average of 15% (from 329±125 m to 377±120 m, $p<0.01$) at similar level of exhaustion on both tests (13/20 on the Borg RPE Scale®). All patients stated breathlessness as limiting factor.
3) QoL remained unchanged.
4) NYHA functional class improved in five of nine patients ($p=0.06$). Echocardiographic variables and plasma NT-proBNP concentration remained unchanged after EECP.
5) Skeletal muscle expression analysed using SAM did not detect any significantly differentially expressed genes after EECP treatment with a false discovery rate cut-off at 10%. Two highly significant networks were identified, one with -log10 p-value of 36 consisting of 20 focus molecules with IGF-1 as central regulator and the second one with a -log10 p-value of 38 consisting of 21 focus molecules with FGF-2 as central regulator by using network analysis. RT-PCR confirmed that both IGF-1 and FGF-2 increased 1.3-fold ($p=0.035$) and 1.2-fold ($p=0.03$) respectively. Comparing with a previously published dataset on endurance type exercise in elderly men, the EECP gene-expression profile found three overlapping transcripts, fewer than expected by chance. Thus, it was concluded that there was no significant resemblance in the gene expression profile with exercise training.

5 DISCUSSION

5.1 Main findings

The overall aim of the present work was to explore possible mechanistic relationships between the failing human heart and the periphery and to investigate variables with possible influence on pathophysiology and prognosis.

1) The distinct walking pattern observed in patients with severe HF had additional prognostic value over HFSS clinical score. The study was innovative in that the quality of exercise was found to be prognostic information.

2) Ten circulating proteins were associated with myocardial function, exercise capacity, and PA at HF, suggesting that these circulating proteins may play a role in the pathophysiology of HF. Eight of these proteins had prognostic value and six after controlling for the established prognostic markers age, eGFR, VO2 peak, and LVEF to predict all-cause mortality.

3) Patients with HF, who were followed for a period of two to four years, were divided into stable or worsening groups based on their clinical outcomes. Ten circulating proteins increased over time in deteriorated but not in stable patients. This suggests that these circulating proteins may play a role in disease progression. Five circulating proteins differed significantly between deteriorated and stable patients at baseline, suggesting that they have prognostic value.

4) RyR1 in skeletal muscle was post-translationally modified at HF. This is a possible mechanism underlying exercise intolerance and reduced functional capacity in HF.

5) External enhanced counterpulsation (EECP) improved functional capacity at HF. Analysis of the skeletal muscle transcriptome revealed no significant similarity to the transcriptome reported after physical training. This suggests that the improvements in functional capacity are most likely not due to transcriptional adaptations in skeletal muscle.

5.2 Physical activity: role in prognosis above physical capacity and clinical scores

The clinician has several tools at his/her disposal when determining prognosis. However, an intriguing and often frustrating fact is that determining prognosis for the individual patient can be misleading. A patient with a dismal prognosis on paper could live for years while a patient with seemingly good prognosis rapidly deteriorates and dies. It is especially challenging to identify patients most risk of rapid deterioration in the grey-zone area of VO_2 peak 10-18 ml/(kg x min) (81). It is important to remember that survival curves for patients with a VO_2 peak in the range 10-14 ml/(kg x min) and 14-18 ml/(kg x min) do not significantly differ (11). It is important for clinicians to identify patients at highest risk of mortality to ensure that these patients receive the optimal treatment. The most advanced treatments, such as heart transplantation or LVAD, are still underutilized and sometimes not thought of until it is too late. Therefore, it is important to find potential candidates at the right time. There are many prognostic models available, but they need to be refined to improve prognostic accuracy in terms of mortality. To put it clearly and simply, there is an urgent need for new and better prognosticators.

Maximal exercise capacity contains prognostic information and VO_2 peak is considered the strongest prognostic indicator available and is commonly used in clinical evaluation for cardiac transplantation and LVADs (17). Recent studies have shown that daily PA also carries prognostic information (137) where patients who are more active have a better prognosis and vice versa (138). This is used in daily clinical practice with the NYHA classification, which is crude but works to sort patients into groups with similar functional capacity and prognosis (92). The approach of measuring PA with accelerometers is rapidly increasing because accelerometers are more accessible and easier to use. Interestingly, studies have shown a strong correlation between PA and VO_2 peak (35). This challenges the notion that PA may provide additional prognostic information. Therefore, we wanted to examine whether PA could be used as a prognostic marker and whether novel aspects of PA could be identified and used as prognosticators in HF. Preferably, one should find a prognostic marker with an additive value and thereby refine the prognostic ability. Our data confirm that time spent in physical activity is associated with prognosis HF. However, when VO_2 peak or HFSS clinical score was added and after correcting for multiple hypothesis testing, time spent in physical activity was not significantly associated with prognosis. We then sought to assess whether other aspects of PA might contain prognostic information. One interesting aspect is that patients with severe HF have a distinctive gait pattern with shorter stride length and frequent stops (36). The reasons for this gait pattern are not known, but we hypothesized that the gait pattern leads to a high degree of variability in PA that could be identified and analysed. Skewness, a new variable that measures the asymmetry of intensity levels of high PA periods, proved to provide additional prognostic information about HFSS. The interesting and novel aspect of this study is that by analysing new aspects of PA we were able to derive prognostic information that goes beyond currently used prognostic tools. Therefore, the use of accelerometers in the clinical assessment of HF patients could be an attractive alternative, as they are easy to use and risk-free for the patient. Another interesting aspect is that skewness has proven its prognostic value in a homogeneous group of patients with severe HF, NYHA III- IV. Since it is easier to identify differences in prognosis in a heterogeneous group, we believe that the results are strong and valid. In our study, we demonstrated strong correlations between skewness and mortality. This opens the possibility of finding even stronger prognosticators in further studies and refining prognostic tools that could be of help to clinicians. By more easily identifying patients in need of advanced treatment, a possible long-term outcome could be to reduce the known underutilization of available therapies. As this is a single centre study and there are questions about external validity, the results need to be validated.

In summary, analysis of accelerometer-derived variables added value to the established prognostic model for predicting all-cause mortality. This finding highlights the use of accelerometer analysis in a clinical setting as it provides additional prognostic information.

Maximal exercise capacity contains prognostic information and VO_2 peak is considered the strongest prognostic indicator available and is commonly used in clinical evaluation for cardiac transplantation and LVADs (17). Recent studies have shown that daily PA also carries prognostic information (137) where patients who are more active have a better prognosis and vice versa (138). This is used in daily clinical practice with the NYHA classification, which is crude but works to sort patients into groups with similar functional

capacity and prognosis (92). The approach of measuring PA with accelerometers is rapidly increasing because accelerometers are more accessible and easier to use. Interestingly, studies have shown a strong correlation between PA and VO_2 peak (35). This challenges the notion that PA may provide additional prognostic information. Therefore, we wanted to examine whether PA could be used as a prognostic marker and whether novel aspects of PA could be identified and used as prognosticators in HF. Preferably, one should find a prognostic marker with an additive value and thereby refine the prognostic ability. Our data confirm that time spent in physical activity is associated with prognosis HF. However, when VO_2 peak or HFSS clinical score was added and after correcting for multiple hypothesis testing, time spent in physical activity was not significantly associated with prognosis. We then sought to assess whether other aspects of PA might contain prognostic information. One interesting aspect is that patients with severe HF have a distinctive gait pattern with shorter stride length and frequent stops (36). The reasons for this gait pattern are not known, but we hypothesized that the gait pattern leads to a high degree of variability in PA that could be identified and analysed. Skewness, a new variable that measures the asymmetry of intensity levels of high PA periods, proved to provide additional prognostic information above HFSS. The interesting and novel aspect of this study is that by analysing new aspects of PA we were able to derive prognostic information that goes beyond currently used prognostic tools. Therefore, the use of accelerometers in the clinical assessment of HF patients could be an attractive alternative, as they are easy to use and risk-free for the patient. Another interesting aspect is that skewness has proven its prognostic value in a homogeneous group of patients with severe HF, NYHA III- IV. Since it is easier to identify differences in prognosis in a heterogeneous group, we believe that the results are strong and valid. In our study, we demonstrated strong correlations between skewness and mortality. This opens the possibility of finding even stronger prognosticators in further studies and refining prognostic tools that could be of help to clinicians. By more easily identifying patients in need of advanced treatment, a possible long-term outcome could be to reduce the known underutilization of available therapies. As this is a single centre study and there are questions about external validity, the results need to be validated.

In summary, analysis of accelerometer-derived variables added value to the established prognostic model for predicting all-cause mortality. This finding highlights the use of accelerometer analysis in a clinical setting as it provides additional prognostic information in HF.

