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TESI DI DOTTORATO

ANTIOXIDATIVE AND ANTISENESCENT EFFECTS OF CARDIAC REHABILITATION IN HEART FAILURE PATIENTS

Coordinatore:
Ch.ma Prof.ssa Amelia Filippelli

Tutor: **Candidata:**
Ch.ma Prof.ssa Amelia Filippelli   **Valentina Manzo**

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ABSTRACT

Exercise-based cardiac rehabilitation (CR) is effectively used as an adjuvant therapy in a number of cardiovascular diseases (CVDs), including chronic heart failure (CHF), and it is recommended by the American and European Society of Cardiology guidelines. Exercise training (ET) increases physical and functional capacity, ameliorates quality of life, decreases symptoms (fatigue and dyspnoea) and, more importantly, reduces the incidence of acute cardiac events, mortality and hospitalization rates. Recently, it has been shown that a moderate exercise is able to induce the recovery of antioxidant defences, whose expression changes with aging and during CVDs. Despite the number of evidences underling the CR-associated cardiovascular protection, CR itself is still an underused medical resource and the mechanisms accounting for such benefits are not completely elucidated yet.

The present study aimed at investigating whether a well-structured rehabilitation program of 4 weeks was able to modify systemic antioxidant potential in HF patients, and at examining the mechanisms by which exercise improves cardiovascular function.

For this purpose, 50 subjects with diagnosis of CHF (NYHA class II and III) were recruited from the Cardiac Rehabilitation Unit of "San Gennaro dei Poveri" Hospital in Naples. On admission, patients underwent case history recording, clinical examination, electrocardiogram, chest X-Ray, echocardiogram, cardiopulmonary stress test and a 6-minute walking test, blood sample collection for routinary and experimental analysis. The CR program consisted in ET of 30' on cycloergometer, respiratory gymnastic along with educational meetings, for a meantime of 4 weeks. Blood samples were collected at baseline and at the end of CR, and oxidants (TBARS and 8-hydroxy-2-deoxyguanosine), antioxidants (catalase, Cat, and superoxide dismutase, SOD), and bioavailability of nitric oxide (NO) were measured in patients' sera, whereas Sirtuin 1 (Sirt1) activity was quantified in patients' lymphocytes.

Human endothelial cells (ECs), exposed or not to H_2O_2-oxidative stress, were conditioned with patients' sera, and cellular redox state and senescence were evaluated. A similar approach in an animal model of post-ischemic HF was used to confirm and assess the effect of exercise on senescence. Finally, inhibitors of Sirt1

(EX-527) and Cat (ATZ) activities were used to investigate the roles of these proteins in modulating endothelial cell senescence.

The results demonstrated that CR stimulated an increase of oxidants with concomitant rise of Sirt1 activity, antioxidants and NO bioavailability. Moreover, CR prevented the ECs senescence via Sirt1 and Cat activation while the inhibition of these enzymes eliminated such effect, both in humans and in the animal model. Lastly, Sirt1 and Cat activities were, respectively, inversely and directly associated with cardiopulmonary stress test duration. Taken together, these findings suggest that CR triggers cellular adaptations leading to enhance systemic antioxidant effectiveness. Circulating levels of Sirt1 and Cat activity are suggested to be promising markers for assessing the efficacy of CR program.

SOMMARIO

Le malattie cardiovascolari, incluso lo scompenso cardiaco, rappresentano ancora oggi la prima causa di morte e di disabilità a livello globale. Numerosi studi hanno dimostrato che l'esercizio fisico influenza favorevolmente la prognosi dei pazienti cardiopatici in quanto è in grado di migliorare le performances fisiche e i sintomi oltre che ridurre l'incidenza di eventi cardiaci acuti, il tasso di mortalità e di riospedalizzazione. In particolare, nei pazienti scompensati sono stati documentati molti dei benefici associati all'esercizio fisico. Recentemente è stato dimostrato che un esercizio moderato è in grado di indurre il recupero delle difese antiossidanti che variano considerevolmente con l'invecchiamento ed in presenza di malattie cardiovascolari. Tuttavia, i meccanismi alla base di tali effetti cardioprotettivi, non sono stati ancora completamente chiariti.

Lo scopo del mio lavoro di tesi è stato valutare, in pazienti con scompenso cardiaco stabile, se un programma di riabilitazione cardiaca (RC) di 4 settimane fosse in grado di modificare il potenziale antiossidante dei pazienti e quali fossero i meccanismi alla base degli effetti protettivi associati all'esercizio.

A tale scopo, sono stati reclutati 50 soggetti con diagnosi di scompenso (classe NYHA II e III) presso l'Unità di Cardiologia Riabilitativa dell'Ospedale "San Gennaro dei Poveri" di Napoli. Al momento del ricovero, i pazienti sono stati sottoposti ad anamnesi, esame clinico obiettivo, elettrocardiogramma, radiografia del torace, ecocardiogramma, test da sforzo cardiopolmonare e prelievo ematico per valutazioni di routine e sperimentali. Il programma di riabilitazione consisteva in un training di 30' su cicloergometro, ginnastica respiratoria ed educazione alimentare, per una durata complessiva di 4 settimane.