5.3 Circulating proteins: role in pathophysiology and prognosis

HF is a complex disease that progressively worsens. Understanding the mechanisms underlying disease progression may open new therapeutic approaches. The rate of disease progression varies both over time and between patients. Why disease progression is faster in some patients or what triggers disease progression is not fully understood. Damage to the heart leads to a cascade of inflammatory and fibrosis pathways. However, the mechanistic links between the failing heart and peripheral organs are still largely unknown. In HF, activation of the neurohormonal and sympathetic systems plays a key role in maintaining cardiac output and counteracting reduced stroke volume. Many circulating proteins increase in HF. Natriuretic peptides are perhaps the best-known example and are widely used in both clinical and research contexts for diagnosis and prognosis.

Exercise capacity, PA and myocardial function all have their own prognostic value in HF, and many circulating proteins covary with these three variables as cardiac dysfunction progresses. Many of these circulating proteins can be quantified with high sensitivity, allowing investigation of a possible clinical role in disease progression beyond potential tools for prognosis. We therefore speculated that circulating proteins provide an ideal platform for detecting and monitoring disease progression and for understanding pathophysiological mechanisms in HF. In **Study II**, we identified 17 circulating proteins that covaried with exercise capacity, PA and cardiac muscle function. Of these, ten circulating proteins (Galectin-4, GDF15, IGFBP7, NT -proBNP, PON3, ST2, TfR1, TRAP5, TNFR1, TNFR) differed between the HF group and controls. We deem that these ten circulating proteins represent a possible link between peripheral function and systolic function. Although causality could not be established with this approach, we believe the likelihood of a biologically significant association is high because covariation was found between all three physiological variables. We then searched for prognostic variables for possible clinical use using Cox regression analysis. Eight circulating proteins (Galectin-4, GDF15, IGFBP7, NT -proBNP, ST2, TfR1, TNFR1, TNFR) correlated with mortality, even when controlling for established prognostic markers.

By incorporating clinical deterioration and repeated measurements of circulating proteins, we aimed to identify the underlying biological processes driving disease progression. To our knowledge, the use of serial measurements of circulating proteins in combination with clinical deterioration in disease progression has not been extensively studied for HF.

We found ten circulating proteins (FABP4, JAMA, MMP-9, PDGF subunit-A, PECAM-1, PLC, SELP, TIMP4, TNFRSF14 and uPAR) with significantly different changes between baseline and follow-up. We hypothesise that these circulating proteins contribute to the pathophysiology of HF and clinical deterioration in patients. However, the potential pathophysiological mechanisms associated with these circulating proteins remain unclear. These circulating proteins have been linked to inflammation, extracellular matrix (ECM) turnover, and cardiac fibrosis, all important components in the pathophysiology of HF and drivers of disease progression. Inflammation plays an important role in the pathophysiology of HF (16). Studies have indicated a possible association between elevated TNF-alpha levels and physical performance and prognosis in HF, which may argue for a biological effect of TNF-alpha in disease progression (19). We found increased levels of TNFRSF14, and it has previously been associated with HF (21). We also found elevated levels of other biomarkers of inflammation, uPAR, PDGF subunit A, TNFRSF14, and elevated levels of MMP-9 and TIMP Metallopeptidase Inhibitor 4 (TIMP4), both of which correlate positively with interstitial fibrosis, diastolic dysfunction, and LVH (139,140). Since a key mechanism of malignant remodelling typical of HF is the progression of myocardial fibrosis, it seems logical that both MMP-9 and TIMP4 play an important role in disease progression HF. The circulating proteins in **Study III** differ from those found in **Study II**. There are several explanations for this. First, the study might be underpowered and therefore fail to detect factors that were significantly associated with prognosis in the larger cohort in **Study II**. But there are also different control groups used in the two studies considering that in the **Study II** a cohort of patients with HF was compared with control subjects, whereas in the **Study III** we studied a cohort of patients with severe HF longitudinally. In summary, two different

cohorts were studied, which could explain the different circulating proteins we found. It seems plausible that the 10 circulating proteins we found could play a role in disease progression. However, causality has not been proven with our studies of HF. These circulating proteins represent a possible link between peripheral function and systolic function in patients and provide insight into disease progression.

In **Study III**, with the aim of studying possible prognostic variables, we also studied associations with circulating proteins at baseline. We identified five circulating proteins that significantly differed at baseline (CD93, CHIT1, IGFBP 7, MB and ST2), and thus carrying prognostic information on disease progression.

In summary, in **Study II** and **III** we identified circulating proteins associated with the pathophysiology of HF as well as prognosis, and we believe these circulating proteins are tightly linked to disease progression and should be studied further.

5.4 Reduced exercise capacity - role of skeletal muscle

Exercise intolerance is a hallmark of HF. Despite the intuitive belief that the symptomatology of HF is derived by the failing heart, many of the symptoms origins from the defective skeletal muscle (58). The skeletal muscle undergoes a variety of alternations in HF, including muscle atrophy, alterations in fibre type, reduced mitochondrial enzymes, and decreased mitochondrial volume density (61–63). However, there are gaps in knowledge behind the muscle pathophysiology in human HF. Muscle contraction is dependent on cytoplasmic Ca^{2+} release. The action potential leads to activation of the RyR1, resulting in Ca^{2+} release into cytoplasm. Ca^{2+} then binds to troponin C at the myofilaments, enabling the formation of actin-myosin cross-bridging (141,142). Animal studies have shown that RyR1 is post-translationally altered with phosphorylation, S-nitrolysation and oxidation of RyR 1 with depletion of the stabilising protein FK506 binding protein 12 (FKBP12 or calstabin1) taking place (143–146). This results in leakage of Ca^{2+} into the cytoplasm, instead of a burst release of Ca^{2+} and is believed to lead to a less effective muscle contraction. We wanted to get a better understanding of the maladaptive calcium metabolism in human HF. Therefore, in **Study IV**, we asked if skeletal muscle in human HF undergoes RyR1 posttranslational remodelling as shown in animal models and if this is associated with reduced exercise capacity.

We demonstrated for the first time in humans the occurrence of the same RyR1 remodelling as previously demonstrated in animal studies. RyR1 was post-translationally altered with phosphorylation, S-nitrolysation and oxidation and with depletion of the stabilising protein FKBP12. A possible mechanistic link could be the activated sympathetic system in HF. In acute stress, the activation of the sympathetic system and release of catecholamines leads to a stronger skeletal and cardiac contraction (147). However, chronic adrenergic response and high levels of catecholamines, as seen in HF, lead in animals to hyperphosphorylation of RyR1 (148). Interestingly, betablockers have been shown to inhibit cardiac RyR2 remodelling (149) and if proven to also have an effect on RyR1, could have a beneficial effect on muscle contraction and attenuate muscle weakness. Betablockers are one of the four medicines (together with ACEi/ARB/ARNI, MRA and SGLT2-inhibitors) that all patients with HFrEF should receive, in the absence of contraindications (2,150,151).

In our study, there was no difference between patients with HF and controls regarding PA, indicating that the disease itself, that is HF, and not inactivity, explains the RyR1 remodelling. The post-translational remodelling has also been linked to ageing and ageing-dependent muscle weakness (146), possibly mediated through mitochondria-derived ROS (152). This could serve as a mechanistic link between age-induced mitochondrial oxidants and age-related muscle weakness. As the HF group in our study was excessively hyperphosphorylated, S-nitrolysated and oxidised compared to age-matched controls, HF seems to worsen the ageing process of the skeletal muscle. Apart from betablockers, new medicines ('rycals') targeting RyR directly are being developed, which will open up new ways of treating HF (153). 'Rycals' have been shown to improve contractile function in both heart and skeletal muscle but are not yet available for patients. There are currently no medications targeting muscle weakness in HF. Hopefully these medicines will eventually be available as they have the potential of increasing muscle contraction (of both skeletal and cardiac muscle) and improving QoL, morbidity and prognosis.

5.5 Treatment by External Enhanced Counterpulsation: role of skeletal muscle

As stated, HF goes with a dismal prognosis and QoL is severely impaired. In HFrEF there is strong scientific evidence on how to treat the patients. However, there are problems with adhering to guidelines and implementation of new scientific findings. Because of the severity and dismal prognosis of HF, new treatments are constantly sought after. External Enhanced Counterpulstation (EECP) has been put forward as an option in HF treatment. Studies showed improvement in functional capacity and improved QoL. The reasons for the improvement in functional capacity remained elusive as no clear central hemodynamic effects had been found at the time for the planning of the current study. Therefore, we asked if the improvement could be mediated by effects on the skeletal muscle. As EECP has been compared with passive training in HF patients and in line with the improved skeletal muscle function seen in physical exercise, the hypothesis seemed plausible. Our study showed an improved walking distance of 48 meters, which might not seem impressive, but has been considered clinically meaningful for patients in other studies (154). An important aspect to consider is if the increase in walking distance could be attributed to the test being repeated at follow-up as the present study lacks a control group. A previous study has shown that repeated tests of 6MWT increase the walking distance by approximately 19 m (155). Interestingly, in patients with HF the walking distance increased only by 9 meters when the test was repeated and it was concluded that 6MWT in HF is highly reliable and that one test is sufficient (108). To be able to account for a possible placebo effect the ideal study design would have been to add a placebo group consisting of sham EECP. Placebo can induce substantial effects, as shown in several studies (70). However, the question of why EECP increased functional capacity remained unanswered. We saw no statistically significant improvements in echocardiographic variables, and NT-proBNP remained unchanged. Moreover, QoL remained unchanged indicating that the improvement in physical capacity was not a result of a general clinical recovery. However, a type II error cannot be excluded for EF that numerically increased from 19 to 24 % by ECCP. A newly published systematic review on the effect of EECP demonstrated that EF increased in response to EECP in HF (90).

Skeletal muscle gene expression analysis by SAM did not detect any significantly differentially expressed genes after EECP treatment with a false discovery rate cut-off at 10%. However, true changes in gene expression could be missed with SAM when using a false discovery rate cut-off at 10% due to false negative results (type II errors). In order to find subtle changes, the data was also analysed with LIMMA robust regression analysis. This has the advantage of finding subtle changes but with the risk of type I errors, that is false positive findings. This method showed 197 genes that were up and downregulated. By using network analysis, two highly significant networks of genes were identified. The networks were focused on fibroblast growth factor 2 (FGF-2) and insulin-like growth factor-1 (IGF-1). The expression of these key molecules was further validated using RT-PCR. FGF-2 and IGF-1 are involved in angiogenesis and anabolic regulation. However, no other genes associated with angiogenesis or anabolic regulation were upregulated. We compared the gene expression results with a previously published dataset on endurance type exercise in elderly men. This analysis showed only three overlapping transcripts, which is fewer than what would have been expected by chance, indicating that there was no significant overlap in the gene expression profile between the previously published data on exercise training and EECP-treatment. Although a small cohort, the statistical power of the number of microarrays and detected variance was large enough to detect smaller changes than those found in previously published data sets on elderly men.

In summary, EECP in patients with HF significantly improved the functional capacity. Analysis of the skeletal muscle transcriptome found no significant resemblance with what has been reported after exercise training, indicating that the improvements in functional capacity cannot be attributed to skeletal muscle transcriptional adaptations.

5.6 Strengths and limitations

In **Study I**, new ways of determining prognosis in HF by analysing new aspects of PA determined by accelerometers have been shown. This not only affected prognosis, but also improved prognostic ability when added to conventional prognostic models. This has the potential to move into clinical use and help physicians with clinical prioritisation. Another strength is the analytic approach used in the II and III studies. There, we used a systems biology strategy to link circulating proteins to important physiological variables to increase knowledge of pathophysiological mechanisms and to find circulating proteins that carry prognostic information.

A limitation is the generalizability of the studies. Patients were recruited in a single centre (Karolinska University Hospital) and were predominantly male, which could limit external validity. A strength was that in **Study II**, the validity of the study group was confirmed after comparison with a broader reference material of outpatient clinic data: patients in the study were similar to the average patient in terms of age, body composition, comorbidities, and underlying diseases. However, there were fewer women, study-patients had worse cardiac function, and were treated more aggressively than in the general heart failure population. The circulatory factors found in **Study III** differed from those found in **Study II**. There are several possible explanations that need to be discussed: The study might be underpowered because only 16 patients were included but the results might be correct considering that in **Study II** a cohort of patients with HF was compared with control subjects, whereas in **Study**

III we studied a cohort of patients with severe HF longitudinally. In summary, two different cohorts were studied, which may explain the different circulatory factors we found.

In **Studies II** and **III**, the proximity extension assay was used to quantify the circulatory factors, which limits the possibility of absolute quantification of the samples. The factors are also prespecified, leading to selection bias, which limits the interpretation of the results.

A limitation of **Study IV** is the small sample size, with eight patients with HF and seven controls. However, the results were compelling and showed a strong significant difference between HF patients and controls. Another limitation is that immunoblots do not allow quantification of the extent of RyR1 remodelling. It seems plausible to assume that the more severe HF the patient has, the more pronounced the RyR1 remodelling. In the absence of associations between the degree of RyR1 remodelling, daily activity, and VO_2 peak, causality could not be established and remains to be investigated. Nevertheless, we believe the results are consistent with findings from previous studies on RyR2 and animal studies on RyR1.

In **Study V**, the lack of a control group is a limitation. It is difficult to have a control group in studies of EECP because the treatment involves inflatable cuffs around the legs, but sham EECP has been tested in some studies, and it would have been better if we had used a similar protocol.

6 CONCLUSIONS

Individuals with severe heart failure exhibit patterns in their daily physical activity characterized by high variance that can be detected by accelerometers. These patterns, which differ from those of healthy, age-matched individuals, are related to disease severity but also have the potential to predict mortality, even when established prognostic markers such as LVEF and peak VO_2 are taken into account. Several circulating proteins involved in inflammation and extracellular matrix remodelling should be considered potential pathophysiological moderators of heart failure severity because they correlate with echocardiographic measures of cardiac function as well as with exercise capacity and daily physical activity. Several of these proteins also have potential clinical utility in the prognostication of patients with severe heart failure, as they contribute significantly to Cox regression models of mortality or have significantly elevated levels in patients who deteriorate clinically over time compared with patients who remain clinically stable. Post-translational modifications of the ryanodine receptor (RyR) in skeletal muscle represent a possible mechanism underlying exercise intolerance and reduced functional capacity in heart failure. Treatment with external enhanced counterpulsation in patients with heart failure results in a modest increase in exercise capacity with no evidence of activation of transcriptional programs associated with improved exercise capacity in skeletal muscle.

7 POINTS OF PERSPECTIVE

Measuring PA over time and studying alterations in PA patterns has the potential of refining prognostic information. A prognostic model that incorporates daily PA could catch the disease progression as cardiac contractile and chronotropic functions deteriorate.

Finding novel circulating proteins that hold prognostic information could improve current prognostic tools. One goal is to find new circulating proteins that could provide additional prognostic information and be used to monitor disease progression, as well as the effect of treatment. It would be desirable to find more, novel cardiac specific biomarkers to refine the prognostic information. Not only blood samples, but also protein molecules like non-coding RNA (e.g. microRNA) have the potential to be novel and to be promising prognostic biomarkers. Circulating proteins could also have a therapeutical effect, opening the way for new treatments.

Finding drugs that target the maladaptive RyR1 in HF and normalize the Ca^{2+} release could be a new therapeutic option to reduce muscle fatigue and improve QoL. The question of why Ca^{2+} release from leaky RyR1 in some cases is harmful and leads to apoptosis and cell damage, while under other circumstances is tolerated by the muscle cells needs to be further studied.

8 ACKNOWLEDGEMENTS

Eric – I´m in complete awe of how gifted you are! Innovative, creative, wise. The most talented person I´ve ever worked with, and your wicked sense of humour has given me many laughs. I´ve had a lot of fun trying to convince you that you on occasion can be wrong but I´ve now finally accepted that you´re always right. You´ve never stopped trying to 'raise me up'. Thank you so much for everything and I´ll owe you 4-ever! I´ve loved every minute of our collaboration!

Thomas – A long time ago you asked me if I wanted to do research. Thank you for the journey that led to, everything I´ve learned from you and for all the good times and laughs we´ve shared! Thank you for all the magical moments at Hybrid!

Inger – My tutor. I´m your biggest fan! Thank you for all your wisdom, your kindness, generosity, guidance and all the wonderful memories! You´re one of a kind and I´m so grateful for knowing you!

Eva – My fourth, unofficial, supervisor! Thank you for your wisdom, your extreme patience with me, your kindness! I´m so impressed by the depth of your knowledge. I couldn´t have done it without you. I´ll be forever in debt. I´ve loved all interaction and I´m so happy to have you as a friend!

Frieder – You´re an exceptional leader, scientist and doctor! I´m so grateful for the opportunity to work with you! Thank you for your support, encouragement, and kindness!