Nei campioni ematici raccolti al basale e dopo 4 settimane sono stati misurati i livelli sierici di perossidazione lipidica (TBARS) e di deossiguanosina (8-OHdG), la biodisponibilità di ossido nitrico (NO) e le attività della Superossido Dismutasi (SOD) e della Catalasi (Cat). Nei linfociti, invece, è stata misurata l'attività della Sirtuina 1 (Sirt1), una deacetilasi NAD^+-dipendente che regola la sopravvivenza cellulare e la risposta allo stress ossidativo. In seguito, il siero dei pazienti è stato utilizzato per eseguire esperimenti *in vitro*, ossia per condizionare cellule umane endoteliali (HUVEC), esposte successivamente alla presenza o

meno di stress ossidativo indotto con H_2O_2. In tali cellule sono stati valutati i livelli di ossidanti e antiossidanti, l'attività di Sirt1 e la senescenza cellulare.

Per analizzare il ruolo svolto dalla Sirt1 e dalla Cat nella modulazione dei meccanismi di senescenza cellulare, le cellule endoteliali, precedentemente condizionate con i sieri dei pazienti, sono state trattate con due inibitori, EX-527 (6-Cloro-2,3,4,9-tetraidro-1H-carbazol-1-carbossammide) e ATZ (3-amino-1,2,3-triazolo), capaci di inibire in maniera selettiva, rispettivamente, Sirt1 e Cat. Infine, lo stesso tipo di esperimento è stato eseguito utilizzando il siero isolato da un modello animale di ratto scompensato sottoposto a un programma di training fisico simile a quello utilizzato nei programmi di RC per i pazienti reclutati.

I risultati ottenuti suggeriscono che un programma di riabilitazione cardiaca di sole 4 settimane migliora la risposta allo stress ossidativo favorendo un adattamento cellulare di tipo ormetico. Infatti, la RC comporta un aumento delle specie ossidanti con un concomitante aumento dell'attività di Sirt1 nei linfociti e degli enzimi antiossidanti e della biodisponibilità di ossido nitrico nel siero dei pazienti. Inoltre, la RC è in grado di prevenire, sia nell'uomo sia nel modello animale, la senescenza delle cellule condizionate attraverso l'attivazione di Sirt1 e Cat, come dimostrato dall'azione degli inibitori specifici di queste proteine. Infine, l'attività di Sirt1 è risultata essere inversamente correlata, mentre l'attività di Cat è direttamente correlata alla durata del test da sforzo cardiopolmonare suggerendo un ruolo determinante di questi fattori nel modulare la tolleranza allo sforzo.

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Abbreviations

8-OH-dG: 8-Hydroxy-2-deoxyguanosine
ACCF: American College of Cardiology Foundation
ACE: Angiotensin-Converting Enzyme
ACSM: American College of Sports Medicine
AHA: American Heart Association
ANGII: Angiotensin II
ARBs: Angiotensin II Receptor Blockers
ATZ: 3-Amino-1:2:4-Triazole
AU: Arbitrary Unit

BMI: Body Mass Index
bpm: beat/minutes

CABG: Coronary Artery Bypass Graft
CAD: Coronary Artery Disease
Cat: Catalase
CHF: Congestive Heart Failure
COPD: Chronic Obstructive Pulmonary Disease
CPX: Cardiopulmonary exercise testing
CR: Cardiac Rehabilitation
CVD: Cardiovascular Disease

DNA-PK: DNA-dependent Protein Kinase
DPB: Diastolic Blood Pressure

ECs: Endothelial Cells
EF: Ejection Fraction
EGTA: Ethylene Glycol Tetraacetic Acid
ESC: European Society of Cardiology
ET: Exercise Training
EX-527: Sirtuin 1 inhibitor

FBS: Foetal Bovine Serum
FOXOs: Forkhead box O transcription factors
FS: Fractional Shortening

GDH: Glutamate dehydrogenase
GPx: Glutathione Peroxidase

HEPES: 4-(2-HydroxyEthyl)-1-PiperazineEthaneSulfonic acid
HF: Heart Failure
HF-ACTION: Heart Failure - A Controlled Trial Investigating Outcomes of Exercise Training
HFpEF: Heart Failure with preserved Ejection Fraction
HFrEF: Heart Failure with reduced Ejection Fraction
HIF-1α: Hypoxia Inducible Factor 1α
HR: Heart Rate
HRQOL: Health-Related Quality Of Life
HRR: Heart Rate Reserve

IDH2: Isocitrate Dehydrogenase 2
IMT: Inspiratory Muscle Training

LAD: Left Anterior Descending coronary artery
LV: Left Ventricle
LVEDD: Left Ventricle End Diastolic Diameter
LVEF: Left Ventricular Ejection Fraction
LVW: Left Ventricle Weight

MCD: Malonyl-CoA Decarboxylase
MI: Myocardial Infarction
MnSOD: Manganese-dependent SuperOxide Dismutase

NAD$^+$: Nicotinamide Adenine Dinucleotide coenzyme
NADPH: Nicotinamide Adenine Dinucleotide Phosphate
NF-κB: Nuclear Factor kappa B
NHEJ: Non-Homologous End Joining
NYHA: New York Heart Association

PDL: Population Doubling Level
PDH: Pyruvate Dehydrogenase
P-ECs: Patient serum-conditioned Endothelial Cells
PPAR: Peroxisome Proliferator-Activated Receptors
PTCA: Percutaneous Transluminal Coronary Angioplasty

RAAS: Renin-Angiotensin-Aldosterone System
RCT: Randomized Controlled Trial
ROS: Reactive Oxygen Species
RP: Rehabilitated Patients
RPE: Rating of Perceived Exertion
RP-ECs: Rehabilitated Patient serum-conditioned Endothelial Cells
RV: Right Ventricle
RVW: Right Ventricle Weight