Jan-Åke – Thank you for mentorship and your kindness to my family!

Anders – You helped me when I needed it the most, and I can never express the gratitude I feel. You´re a remarkable leader. Thank you for everything!

Eva M. – Thank you so much for your support and kindness! You´re an exceptional person. My favourite boss!

Cecilia, Fredrik – Thank you for your inspirational leadership and meaningful discussions!

Lars – You´re so extremely talented and inspirational. It´s truly an honour to work with you!

John P. - Truly exceptional. Thank you!

Lars R. – Thank you for expertise!

Christer, Mats, Karin –Thank you!

Gianluigi - It´s a privilege to work with you! Thank you for everything!

Camilla H. – It´s an honour to work with you!

Linda, Nawsad – Thank you for your support and great times! You have my deepest respect!

Peder – I really appreciate and value our friendship, your kindness and support! Thanks for great talks over a beer (or two)!

Anne, Annelie, Anne-Marie, Carolin, Francisca, Jila, Maryam, Åsa – Thank you for everything! I love working with you!

Linn, Jennifer, Marie, Jessica, Annika, Benjamin, Björn, Marie, Jon, Anna and all the wonderful colleagues working in Ward 2 Huddinge – thank you for making my working place a joy!

Katarzyna, Ewa – So impressed by your ability to navigate in this crazy world! I´ve loved all contact and wonderful times we´ve had. Forever a big fan!

Ali, Anna W, Brun, Karin, Lena, Mikael A., Rodrigo, Tommy – So talented and a wonderful research team to have as support! Thank you for collaboration and kindness!

Moa – Thank you for all your support and nice times!

Mats, Amo, Håkan, Seher – Thank you for great times together, our friendship and all your support!

Anna S. – So smart and with a wonderful sense of humour! I always love to talk to you!

Mirko, Alen – Looking forward to seeing the both of you prosper, dr Mandic´ and dr Levric! Talented and wonderful colleagues - I´ve really loved working with you!

All my colleagues and friends at Södertälje Hospital, especially Gloria, Johannes, Sara, Lars, Imad. Thank you for great times! Annicka – I miss you!

Hans P. and Karin M. – Thank you!

Magnus D., Peter – The best thoracic surgeons in the world. It´s truly an honour to work with you!

Martin T., Johan R. – Thank you for your professionalism, all the help you given me and great friendship!

Lars W., Helena G., Johan N., John S. – I´m always impressed by your expertise and for being such wonderful colleagues and people!

Alexander, Daniel, Helena, Jenny, Kristina – Thank you!

Anette, Maria E. – Thank you for your excellence and willingness to share your knowledge! Thank you for interesting conversations!

Tia, Adrian – What gifted colleagues you are! Thank you so much for laughter and serious conversations!

Goran, Martin A. – Thank you for being such great friends!

Martin E. – I really appreciate our talks and I love your humour! Looking forward to exploring the restaurants in Stockholm with you!

Anders, Andreas, Francesca, Jan, Jesper, Malin, Susanne – I love working with you and I´m so impressed by your knowledge!

Alicia – Thank you for nice conversations!

Birgitta W., Estrella, Frida, Kerstin, Kicki, Maria, Susanne – Thank you for always being there with support and friendship!

Birgitta, Gunilla, Margareta – Thank you for all your help!

Eva W. – Thank you for all your help! You´re wonderful!

Karin, Terhi – Thank you for all the help during the years and our friendship! You´re the best!

Anna, Andreas R., Angela, Anne, Arnar, Astrid, Ayman, Ayse-Gul, Christian, Dimitrios, Dinos, Emma, Fahd, Felix, Finn, Georgios, Gutte, Hamid, Helga, Jari, Josefin, Juliane, Kari, Katarina E., Layth, Loghman, Liew, Magnus S., Mikael, Moa, Mona, Moayad, Nikola, Nondita, Patrik N., Pontus, Rebecca, Robert, Shams, Staffan, Stefan, Susanna, Tara, Thomas F., Tigist – What wonderful, gifted colleagues! Thank you!
Göran – Thank you for being a good friend and wise colleague! I appreciate your mentorship!

Carl, Ulrika, Jonas – Thank you for friendship and all the great fun I have with you!

Bita – Thank you for being an exceptional colleague and friend! I love your humour.

Agneta, Ann, Anna K., Anna S., Annika, Aristomenis, Ashwin, Ben, Camilla S, Daniel A, David, Dmitri, Emil, Erica, Eva, Fauzia, Filippa, Frida, Gunilla, Helena, Jenny, Johanna, Jonas, Karin, Karolina, Katarina, Magnus N., Madeleine A., Madeleine G., Noella, Per, Petros, Tonje, Ulla – Truly the best team in the world! You´re all so dedicated and talented. It´s a true honour to work with all of you, and I love having you as colleagues! Thank you! And especially a big thank you to Ida! We go back a long way, and I want to thank you so much for your never-ending support and kindness!

Peter and Katarina – thank you for your friendship!

All our friends in Vällnora – Gunnel, Urs, Ingela and Peter. Thank you for wonderful memories.

Birgitta, Hans and Karin S.– Thank you for wonderful experiences both related to work and in private! You´re truly wonderful! I love your company!

Laura – Thank you for being such a wonderful person and friend! I always love our discussions and I´m impressed by your expertise! Italians do it better!

Patrik – a wise man said that it was right of me to put you on a pedestal! Extremely knowledgeable and smart, and I´ve always enjoyed working with you. A true professional!

Fredrik - Thank you for all the hilarious moments and interesting talks! I love your humour!

Magnus J. – I truly value our friendship extremely much! You´re so smart and successful in everything you do! And to me you´ve always been so extremely kind and supportive. Thank you!

Erik H. – who knew that a young, smart doctor that I was supposed to mentor turned out to be a very, very dear friend! I really appreciate our friendship!

Linnea, Fia - Thank you for your friendship and support, and all the good talks we´ve had! I wish I had more time to hang with you because you always brighten my day!

Viktoria och Mikaela – So talented and gifted! You´re the most amazing friends! I can always count on you being there, and that means incredibly much to me! I love all our adventures!

Sanna – My best friend! You´ve always been there for me! I love our talks, your wise advice ('Fjärran vore det mig att råda, MEN…'!!!), and your laughter. You help me become a better person (and that´s not an easy task!). You´re truly a remarkable friend and a wonderful human being! THANK YOU!

Margareth and Calle, Jeanette and Johan, Madeleine, Helena – My wonderful family! Thank you for happy memories! And especially much, much love to Margareth!

Annalisa and Carl, Kirsti and Gunnar – I wish that you could have been here to share this with me. I miss you.

Mie, Robin and Emma, Fabian – My wonderful family! Thank you for all the memories we share, the laughter we´ve had and all the laughter yet to come!

Carina and Philippe, Emilie, Sebastian - Such amazing friends! I can never pay back what you´ve given me through the years! I love you 4-ever!
Leni – We go back a long way, and words cannot express how much I and my closest family appreciate you. You´re so kind to us, and we love you enormously! Thank you for all the ongoing, interesting discussions.

Ewa, Per-Olof, David, Fredrika, Alexa, Harriet, Christoffer, Johanna, Helga, Gunnar, Lena, Rebecka, Karl, Gustaf - The best family in the world! Thank you for all the wonderful memories! And especially, a big thank you and much, much love to Ewa!

Markus, Nico – My two wonderful 'son-in-laws'!

Cleo – What a wonderful person you are! I love you so much!

Cissi and Ella – My two wonderful daughters! You are the greatest gift of my life! I'm so proud of you!

Aino – The love of my life! Thank you for our precious girls, for your love and support through the years! To me you mean everything and without you, life would be empty. And I love that the conversation between us never ends…

9 REFERENCES

1. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JGF, Coats AJS, et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. Eur Heart J. 2016;37(27):2129–200.

2. McDonagh TA, Metra M, Adamo M, Gardner RS, Baumbach A, Böhm M, et al. 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure. Eur Heart J. 2021;ehab368.

3. James SL, Abate D, Abate KH, Abay SM, Abbafati C, Abbasi N, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. The Lancet. 2018;392(10159):1789–858.

4. Savarese G, Lund LH.Global Public Health Burden of Heart Failure. Card Fail Rev. 2017;03(01):7-11.

5. Jenkinson C, Jenkinson D, Shepperd S, Richard L, Petersen S. Evaluation of treatment for congestive heart failure in patients aged 60 years and older using generic measures of health status (SF-36 and COOP charts). Age Ageing. 1997;26(1):7–13.

6. Blyth FM, Lazarus R, Ross D, Price M, Cheuk G, Leeder SR. Burden and outcomes of hospitalisation for congestive heart failure. Med J Aust. 1997;167(2):67–70.

7. Mosterd A. The prognosis of heart failure in the general population. The Rotterdam Study. Eur Heart J. 2001;22(15):1318–27.

8. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, et al. Heart Disease and Stroke Statistics—2017 Update: A Report From the American Heart Association. Circulation [Internet]. 2017;135(10). https://www.ahajournals.org/doi/10.1161/CIR.0000000000000485

9. Thorvaldsen T, Benson L, Dahlström U, Edner M, Lund LH. Use of evidence-based therapy and survival in heart failure in Sweden 2003-2012: Evidence-based therapy and survival in HF in Sweden. Eur J Heart Fail. 2016;18(5):503–11.

10. Aaronson KD, Cowger J. Heart Failure Prognostic Models: Why Bother? Circ Heart Fail. 2012;5(1):6–9.

11. Opasich C, Pinna GD, Bobbio M, Sisti M, Demichelis B, Febo O, et al. Peak Exercise Oxygen Consumption in Chronic Heart Failure: Toward Efficient Use in the Individual Patient. J Am Coll Cardiol. 1998;31(4):766–75.

12. Jessup Likoff M, Chandler SL, Kay HR. Clinical determinants of mortality in chronic congestive heart failure secondary to idiopathic dilated or to ischemic cardiomyopathy. Am J Cardiol. 1987;59(6):634–8.

13. Parameshwar J, Keegan J, Sparrow J, Sutton GC, Poole-Wilson PA. Predictors of prognosis in severe chronic heart failure. Am Heart J. 1992;123(2):421–6.

14. Franciosa JA, Park M, Barry Levine T. Lack of correlation between exercise capacity and indexes of resting left ventricular performance in heart failure. Am J Cardiol. 1981;47(1):33–9.

15. Agra Bermejo R, Cordero A, García-Acuña JM, Gómez Otero I, Varela Román A, Martínez Á, et al. Determinants and Prognostic Impact of Heart Failure and Left Ventricular Ejection Fraction in Acute Coronary Syndrome Settings. Rev Esp Cardiol Engl Ed. 2018;71(10):820–8.

16. Nicod P, Gilpin E, Dittrich H, Chappuis F, Ahnve S, Engler R, et al. Influence on prognosis and morbidity of left ventricular ejection fraction with and without signs of left ventricular failure after acute myocardial infarction. Am J Cardiol. 1988;61(15):1165–71.

17. Mancini DM, Eisen H, Kussmaul W, Mull R, Edmunds LH, Wilson JR. Value of peak exercise oxygen consumption for optimal timing of cardiac transplantation in ambulatory patients with heart failure. Circulation. 1991;83(3):778–86.

18. Levy WC, Mozaffarian D, Linker DT, Sutradhar SC, Anker SD, Cropp AB, et al. The Seattle Heart Failure Model: Prediction of Survival in Heart Failure. Circulation. 2006;113(11):1424–33.

19. Aaronson KD, Schwartz JS, Chen T-M, Wong K-L, Goin JE, Mancini DM. Development and Prospective Validation of a Clinical Index to Predict Survival in Ambulatory Patients Referred for Cardiac Transplant Evaluation. Circulation. 1997;95(12):2660–7.

20. Lund LH, Aaronson KD, Mancini DM. Validation of peak exercise oxygen consumption and the Heart Failure Survival Score for serial risk stratification in advanced heart failure. Am J Cardiol. 2005;95(6):734–41.

21. Anker SD, Ponikowski P, Varney S, Chua TP, Clark AL, Webb-Peploe KM, et al. Wasting as independent risk factor for mortality in chronic heart failure. The Lancet. 1997;349(9058):1050–3.

22. Drexler H. Reduced exercise tolerance in chronic heart failure and its relationship to neurohumoral factors. Eur Heart J. 1991;12(suppl C):21–8.

23. Coats A. The “Muscle Hypothesis” of Chronic Heart Failure. J Mol Cell Cardiol. 1996;28(11):2255–62.

24. Myers J, Arena R, Dewey F, Bensimhon D, Abella J, Hsu L, et al. A cardiopulmonary exercise testing score for predicting outcomes in patients with heart failure. Am Heart J. 2008;156(6):1177–83.

25. Balady GJ, Arena R, Sietsema K, Myers J, Coke L, Fletcher GF, et al. Clinician's Guide to Cardiopulmonary Exercise Testing in Adults: A Scientific Statement From the American Heart Association. Circulation. 2010;122(2):191–225.

26. Francis D. Cardiopulmonary exercise testing for prognosis in chronic heart failure: continuous and independent prognostic value from VE/VCO_2slope and peak VO_2. Eur Heart J. 2000;21(2):154–61.

27. Poggio R, Arazi HC, Giorgi M, Miriuka SG. Prediction of severe cardiovascular events by VE/VCO_2 slope versus peak VO_2 in systolic heart failure: a meta-analysis of the published literature. Am Heart J. 2010;160(6):1004–14.

28. Davies LC, Wensel R, Georgiadou P, Cicoira M, Coats AJS, Piepoli MF, et al. Enhanced prognostic value from cardiopulmonary exercise testing in chronic heart failure by non-linear analysis: oxygen uptake efficiency slope. Eur Heart J. 2006;27(6):684–90.

29. Arslan S, Erol MK, Gundogdu F, Sevimli S, Aksakal E, Senocak H, et al.Prognostic value of 6-minute walk test in stable outpatients with heart failure. Tex Heart Inst J. 2007;34(2):166–9.

30. Braunschweig F, Mortensen PT, Gras D, Reiser W, Lawo T, Mansour H, et al. Monitoring of Physical Activity and Heart Rate Variability in Patients With Chronic Heart Failure Using Cardiac Resynchronization Devices. Am J Cardiol. 2005;95(9):1104–7.

31. Cowie A, Thow MK, Granat MH, Mitchell SL. A comparison of home and hospital-based exercise training in heart failure: immediate and long-term effects upon physical activity level. Eur J Cardiovasc Prev Rehabil. 2011;18(2):158–66.

32. Cao Z-B, Miyatake N, Higuchi M, Miyachi M, Tabata I. Predicting VO_2max with an objectively measured physical activity in Japanese men. Eur J Appl Physiol. 2010;109(3):465–72.

33. Jehn M, Schmidt-Trucksäss A, Schuster T, Weis M, Hanssen H, Halle M, et al. Daily walking performance as an independent predictor of advanced heart failure. Am Heart J. 2009;157(2):292–8.

34. Doukky R, Mangla A, Ibrahim Z, Poulin M-F, Avery E, Collado FM, et al. Impact of Physical Inactivity on Mortality in Patients With Heart Failure. Am J Cardiol. 2016;117(7):1135–43.

35. da Silva VZM, Lima AC, Vargas FT, Cahalin LP, Arena R, Cipriano G. Association Between Physical Activity Measurements and Key Parameters of Cardiopulmonary Exercise Testing in Patients With Heart Failure. J Card Fail.

2013;19(9):635–40.

36. Davies SW, Greig CA, Jordan SL, Grieve DW, Lipkin DP. Short-stepping gait in severe heart failure. Heart. 1992;68(11):469–72.

37. Hartupee J, Mann DL. Neurohormonal activation in heart failure with reduced ejection fraction. Nat Rev Cardiol. 2017;14(1):30–8.

38. Jackson G. ABC of heart failure: Pathophysiology. BMJ. 2000;320(7228):167–70.

39. Swedberg K. Importance of neuroendocrine activation in chronic heart failure. Impact on treatment strategies. Eur J Heart Fail. 2000;2(3):229–33.

40. Francis GS, McDonald KM, Cohn JN. Neurohumoral activation in preclinical heart failure. Remodeling and the potential for intervention. Circulation. 1993;87(5 Suppl):IV90-96.

41. Doust JA, Pietrzak E, Dobson A, Glasziou P. How well does B-type natriuretic peptide predict death and cardiac events in patients with heart failure: systematic review. BMJ. 2005;330(7492):625.

42. Gardner R. N-terminal pro-brain natriuretic peptide A new gold standard in predicting mortality in patients with advanced heart failure. Eur Heart J. 2003;24(19):1735–43.

43. Eriksson B, Wändell P, Dahlström U, Näsman P, Lund LH, Edner M. Limited value of NT-proBNP as a prognostic marker of all-cause mortality in patients with heart failure with preserved and mid-range ejection fraction in primary care: A report from the swedish heart failure register. Scand J Prim Health Care. 2019;37(4):434–43.