S: Sedentary post-myocardial infarction heart failure rats
SA-β-gal: Senescence-Associated β-galactosidase
SBP: Systolic Blood Pressure
S-ECs: Sedentary post-myocardial infarction heart failure rats' serum-conditioned Endothelial Cells
Sh: Sham-operated rats
Sh-ECs: Sham-operated rats' serum-conditioned Endothelial Cells
Sirt1: Sirtuin 1
SOD: SuperOxide Dismutase

T: Trained post-myocardial infarction heart failure rats
TBARS: thiobarbituric acid-reactive substances
TCA: tricarboxylic acid cycle
T-ECs: Trained post-myocardial infarction heart failure rats' serum-conditioned Endothelial Cells

VAD: Ventricular Assist Device
VO$_2$: maximal oxygen consumption

X-gal: 5-bromo-4 chloro-3-indolyl β-D-galactoside

CHAPTER 1

1.1 Heart Failure

Cardiovascular disease (CVD) is one of the major causes of morbidity and mortality in Western countries, with over 4 million deaths per year, half of them in Europe (Nichols et al., 2014). Its prevalence increases with age and is higher in men than in women (Ho et al., 1993). Among CVDs, Heart Failure (HF) represents a very high social burden.

In North America and Europe, few patients with HF are 50 years of age or younger and more than 80% are 65 years of age or older. Therefore, the number of such patients is predicted to increase in countries with ageing populations (Bui et al., 2011; Go et al., 2014).

During the past half-century, the advances in the prevention, diagnosis and management of CVDs have been of noteworthy relevance. Nevertheless, while the rate of mortality associated with acute coronary syndromes, valvular and congenital heart disease, uncontrolled hypertension and many arrhythmias was decreased, HF is still one of the most common cause of hospitalization (Braunwald, 2013) and mortality (Guha and McDonagh, 2013).

Several studies estimate the overall prevalence of HF in the US population to be about 2%-3%, whereas in Western European populations it is estimated to range from 0.4% to 2% (Bui et al., 2011; WRITING GROUP MEMBERS et al., 2010). Definitively, over 26 million adults worldwide suffer from HF and over 3.5 million people are newly diagnosed every year in Europe (Bui et al., 2011).

HF has the greatest negative impact on quality of life when compared with other major chronic disease, such as diabetes, arthritis and hypertension and, in term of disability, the end stage is comparable to that of terminal cancer (Ponikowski et al., 2014).

The prognosis associated with HF is poor (Mahjoub et al., 2008); half of all patients die within 4 years, and the survival rate (about 5-year) is lower than that associated with myocardial infarction and the majority of the most common malignancies.

The economic cost of HF is estimated in billions of dollars per year; in fact, the need for repeated hospitalization is the most powerful contributing factor to direct

costs associated with such disease. Moreover, the longer life expectancy of the population and the increase in risk factors for ischemic heart disease, particularly in countries with economies in transition, account for a growing incidence and prevalence of HF around the world. Indeed, more patients with heart disease are surviving compared to the past, but those who survive are at high risk of going on to develop HF (Ambrosy et al., 2014).

The only effective way to decrease the oncoming pandemic is by reducing the risk factors for CVD through education of the population, adoption of a healthier lifestyle and optimal pharmacological treatment.

1.2 Pathophysiology/Aetiology of Heart Failure

HF is a chronic condition in which the heart is unable to pump enough blood to meet the body's needs for oxygen and nutrients (American Heart Association "About Heart Failure", 2015).

In order to compensate for reduction in blood flow and increase in demand from surrounding body tissue, the heart will typically undergo enlargement, increase in muscle mass and/or in pumping action; likewise, blood vessels can narrow to maintain blood pressure or divert blood to the vital organs (i.e. the heart and brain) (Mefford, 2013).

European Society of Cardiology (ESC) guidelines define HF as "a syndrome in which patients have typical symptoms (e.g. breathlessness, ankle swelling, and fatigue) and signs (e.g. elevated jugular venous pressure, pulmonary crackles, and displaced apex beat) resulting from an abnormality of cardiac structure or function" (McMurray et al., 2012) (**Table 1.1**).

HF may be associated with a wide spectrum of left ventricular (LV) functional abnormalities that may impair the functional capacity and the quality of life of affected individuals; however, they don't necessarily dominate the clinical picture (Givertz et al., 2001).

Furthermore, many of the typical signs and symptoms of HF are not directly connected to the cardiac malfunction, but rather arise secondarily to abnormalities that occur in other organs or tissues. Such dysfunction cannot be explained

exclusively by decreased perfusion pressure, suggesting that other systemic processes (e.g. neurohormonal activation) contribute to HF.

As some patients present HF without signs or symptoms of volume overload, the term "heart failure" is preferred over "congestive heart failure". Congestive HF is an outdated term that reflects the original clinical observation that patients with HF were frequently oedematous or volume-overloaded.

The most common aetiologies of HF are ischemic heart disease, coronary artery disease (CAD), hypertension and diabetes (Roger et al., 2011). Three quarters of all HF patients have pre-existing hypertension, and this risk factor alone doubles the risk of developing HF compared to normotensive patients (Lloyd-Jones et al., 2002). Less common, but important, causes of HF are cardiomyopathies, infections (e.g., viral myocarditis, Chagas' disease), toxins (e.g., alcohol, cytotoxic drugs), valvular disease and prolonged arrhythmias. However, it is very difficult to establish what is the primary aetiology of HF in a patient with multiple potential causes (Kemp and Conte, 2012; Rahman et al., 2014). Although the aetiology is highly variable, HF represents a global derangement of the interplay among cardiac, renal and vascular systems. Without successful intervention, inadequate systolic or diastolic cardiac function leads to poor systemic blood flow with compensatory neurohormone release, vasoconstriction and fluid retention (Yang et al., 2015).