44. Hrynchyshyn N, Jourdain P, Desnos M, Diebold B, Funck F. Galectin-3: A new biomarker for the diagnosis, analysis and prognosis of acute and chronic heart failure. Arch Cardiovasc Dis. 2013;106(10):541–6.

45. Chisalita SI, Dahlström U, Arnqvist HJ, Alehagen U. Increased IGF1 levels in relation to heart failure and cardiovascular mortality in an elderly population: impact of ACE inhibitors. Eur J Endocrinol. 2011;165(6):891–8.

46. Markousis-Mavrogenis G, Tromp J, Ouwerkerk W, Devalaraja M, Anker SD, Cleland JG, et al. The clinical significance of interleukin-6 in heart failure: results from the BIOSTAT-CHF study. Eur J Heart Fail. 2019;21(8):965–73.

47. Peacock WF. Novel biomarkers in acute heart failure: MR-pro-adrenomedullin. Clinical Chemistry and Laboratory Medicine (CCLM), 2014;52(10): 1433-1435. https://www.degruyter.com/document/doi/10.1515/cclm-2014-0222/html

48. Gül İ, Yücel O, Zararsız A, Demirpençe Ö, Yücel H, Zorlu A, Yılmaz MB. Prognostic role of soluble suppression of tumorigenicity-2 on cardiovascular mortality in outpatients with heart failure. Anatol J Cardiol. 2017 Sep;18(3):200-205.49. Dunlay SM, Weston SA, Redfield MM, Killian JM, Roger VL. Tumor Necrosis Factor-α and Mortality in Heart Failure: A Community Study. Circulation. 2008;118(6):625–31.

50. Ritt LE, Oliveira RB, Myers J, Arena R, Peberdy MA, Bensimhon D, et al. Patients With Heart Failure in the "Intermediate Range" of Peak Oxygen Uptake: ADDITIVE VALUE OF HEART RATE RECOVERY AND THE MINUTE VENTILATION/CARBON DIOXIDE OUTPUT SLOPE IN PREDICTING MORTALITY. J Cardiopulm Rehabil Prev. 2012;32(3):141–6.

51. Walke LM. Range and Severity of Symptoms Over Time Among Older Adults With Chronic Obstructive Pulmonary Disease and Heart Failure. Arch Intern Med. 2007;167(22):2503-2508.

52. Alpert CM, Smith MA, Hummel SL, Hummel EK. Symptom burden in heart failure: assessment, impact on outcomes, and management. Heart Fail Rev. 2017;22(1):25–39.

53. Wilson JR, Rayos G, Yeoh TK, Gothard P, Bak K. Dissociation Between Exertional Symptoms and Circulatory Function in Patients With Heart Failure. Circulation. 1995;92(1):47–53.

54. Myers J, Gullestad L, Vagelos R, Do D, Bellin D, Ross H, et al. Cardiopulmonary exercise testing and prognosis in severe heart failure: 14 mL/kg/min revisited. Am Heart J. 2000;139(1):78–84.

55. Arena R, Myers J, Abella J, Peberdy MA, Bensimhon D, Chase P, et al. Development of a Ventilatory Classification System in Patients With Heart Failure. Circulation. 2007;115(18):2410–7.

56. Sullivan MJ, Hawthorne MH. Exercise intolerance in patients with chronic heart failure. Prog Cardiovasc Dis. 1995;38(1):1–22.

57. Szlachcic J, Masse BM, Kramer BL, Topic N, Tubau J. Correlates and prognostic implication of exercise capacity in chronic congestive heart failure. Am J Cardiol. 1985;55(8):1037–42.

58. Clark AL. Origin of symptoms in chronic heart failure. Heart. 2006;92(1):12–16.

59. Bassett DR Jr, Howley ET. Limiting factors for maximum oxygen uptake and determinants of endurance performance. Med Sci Sports Exerc. 2000 Jan;32(1):70-84.

60. Belardinelli R, Georgiou D, Scocco V, Barstow TJ, Purcaro A. Low intensity exercise training in patients with chronic heart failure. J Am Coll Cardiol. 1995;26(4):975–

82.

61. Drexler H, Riede U, Münzel T, König H, Funke E, Just H. Alterations of skeletal muscle in chronic heart failure. Circulation. 1992;85(5):1751–9.

62. Harrington D, Anker SD, Chua TP, Webb-Peploe KM, Ponikowski PP, Poole-Wilson PA, et al. Skeletal Muscle Function and Its Relation to Exercise Tolerance in Chronic Heart Failure. J Am Coll Cardiol. 1997;30(7):1758–64.

63. Mancini DM, Walter G, Reichek N, Lenkinski R, McCully KK, Mullen JL, et al. Contribution of skeletal muscle atrophy to exercise intolerance and altered muscle metabolism in heart failure. Circulation. 1992;85(4):1364–73.

64. Buller NP, Jones D, Poole-Wilson PA. Direct measurement of skeletal muscle fatigue in patients with chronic heart failure. Heart. 1991;65(1):20–4.

65. Takeshima H, Nishimura S, Matsumoto T, Ishida H, Kangawa K, Minamino N, et al. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. Nature. 1989;339(6224):439–45.

66. Zorzato F, Fujii J, Otsu K, Phillips M, Green NM, Lai FA, et al. Molecular cloning of cDNA encoding human and rabbit forms of the Ca2+ release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. J Biol Chem. 1990;265(4):2244–56.

67. Nakai J, Imagawa T, Hakamata Y, Shigekawa M, Takeshima H, Numa S. Primary structure and functional expression from cDN A of the cardiac ryanodine receptor/calcium release channel. FEBS Lett. 1990;271(1–2):169–77.

68. Otsu K, Willard HF, Khanna VK, Zorzato F, Green NM, MacLennan DH. Molecular cloning of cDNA encoding the Ca2+ release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. J Biol Chem. 1990;265(23):13472–83.

69. Hakamata Y, Nakai J, Takeshima H, Imoto K. Primary structure and distribution of a novel ryanodine receptor/calcium release channel from rabbit brain. FEBS Lett. 1992;312(2–3):229–35.

70. Reiken S, Lacampagne A, Zhou H, Kherani A, Lehnart SE, Ward C, et al. PKA phosphorylation activates the calcium release channel (ryanodine receptor) in skeletal muscle. J Cell Biol. 2003;160(6):919–28.

71. Ward CW, Reiken S, Marks AR, Marty I, Vassort G, Lacampagne A. Defects in ryanodine receptor calcium release in skeletal muscle from postmyocardial infarcted rats. FASEB J. 2003;17(11):1–18.

72. The Consensus Trial Study Group. Effects of Enalapril on Mortality in Severe Congestive Heart Failure. N Engl J Med. 1987;316(23):1429–35.

73. The SOLVD Investigators. Effect of Enalapril on Survival in Patients with Reduced Left Ventricular Ejection Fractions and Congestive Heart Failure. N Engl J Med. 1991;325(5):293–302.

74. McMurray JJV, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, et al. Angiotensin–Neprilysin Inhibition versus Enalapril in Heart Failure. N Engl J Med. 2014;371(11):993–1004.

75. Hjalmarson Å, Goldstein S, Fagerberg B, Wedel H, Waagstein F, Kjekshus J, et al. Effects of Controlled-Release Metoprolol on Total Mortality, Hospitalizations, and Well-being in Patients With Heart Failure: The Metoprolol CR/XL Randomized Intervention Trial in Congestive Heart Failure (MERIT-HF). JAMA. 2000;283(10):1295-1302.

76. Poole-Wilson PA. The Cardiac Insufficiency Bisoprolol Study II. The Lancet. 1999;353(9146): 9-13.

77. Packer M, Bristow MR, Cohn JN, Colucci WS, Fowler MB, Gilbert EM, et al. The Effect of Carvedilol on Morbidity and Mortality in Patients with Chronic Heart Failure. N Engl J Med. 1996;334(21):1349–55.

78. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, et al. The Effect of Spironolactone on Morbidity and Mortality in Patients with Severe Heart Failure. N Engl J Med. 1999;341(10):709–17.

79. Zannad F, McMurray JJV, Krum H, van Veldhuisen DJ, Swedberg K, Shi H, et al. Eplerenone in Patients with Systolic Heart Failure and Mild Symptoms. N Engl J Med. 2011;364(1):11–21.

80. Moss AJ, Zareba W, Hall WJ, Klein H, Wilber DJ, Cannom DS, et al. Prophylactic Implantation of a Defibrillator in Patients with Myocardial Infarction and Reduced Ejection Fraction. N Engl J Med. 2002;346(12):877–83.