The terminology currently used to describe HF is based on measurement of LV ejection fraction (EF), essential in determining both the diagnosis and prognosis. Mathematically, EF is the stroke volume (which is the end-diastolic volume minus the end-systolic volume) divided by the end-diastolic volume.

Patients could have HF with depressed or reduced EF (HFrEF, commonly referred to as systolic failure), or HF with a normal EF or preserved EF (HFpEF, sometimes described as "diastolic failure") (McMurray et al., 2012) (**Table 1.2**). However, a significant proportion of patients with HF happen to have a normal ventricular EF at echocardiography during examination.

Epidemiological studies have also shown that patients may have significant abnormalities of LV contraction and relaxation and yet have no symptoms; in this case patients are referred to as having asymptomatic HF.

According to the American Heart Association (AHA) and American College of Cardiology (ACC) guidelines, diastolic HF is defined as (1) exhibiting clinical symptoms of congestive HF, (2) having normal LV systolic function (LVEF of 45 to 50% and above), and (3) having abnormal LV diastolic dysfunction, which can be determined by Doppler echocardiography or cardiac catheterization. Using this threshold, EF is preserved in more than half of failing patients (Owan et al., 2006; Vasan and Levy, 2000).

The diagnosis of HFpEF is more difficult than that of HFrEF, because one should first exclude other potential non cardiac causes of symptoms suggestive of HF (such as anaemia or chronic lung disease). In the general population, patients with HFpEF are usually older women with a history of hypertension. Obesity, CAD, diabetes mellitus, atrial fibrillation and hyperlipidaemia are highly prevalent in HFpEF patients as reported in many studies and registries (Lee et al., 2009; Owan et al., 2006).

In addition, several studies indicate that the number of patients with HFpEF is similar or even higher compared to the number of patients with HFrEF (Brouwers et al., 2013; Steinberg et al., 2012); nevertheless, mortality and morbidity rates of these two groups are nearly equal (Meta-analysis Global Group in Chronic Heart Failure (MAGGIC), 2012).

Importantly, while a decline in mortality in HFrEF patients treated following the recommendations of current guidelines was recorded, trials resulting in a comparable improved outcome in HFpEF patients are still lacking.

Table 1.1. Symptoms and signs typical of HF. (Adapted from McMurray et al., 2012)

Symptoms		Signs	
Typical	**Less typical**	**Specific**	**Less specific**
• Breathlessness • Orthopnoea • Paroxysmal nocturnal dyspnoea • Reduced exercise tolerance • Fatigue, tiredness, increased time to recover after exercise • Ankle swelling	• Nocturnal cough • Wheezing • Weight gain or weight loss (in advanced heart failure) • Bloated feeling • Loss of appetite • Confusion • Depression • Palpitations • Syncope	• Elevated jugular venous pressure • Hepatojugular reflux • Third heart sound (gallop rhythm) • Laterally displaced apical impulse • Cardiac murmur	• Peripheral oedema (ankle, sacral, scrotal) • Pulmonary crepitations • Reduced air entry and dullness to percussion at lung bases (pleural effusion) • Tachycardia • Tachypnoea (>16 breaths/min) • Hepatomegaly • Ascites • Cachexia

Table 1.2. Differences of HF related to left ventricular ejection fraction. (Adapted from McMurray et al., 2012)

Patients with reduced EF (HFrEF) have:	Patients with preserved EF (HFpEF) have:
• Symptoms typical of HF • Signs typical of HF* • Reduced LVEF	• Symptoms typical of HF • Signs typical of HF* • Normal or only mildly reduced LVEF and LV not dilated • Relevant structural heart disease (LV hypertrophy/LA enlargement) and/or diastolic dysfunction

* Signs may not be present in the early stages of HF (especially in HFpEF) and in patients treated with diuretics

The diagnosis of HFrEF requires three conditions to be satisfied, while the diagnosis of HFpEF requires four conditions. LA= left atrial; LV= left ventricular; LVEF= left ventricular ejection fraction.

1.3 Classification and Staging of Heart Failure

HF is commonly classified using the New York Heart Association (NYHA) (Dolgin and New York Heart Association. Criteria Committee, 1994) and the ACC/AHA classification system (Williams et al., 1995).

Both the ACC/AHA and the NYHA classification provide useful and complementary information about the severity of HF. The ACC/AHA staging emphasizes the development and progression of disease, whereas the NYHA classes focus on exercise capacity and the symptomatic status of HF patients.

In the NYHA scale, one of the oldest attempts to grade the severity of functional limitation among failing patients, patients are divided into four functional groups (class I - least severe to class IV - most severe), based on individual physical ability.

Patients with NYHA Class I have cardiac disease without any limitations or symptoms with ordinary activity. Those in NYHA Class II have a slight limitation of physical activity; they are comfortable at rest, but ordinary physical activity will result in fatigue, palpitation, dyspnoea, or angina. NYHA Class III patients are still comfortable at rest, but have marked limitation of physical activity with symptoms occurring during less than ordinary activity. NYHA Class IV patients may have symptoms at rest and are unable to carry out any physical activity without symptoms. According to the National Heart, Lung, and Blood Institute, 35% of patients have Class I, followed by 35% with Class II, 25% with Class III, and 5% with Class IV HF; mortality rises as patients progress through the various NYHA classifications.

The ACC and the AHA first published guidelines for the evaluation and management of HF in 1995; recently, these guidelines have been updated (Hunt et al., 2001, 2009).

The ACC/AHA classification identifies four stages and establishes the risk factors as well as structural prerequisites for the development of HF. The first two stages (A and B) are clearly not HF but include patients who are at risk for developing disease. Patients in stage A have hypertension, diabetes mellitus, a history of cardiotoxic drug therapy, alcohol abuse, a history of rheumatic fever, or a family history of cardiomyopathy, conditions strongly associated with the development of HF but do not have any heart structural disorder. Stage B patients have structural

disorder without any symptoms of HF. These patients have a prior myocardial infarction (MI), LV hypertrophy or fibrosis, LV dilatation or hypo-contractility, or asymptomatic valvular heart disease. Those in Stage C have past or current symptoms of HF, which are associated with underlying structural heart disease. Stage D patients are those who have end-stage HF who require specialized treatment strategies.

There are some overlaps between ACC/AHA and NYHA classification systems; actually, stage A corresponds to pre-HF, Stage B to NYHA Class I, Stage C to NYHA Class II and III, and Stage D to NYHA Class IV HF (**Table 1.3**).

This four-stage classification scheme highlights the principle that therapeutic interventions before the occurrence of LV dysfunction can result in a reduction in morbidity and mortality.

Table 1.3. Comparison of American College of Cardiology/American Heart Association (ACC/AHA) stages and NYHA functional classification of HF.

ACC/AHA Heart Failure Stage	NYHA Functional Class
Stage A Patients at high risk of developing HF but without any structural disorders of the heart or symptoms.	**Pre-HF**
Stage B Patients are asymptomatic but have developed structural heart disease.	**Class I** No limitation of physical activity. Ordinary physical activity does not cause fatigue, dyspnea, palpitations, or anginal pain.
Stage C Patients have symptoms with mild to moderate activities and have structural heart disease.	**Class II** Slight limitation of physical activity. No symptoms at rest, but ordinary physical activity results in fatigue, palpitation, dyspnea. **Class III** Marked limitation of physical activity. Comfortable at rest. Ordinary physical activity cause fatigue, dyspnea, palpitations.
Stage D Patients have symptoms at rest and have advanced structural heart disease. Specialized interventions are required.	**Class IV** Unable to carry out any physical activity without symptoms. Symptoms of cardiac insufficiency or of angina may be present at rest.

Stage A of ACC/AHA classification corresponds to very early stage of HF (pre-HF), Stage B to NYHA Class I, Stage C to NYHA Class II and III, and Stage D to NYHA Class IV.

1.4 Pharmacotherapy of Heart Failure

The goals of HF therapy are the reduction of symptoms, decrease in the rate of hospitalization and prevention of premature death. Treatment ideally begins with prevention of cardiac dysfunction via the identification of risk factors for development of structural heart disease. Medical care for HF includes lifestyle management, drugs, cardiac surgery, pacemakers, implantable cardioverter-defibrillators or cardiac resynchronization therapy. When end-stage of HF occurs despite maximal medical therapy, when the prognosis is poor, and when there is no viable therapeutic alternative, the standard therapeutic approach is heart transplantation.

Depending on the severity of illness, non-pharmacologic therapies including both lifestyle modification, such as dietary sodium and fluid restriction, could be effective. Patients should be encouraged to lose excess weight, to abstain from smoking and alcohol consumption, and to improve their physical condition through physical exercise when possible.

HF diagnosis is usually confirmed by physical examination, patient history and clinical (biochemical and haematological) and instrumental (e.g. echocardiogram, electrocardiogram) tests in order to detect abnormal function of the left ventricle and/or heart valves and to define the severity and prognosis of disease. In particular, as the signs and symptoms of HF are quite non-specific, many patients with suspected HF eligible for echocardiography have no important cardiac abnormality. Where the availability of echocardiography is limited, an alternative approach to diagnosis is to measure blood concentration of two natriuretic peptides (B-type natriuretic peptide, BNP, and N-terminal pro B-type natriuretic peptide, NT-proBNP) (Cowie et al., 1997; Fuat et al., 2006; Kelder et al., 2011a, 2011b; Krishnaswamy et al., 2001; Nielsen et al., 2004; Yamamoto et al., 2000), which are hormones secreted in high concentration when the heart is compromised or the load on any chamber is increased (e.g. by AF, pulmonary embolism, renal failure) (Doust et al., 2004; Ewald et al., 2008; Maisel et al., 2008; Zaphiriou et al., 2005).

In the past, drug therapies targeted the endpoints of this syndrome such as volume overload (congestion) and myocardial dysfunction (pump failure). As a consequence, diuretics and cardiac glycosides have dominated the medical

management of HF for more than 40 years. These drugs remain effective for symptom relief and in stabilizing patients with hemodynamic decompensation but do not improve long-term survival. The contemporary conception of HF as a disorder encompassing circulatory hemodynamics abnormalities, pathologic cardiac remodelling and increased arrhythmogenic instability has boosted the development of novel pharmacotherapies aiming at reducing HF-associated morbidity and mortality rates.

In general, medical therapies focus on the treatment of hypertension, dyslipidaemia, diabetes, and arrhythmias but, despite pharmacologic advances, many patients remain symptomatic on maximally tolerated doses (Hare, 2002).