81. Cazeau S, Leclercq C, Lavergne T, Walker S, Varma C, Linde C, et al. Effects of Multisite Biventricular Pacing in Patients with Heart Failure and Intraventricular Conduction Delay. N Engl J Med. 2001;344(12):873–80.

82. Swedberg K, Komajda M, Böhm M, Borer JS, Ford I, Dubost-Brama A, et al. Ivabradine and outcomes in chronic heart failure (SHIFT): a randomised placebo-controlled study. The Lancet. 2010;376(9744):875–85.

83. McMurray JJV, Solomon SD, Inzucchi SE, Køber L, Kosiborod MN, Martinez FA, et al. Dapagliflozin in Patients with Heart Failure and Reduced Ejection Fraction. N Engl J Med. 2019;381(21):1995–2008.

84. Packer M, Anker SD, Butler J, Filippatos G, Pocock SJ, Carson P, et al. Cardiovascular and Renal Outcomes with Empagliflozin in Heart Failure. N Engl J Med. 2020;383(15):1413–24.

85. Rose EA, Gelijns AC, Moskowitz AJ, Heitjan DF, Stevenson LW, Dembitsky W, et al. Long-Term Use of a Left Ventricular Assist Device for End-Stage Heart Failure. N Engl J Med. 2001;345(20):1435–43.

86. Piano MR, Prasun MA, Stamos T, Groo V. Flexible Diuretic Titration in Chronic Heart Failure: Where Is the Evidence? J Card Fail. november 2011;17(11):944–54.

87. Moss AJ, Hall WJ, Cannom DS, Klein H, Brown MW, Daubert JP, et al. Cardiac-Resynchronization Therapy for the Prevention of Heart-Failure Events. N Engl J Med. 2009;361(14):1329–38.

88. O'Connor CM, Whellan DJ, Lee KL, Keteyian SJ, Cooper LS, Ellis SJ, et al. Efficacy and Safety of Exercise Training in Patients With Chronic Heart Failure: HF-ACTION Randomized Controlled Trial. JAMA. 2009;301(14):1439-1450.

89. Aronow WS, Shamliyan TA. Exercise for Preventing Hospitalization and Readmission in Adults With Congestive Heart Failure: Cardiol Rev. 2019;27(1):41–8.

90. Zhou Z-F, Wang D, Li X-M, Zhang C-L, Wu C-Y. Effects of enhanced external counterpulsation on exercise capacity and quality of life in patients with chronic heart failure: A meta-analysis. Medicine (Baltimore). 2021;100(27):e26536.

91. Rampengan SH, Prihartono J, Siagian M, Immanuel S. The Effect of Enhanced External Counterpulsation Therapy and Improvement of Functional Capacity in Chronic Heart Failure patients: a Randomized Clinical Trial. Acta Medica Indones. 2015;47(4):275–82.

92. Harvey RM, Doyle EF, Ellis K, Farber SJ, Ferrer MI, et al. Major Changes Made by Criteria Committee of the New York Heart Association. Circulation. 1974;49(3):390–390.

93. Scrutenid D, Lagioia R, Ricci A, Clemente M, Boni L, Rizzon P. Prediction of mortality in mild to moderately symptomatic patients with left ventricular dysfunction. Eur Heart J. 1994;15(8):1089–95.

94. Madsen BK, Hansen JF, Stokholm KH, Brøns J, Husum D, Mortensen LS. Chrome congestive heart failure. Eur Heart J. 1994;15(3):303–10.

95. Muntwyler J. One-year mortality among unselected outpatients with heart failure. Eur Heart J. 2002;23(23):1861–6.

96. Edler I, Lindström K. The history of echocardiography. Ultrasound Med Biol. 2004;30(12):1565–644.

97. Teichholz LE, Kreulen T, Herman MV, Gorlin R. Problems in echocardiographic volume determinations: Echocardiographic-angiographic correlations in the presence or absence of asynergy. Am J Cardiol. 1976;37(1):7–11.

98. Folland ED, Parisi AF, Moynihan PF, Jones DR, Feldman CL, Tow DE. Assessment of left ventricular ejection fraction and volumes by real-time, two-dimensional echocardiography. A comparison of cineangiographic and radionuclide techniques. Circulation. 1979;60(4):760–6.

99. Lang RM, Badano LP, Mor-Avi V, Afilalo J, Armstrong A, Ernande L, et al. Recommendations for Cardiac Chamber Quantification by Echocardiography in Adults: An Update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. J Am Soc Echocardiogr. 2015;28(1):1-39.e14.

100. Mertens LL, Friedberg MK. Imaging the right ventricle—current state of the art. Nat Rev Cardiol. 2010;7(10):551–63.

101. Kaul S, Tei C, Hopkins JM, Shah PM. Assessment of right ventricular function using two-dimensional echocardiography. Am Heart J. 1984;107(3):526–31.

102. Appleton CP, Hatle LK, Popp RL. Relation of transmitral flow velocity patterns to left ventricular diastolic function: New insights from a combined hemodynamic and Doppler echocardiographic study. J Am Coll Cardiol. 1988;12(2):426–40.

103. Nagueh SF, Smiseth OA, Appleton CP, Byrd BF, Dokainish H, Edvardsen T, et al. Recommendations for the Evaluation of Left Ventricular Diastolic Function by Echocardiography: An Update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. J Am Soc Echocardiogr. 2016;29(4):277–314.

104. Currie PJ, Seward JB, Chan K-L, Fyfe DA, Hagler DJ, Mair DD, et al. Continuous wave doppler determination of right ventricular pressure: A simultaneous Doppler-catheterization study in 127 patients. J Am Coll Cardiol. 1985;6(4):750–6.

105. Yock PG, Popp RL. Noninvasive estimation of right ventricular systolic pressure by Doppler ultrasound in patients with tricuspid regurgitation. Circulation. 1984;70(4):657–62.

106. Parasuraman S, Walker S, Loudon BL, Gollop ND, Wilson AM, Lowery C, et al. Assessment of pulmonary artery pressure by echocardiography—A comprehensive review. IJC Heart Vasc. 2016;12:45–51.

107. Yap J, Lim FY, Gao F, Teo LL, Lam CSP, Yeo KK. Correlation of the New York Heart Association Classification and the 6-Minute Walk Distance: A Systematic Review: Correlation of NYHA and 6MWD. Clin Cardiol. 2015;38(10):621–8.

108. Lans C, Cider Å, Nylander E, Brudin L. Test–retest reliability of six-minute walk tests over a one-year period in patients with chronic heart failure. Clin Physiol Funct Imaging. 2020;40(4):284–9.

109. Arena R, Myers J, Aslam SS, Varughese EB, Peberdy MA. Peak VO_2 and

VE/VCO2 slope in patients with heart failure: a prognostic comparison. Am Heart J. 2004;147(2):354–60.

110. Guazzi M, Bandera F, Ozemek C, Systrom D, Arena R. Cardiopulmonary Exercise Testing. J Am Coll Cardiol. 2017;70(13):1618–36.

111. Ingle L, Goode K, Carroll S, Sloan R, Boyes C, Cleland JGF, et al. Prognostic value of the VE/VCO2 slope calculated from different time intervals in patients with suspected heart failure. Int J Cardiol. 2007;118(3):350–5.

112. Cahalin LP, Mathier MA, Semigran MJ, Dec GW, DiSalvo TG. The Six-Minute Walk Test Predicts Peak Oxygen Uptake and Survival in Patients With Advanced Heart Failure. Chest. 1996;110(2):325–32.

113. Westerterp K. Physical activity assessment with accelerometers. Int J Obes. 1999;23(S3):S45–9.

114. Melczer C, Melczer L, Goják I, Oláh A, Ács P. A comparative analysis between external accelerometer and internal accelerometer's physical activity data from implanted resynchronization devices in patients with heart failure. Eur J Integr Med. 2016;8:18–22.

115. Pressler A, Danner M, Esefeld K, Haller B, Scherr J, Schömig A, et al. Validity of cardiac implantable electronic devices in assessing daily physical activity. Int J Cardiol. 2013;168(2):1127–30.

116. Sylvia LG, Bernstein EE, Hubbard JL, Keating L, Anderson EJ. Practical Guide to Measuring Physical Activity. J Acad Nutr Diet. 2014;114(2):199–208.

117. Troiano RP. Large-Scale Applications of Accelerometers: New Frontiers and New Questions. Med Sci Sports Exerc. 2007;39(9):1501.