The 2013 ACC/AHA updated (Yancy et al., 2013) guidelines, the 2010 Heart Failure Society of America (HFSA) (Heart Failure Society of America et al., 2010) and the 2008 ESC guidelines (Dickstein et al., 2008), recommend to use:
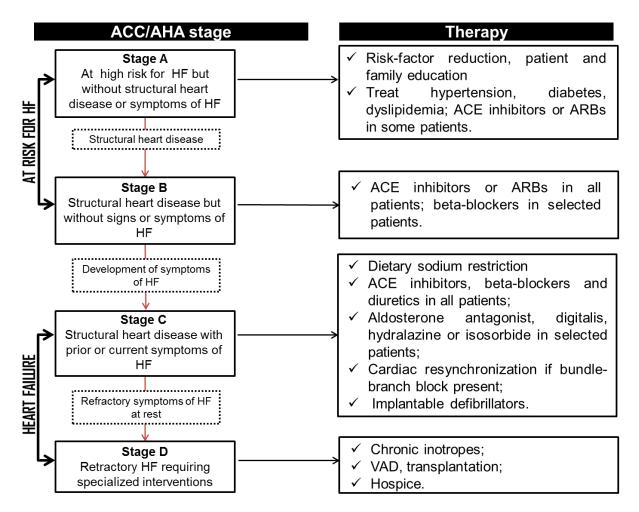
1) Diuretics to attenuate oedema and fluid retention by reduction of blood volume and venous pressures;

2) Angiotensin-converting enzyme inhibitors (ACEIs), primarily used in the treatment of hypertension, for neurohormonal modification, vasodilatation, LVEF improvement and survival increase. In patients intolerant to ACE inhibitors, angiotensin II receptors blockers are an alternative;

3) Angiotensin II Receptors Blockers (ARBs), also known as AT_1 receptor antagonists, as ACEIs alternative, commonly prescribed medications in the treatment of hypertension, to relax the blood vessels and lower blood pressure, allowing the heart to pump blood with greater ease;

4) β adrenergic receptor antagonists, commonly known as beta-blockers, to reduce blood pressure by shutting down sympathetic stimulation, thereby reducing heart rate. β blockers have proven effective in reducing overall mortality among HF patients, as well as improving LV function. They are indicated for patients with prior MI or hypertension and for control of ventricular rate in those with atrial fibrillation;

5) Aldosterone antagonists or receptor blockers for additive diuresis, HF symptom control, improvement of heart rate variability, decrease of

ventricular arrhythmias, reduction in cardiac workload, improvement of LVEF, and increase in survival.

Furthermore, other drugs (vasodilators, inotropic agents, anticoagulant) can be used to relieve symptoms, ventricular function, exercise capacity, cardiac hemodynamics and function through preload and afterload reduction and enhanced inotropism (i.e., myocardial contractility).

It is important to note that while pharmacologic intervention can improve the clinical status of failing patients, cardiac function and organ perfusion are generally not restored to normal values. In the late stages of chronic HF or in severe acute HF, patients may be refractory to drug therapy. In these cases, the only option is surgery consisting in mechanical cardiac assist devices or heart transplant (**Figure 1.1**).

Figure 1.1. Stages of HF and recommended therapy by stage. (Adapted from Hunt et al., 2001)

ACC/AHA stage	Therapy
Stage A At high risk for HF but without structural heart disease or symptoms of HF	✓ Risk-factor reduction, patient and family education ✓ Treat hypertension, diabetes, dyslipidemia; ACE inhibitors or ARBs in some patients.
Structural heart disease	
Stage B Structural heart disease but without signs or symptoms of HF	✓ ACE inhibitors or ARBs in all patients; beta-blockers in selected patients.
Development of symptoms of HF	
Stage C Structural heart disease with prior or current symptoms of HF	✓ Dietary sodium restriction ✓ ACE inhibitors, beta-blockers and diuretics in all patients; ✓ Aldosterone antagonist, digitalis, hydralazine or isosorbide in selected patients; ✓ Cardiac resynchronization if bundle-branch block present; ✓ Implantable defibrillators.
Refractory symptoms of HF at rest	
Stage D Retractory HF requiring specialized interventions	✓ Chronic inotropes; ✓ VAD, transplantation; ✓ Hospice.

AT RISK FOR HF — HEART FAILURE

ACE= Angiotensin-converting enzyme; ARBs= angiotensin receptor blockers; VAD= ventricular assist device.

CHAPTER 2

2.1 Cardiac Rehabilitation

Cardiac rehabilitation (CR) is an integral part of the standard care in modern cardiology. It is a multidisciplinary approach, which focuses on patient education to a healthier lifestyle and management of the CVD risk factors, including overweight control, smoking habits cessation and treatment of clinical condition such as hyperlipidaemia, hypertension and diabetes (Mampuya, 2012).

CR consists in a medically supervised program that helps people who have CVD (CAD, HF, angina or heart attacks) or who have undergone heart surgery or percutaneous coronary intervention (coronary angioplasty and stenting, valve replacement, pacemaker or implantable cardioverter defibrillator) to improve health and quality of life, by reducing cardiac symptoms and dampening the risk of future heart diseases.

According to the US Public Health Service, CR provides medical evaluation, monitored physical activity program and education/counselling services and should supply an individualized therapeutic approach.

Experimental data clearly establish that a regular physical activity and a structured exercise training (ET) provide many benefits including symptoms relief, improved exercise tolerance, cardiorespiratory fitness, reduction of depression and anxiety and, as a consequence, they increase life expectancy of the trained patient. Moreover, exercise and physical activity decrease the risk of developing CHD, stroke, type 2 diabetes (Bassuk and Manson, 2005), and some forms of cancer (e.g., colon and breast cancers) (Lee, 2003; Physical Activity Guidelines Advisory Committee, 2008).

Exercise-based CR programmes are now recommended (Class I) by the European Society of Cardiology, the American Heart Association, and the American College of Cardiology for the treatment of patients with CAD and CHF.

CR encompasses short-term and long-term goals pointing at reducing the risk of sudden cardiac arrest or reinfarction (Coll-Fernández et al., 2014; Kirk et al., 2014), at limiting both physiologic and psychological negative effects of CVD, at stabilizing or reverting the atherosclerotic process and at enhancing the

psychological status of the patient together with the promotion of heart-healthy behaviours.

CR programmes equally benefit women and men (Cannistra et al., 1992) and elderly patients can also achieve significant relief.

Initially, CR was designed for low-risk cardiac patients, but now, since the efficacy and safety of exercise have been documented in patients previously stratified to the high-risk category, such as those with CHF, CR indications have been expanded to include such patients.

Subjects with limitations due to chronic obstructive pulmonary disease (COPD), peripheral vascular disease, stroke, and orthopaedic conditions can follow the CR program using special techniques and adaptive equipment (e.g. arm-crank ergometer). The indications and contraindications in participating to an exercise-based CR are summarized in **table 2.1** (Arena et al., 2007; Balady et al., 2007; Carrel and Mohacsi, 1998; Herdy et al., 2008; Lavie et al., 2009; Marwick et al., 2009; Myers et al., 2009; Naughton, 1992; Wenger, 2008).

Unfortunately, despite a well-documented benefit, CR remains an underutilized therapeutic approach and its effects on all-cause and cardiac mortality continue to be discussed. Several systematic reviews and meta-analyses of small studies have shown that physical training improves exercise tolerance, health-related quality of life (HRQOL), and hospitalization. Results of EXTRA-MATCH case-based meta-analysis, including a total of 395 exercise-trained patients with HFrEF and 406 control patients, showed a significant 35% reduction in all-cause mortality and 28% reduction in the combined end-point of death and hospital admission (Piepoli et al., 2004).

Many randomized controlled trials (RCTs) exploring the efficacy of ET in HF have been conducted, but the statistical power of such trials was often too low. Recently, a single large RCT [Heart Failure: A Controlled Trial Investigating Outcomes of Exercise Training (HF-ACTION)] investigated the effects of ET in 2,331 subjects (72% men, mean age 59 years) who were medically stable with mild to moderately severe symptoms (NYHA class II 63% and class III 35%) and with LVEF ≤35% (O'Connor et al., 2009).

Patients were randomized either to the group who performed a program of 36 sessions of supervised, moderate-intensity training in the initial 3 months followed by home-based training, or to the group treated with conventional therapy.

At the end of the trial, unadjusted analyses demonstrated no significant difference in total mortality or hospitalization rates between the two groups analysed. Conversely, after a median follow-up time of 30 months and an adjustment analysis for predefined prognostic predictors of mortality (duration of the cardiopulmonary exercise test; LVEF; Beck Depression Inventory II score; history of atrial fibrillation), ET statistically led to, even if modest, reduction in all-cause and cardiovascular mortality and HF hospitalization (O'Connor et al., 2009). However, it is important to emphasize that the adherence of patients to exercise routine declined substantially after the first period of the supervised training. Based on ET safety and reduction in clinical events in addition to the modest increase in HRQOL, the HF-ACTION results support the efficacy of an ET program for the treatment of patients with reduced LV function and HF symptoms.

Although more than 50% of patients with HF have preserved EF (diastolic HF), only two similar randomized clinical trials involving patients with HF symptoms and LVEF ≥50% have been recently performed (Edelmann et al., 2011; Kitzman et al., 2010). The results of such studies showed an improvement of peak VO_2, LV diastolic function and quality of life in the exercise group. Therefore, patients with diastolic HF may represent a group of patients in whom ET can potentially improve clinical outcomes.

In the clinical practice, CR training protocols vary for type (aerobic, anaerobic and mixed aerobic/anaerobic), intensity/workload (endurance, resistance, and strength), protocol (continuous and intermittent/interval, supervised and non-supervised), and setting (hospital/centre- and home-based) and are recommended for stable, motivated patients who have not comorbidities limiting exercise capacity.

The 6-min walk test, used to evaluate exercise capacity in patients with marked LV dysfunction or peripheral arterial occlusive disease that cannot perform cycle ergometer, and a variety of treadmill and bicycle protocols allow objective evaluation of exercise capacity and symptoms, such as dyspnoea and fatigue. Gas exchange analysis helps to differentiate between cardiac and respiratory

causes of dyspnoea, shows whether the anaerobic threshold has been reached, and provides prognostic information (e.g. peak oxygen consumption is often measured as part of the assessment of candidates for heart transplantation). Over the last 30 years, cardiopulmonary exercise testing (CPX) has been recognized as the gold standard for the assessment of exercise capacity and prognostic stratification in both normal subjects and patients with cardiovascular and some non-cardiovascular diseases in stable clinical conditions. CPX is a type of exercise testing, which includes parameters such as ventilation, oxygen consumption and carbon dioxide production in addition to the conventional parameters, i.e. ECG and blood pressure (Guazzi et al., 2012).

The Working Group on Cardiac Rehabilitation and Exercise Physiology of ESC categorized ET in endurance aerobic (continuous and interval), strength/resistance and respiratory training (Corrà et al., 2005).