118. Mehra MR, Canter CE, Hannan MM, Semigran MJ, Uber PA, Baran DA, et al. The 2016 International Society for Heart Lung Transplantation listing criteria for heart transplantation: A 10-year update. J Heart Lung Transplant. 2016;35(1):1–23.

119. Pocock SJ, Ariti CA, McMurray JJV, Maggioni A, Køber L, Squire IB, et al. Predicting survival in heart failure: a risk score based on 39 372 patients from 30 studies. Eur Heart J. 2013;34(19):1404–13.

120. Szczurek W, Szyguła-Jurkiewicz B, Siedlecki Ł, Gąsior M. Prognostic scales in advanced heart failure. Pol J Cardio-Thorac Surg. 2018;15(3):183–7.

121. Green CP, Porter CB, Bresnahan DR, Spertus JA. Development and evaluation of the Kansas City Cardiomyopathy Questionnaire: a new health status measure for heart failure. J Am Coll Cardiol. 2000;35(5):1245–55.

122. Rector TS, Cohn JN. Assessment of patient outcome with the Minnesota Living with Heart Failure questionnaire: Reliability and validity during a randomized, double-blind, placebo-controlled trial of pimobendan. Am Heart J. 1992;124(4):1017–25.

123. Parajón T, Lupón J, González B, Urrutia A, Altimir S, Coll R, et al. Use of the "Minnesota Living With Heart Failure" Quality of Life Questionnaire in Spain. Rev Esp Cardiol Engl Ed. 2004;57(2):155–60.

124. Lawson WE, Hui JCK, Barsness GW, Kennard ED, Kelsey SF. Effectiveness of enhanced external counterpulsation in patients with left main disease and angina: EEEC in patients with left main disease. Clin Cardiol. 2004;27(8):459–63.

125. Lawson WE, Hui JCK, Soroff HS, Zheng ZS, Kayden DS, Sasvary D, et al. Efficacy of enhanced external counterpulsation in the treatment of angina pectoris. Am J Cardiol. 1992;70(9):859–62.

126. Michaels AD, Accad M, Ports TA, Grossman W. Left Ventricular Systolic Unloading and Augmentation of Intracoronary Pressure and Doppler Flow During Enhanced External Counterpulsation. Circulation. 2002;106(10):1237–42.

127. Shea ML, Conti CR, Arora RR. An update on enhanced external counterpulsation. Clin Cardiol. 2005;28(3):115–8.

128. Soran O. A New Treatment Modality in Heart Failure: Enhanced External Counterpulsation (EECP). Cardiol Rev. 2004;12(1):15–20.

129. Stys T, Lawson WE, Hui JCK, Lang G, Liuzzo J, Cohn PF. Acute Hemodynamic Effects and Angina Improvement with Enhanced External Counterpulsation. Angiology. 2001;52(10):653–8.

130. Werner D, Michalk F, Hinz B, Werner U, Voigt J-U, Daniel WG. Impact of Enhanced External Counterpulsation on Peripheral Circulation. Angiology. 2007;58(2):185–90.

131. Bergström J. Percutaneous Needle Biopsy of Skeletal Muscle in Physiological and Clinical Research. Scand J Clin Lab Invest. 1975;35(7):609–16.

132. Rio DC, Ares M, Hannon GJ, Nilsen TW. Purification of RNA Using TRIzol (TRI Reagent). Cold Spring Harb Protoc. 2010;2010(6):pdb.prot5439.

133. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc Ser B Methodol. 1995;57(1):289–300.

134. Benjamini Y, Hochberg Y. On the Adaptive Control of the False Discovery Rate in Multiple Testing with Independent Statistics. J Educ Behav Stat. 2000;25(1):60.

135. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci. 2001;98(9):5116–21.

136. Krämer A, Green J, Pollard J, Tugendreich S. Causal analysis approaches in Ingenuity Pathway Analysis. Bioinformatics. 2014;30(4):523–30.

137. Conraads VM, Spruit MA, Braunschweig F, Cowie MR, Tavazzi L, Borggrefe M, et al. Physical Activity Measured With Implanted Devices Predicts Patient Outcome in Chronic Heart Failure. Circ Heart Fail. 2014;7(2):279–87.

138. Jehn M, Prescher S, Koehler K, von Haehling S, Winkler S, Deckwart O, et al. Tele-accelerometry as a novel technique for assessing functional status in patients with heart failure: feasibility, reliability and patient safety. Int J Cardiol. 2013;168(5):4723–8.

139. Krebber MM, van Dijk CGM, Vernooij RWM, Brandt MM, Emter CA, Rau CD, et al. Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases in Extracellular Matrix Remodeling during Left Ventricular Diastolic Dysfunction and Heart Failure with Preserved Ejection Fraction: A Systematic Review and Meta-Analysis. Int J Mol Sci. 2020;21(18):6742.

140. Lindsay MM, Maxwell P, Dunn FG. TIMP-1: a marker of left ventricular diastolic dysfunction and fibrosis in hypertension. Hypertension, 2002;40(2):136–41.

141. Allen DG, Lamb GD, Westerblad H. Skeletal Muscle Fatigue: Cellular Mechanisms. Physiol Rev. 2008;88(1):287–332.

142. Zalk R, Lehnart SE, Marks AR. Modulation of the Ryanodine Receptor and Intracellular Calcium. Annu Rev Biochem. 2007;76(1):367–85.

143. Reiken S, Lacampagne A, Zhou H, Kherani A, Lehnart SE, Ward C, et al. PKA phosphorylation activates the calcium release channel (ryanodine receptor) in skeletal muscle. J Cell Biol. 2003;160(6):919–28.

144. Bellinger AM, Reiken S, Carlson C, Mongillo M, Liu X, Rothman L, et al. Hypernitrosylated ryanodine receptor calcium release channels are leaky in dystrophic muscle. Nat Med. 2009;15(3):325–30.

145. Bellinger AM, Reiken S, Dura M, Murphy PW, Deng S-X, Landry DW, et al. Remodeling of ryanodine receptor complex causes" leaky" channels: A molecular mechanism for decreased exercise capacity. Proc Natl Acad Sci. 2008;105(6):2198–202.

146. Andersson DC, Betzenhauser MJ, Reiken S, Meli AC, Umanskaya A, Xie W, et al. Ryanodine Receptor Oxidation Causes Intracellular Calcium Leak and Muscle Weakness in Aging. Cell Metab. 2011;14(2):196–207.

147. Cairns SP, Borrani F. β-Adrenergic modulation of skeletal muscle contraction: key role of excitation-contraction coupling: β-Adrenergic processes and muscle contraction. J

Physiol. 2015;593(21):4713–27.

148. Bellinger AM, Mongillo M, Marks AR. Stressed out: the skeletal muscle ryanodine receptor as a target of stress. J Clin Invest. 2008;118(2):445–53.

149. Reiken S, Wehrens XHT, Vest JA, Barbone A, Klotz S, Mancini D, et al. β-Blockers Restore Calcium Release Channel Function and Improve Cardiac Muscle Performance in Human Heart Failure. Circulation. 2003;107(19):2459–66.

150. McMurray JJV, Solomon SD, Inzucchi SE, Køber L, Kosiborod MN, Martinez FA, et al. Dapagliflozin in Patients with Heart Failure and Reduced Ejection Fraction. N Engl J Med. 2019;381(21):1995–2008.

151. Zannad F, Ferreira JP, Pocock SJ, Anker SD, Butler J, Filippatos G, et al. SGLT2 inhibitors in patients with heart failure with reduced ejection fraction: a meta-analysis of the EMPEROR-Reduced and DAPA-HF trials. The Lancet. 2020;396(10254):819–29.

152. Umanskaya A, Santulli G, Xie W, Andersson DC, Reiken SR, Marks AR. Genetically enhancing mitochondrial antioxidant activity improves muscle function in aging. Proc Natl Acad Sci. 2014;111(42):15250–5.

153. Andersson DC, Marks AR. Fixing ryanodine receptor Ca2+ leak – a novel therapeutic strategy for contractile failure in heart and skeletal muscle. Drug Discov Today Dis Mech. 2010;7(2):e151–7.

154. Shoemaker MJ, Curtis AB, Vangsnes E, Dickinson MG. Clinically meaningful change estimates for the six-minute walk test and daily activity in individuals with chronic heart failure. Cardiopulm Phys Ther J. 2013;24(3):21–9.

155. Spencer L, Zafiropoulos B, Denniss W, Fowler D, Alison J, Celermajer D. Is there a learning effect when the 6-minute walk test is repeated in people with suspected pulmonary hypertension? Chron Respir Dis. 2018;15(4):339–46.