As highlighted in the consensus document of the Heart Failure Association and the European Association for Cardiovascular Prevention and Rehabilitation (2011) there is no universal agreement on exercise prescription in CHF (Piepoli et al., 2011). Traditionally, exercise programmes include the use of treadmills, steppers, rowers, weights, elliptical trainers, exercise bikes, dumbbells etc., whereas swimming pools can be very helpful for the training of highly debilitated patients.

Aerobic or endurance training (i.e. cycling, walking, rowing) is the most investigated training modality in CHF patients and is recommended as baseline activity.

Continuous aerobic training is typically performed at moderate to high exercise intensities in steady-state aerobic conditions, which allows the patient to perform prolonged training sessions. Patients start exercise at low intensity and with short sessions (5-10 minutes) twice a week; then, if the training is well tolerated, the numbers and duration of sessions per week are increased (up to 20–60 minutes on 3-5 days a week) at moderate-to-high intensity. Aerobic training is the best-described and established form of training, because of its well-demonstrated efficacy and safety, and because it is easily performed by patients, usually on a cycle ergometer or a treadmill. The gold standard method for exercise intensity assessment is the use of directly determined physiological descriptors of metabolic

effort intensity, i.e. peak oxygen consumption (VO_2 peak) by symptom-limited CPX. If CPX is not available, heart rate (HR) or HR reserve (HRR= difference between the basal and peak HR) and rating of perceived exertion (RPE) are determined during a conventional stress test or a 6 min walking test. A 'training HRR range' of 40–70% HRR and 10/20–14/20 of the Borg RPE are recommended.

Recently, interval (or intermittent) endurance training has been proposed to be more effective than sessions of continuous exercise for improving exercise capacity. Short moderate–high intensity bouts (10–30 s) (50–100% of peak VO_2) are interspersed with 1-3 min recovery periods at no or low intensity. Typically high- and low-intensity interval training programmes can be used according to the patient's capabilities.

Resistance/strength training is a type of physical exercise that leads the muscles to contract against an external resistance with the expectation of increases in strength, tone, mass, and/or endurance. It gradually and progressively overloads the musculoskeletal system, so it strengthens and tones muscles and increases bone mass. It has been proposed as an anabolic intervention to prevent the wasting syndrome, particularly frequent in elderly HF patients.

Resistance/strength training is divided in three progressive steps.

- The first step ("instruction phase") should be performed to allow the patient to learn and to improve the modality of the exercise and muscular coordination. These preparatory exercises must be conducted slowly, without or at a very low resistance (<30% 1-RM) until the patient is confident with the course of the movements. One repetition maximum (1-RM) is used for determination of the training intensity even if not suitable for CHF patients, in fact a graded stress test is preferred. The training intensity should be set at the level of resistance at which the patient can perform 10 repetitions without abdominal straining and without symptoms. When assessing the load for a patient, the Borg RPE scale provides the patient's stress perceptions. In patients with moderate risk, stress perception should be at a maximum RPE of 15.

- The second step is called "resistance/endurance phase' and can be started with a high number of repetitions (12–25) and a low intensity (30–40% 1-RM), corresponding rather to a combination of endurance and resistance because of a low haemodynamic load.

- The last step is the "strength phase" and consists of 8-15 repetition at higher intensity (40–60% 1-RM) in order to increase muscle mass.

During each step, the risk of the abdominal straining (Valsalva manoeuvre) and consequent blood pressure elevations could occur, thus it is important to prescribe the appropriate level of training according to the patient's motivation level, personality, and previous experience with resistance/strength training.

On the contrary, the American College of Sports Medicine (ACSM) recommends that resistance training should be progressive, individualized, and provide a stimulus for all the major muscle groups (chest, back, shoulders, arms, abdominals, and legs). Moreover, ACSM recommends that beginners perform one set of 8 to 10 exercises for the major muscle groups, 8 to 12 repetitions to fatigue, 2 to 3 days per week. For older and particularly frail people (approximately 50-60 years of age and above), 10-15 repetitions may be more appropriate (Garber et al., 2011).

Both aerobic endurance exercises and resistance training can promote substantial benefits in physical fitness and health-related factors (Pollock et al., 2000). Aerobic endurance training effectively modifies cardiovascular risk factors associated with the development of CAD. Resistance training offers greater development of muscular strength, endurance, and mass. It also assists in the maintenance of basal metabolic rate (to complement aerobic training for weight control), and can be beneficial in the prevention and management of other chronic conditions, e.g. low back pain, osteoporosis, overweight and obesity, sarcopenia, diabetes mellitus, and impaired physical function in frail and elderly persons, as well as in the prevention and rehabilitation from orthopaedic injuries. Although the mechanisms may be different, both aerobic/endurance exercise and resistance training appear to have similar effects on bone mineral density, glucose tolerance, and insulin sensitivity (Eriksson et al., 1998; Pollock and Vincent, 1996).

For weight control, aerobic exercise is considered a significant calories burner, whereas resistance training assists the body in expending calories via an increase in lean body mass and basal metabolism. Thus, resistance training exercise is strongly recommended for implementation in primary and secondary cardiovascular disease–prevention programmes (Pollock et al., 2000).

Respiratory Muscle Training, can be defined as a technique that aims at improving the function of the respiratory muscles through specific exercises. It consists of a series of exercises, breathing and other, to increase strength and endurance of the respiratory muscles and therefore improve respiration. Respiratory muscle dysfunction, characterized by respiratory muscle fibre atrophy, deoxygenation and impaired mitochondrial oxidative capacity has been predominantly observed in patients with advanced HF (Wong et al., 2011).
Winkelmann and colleagues demonstrated in 2009 that the addition of inspiratory muscle training (IMT), using respiratory muscle-specific training devices, to aerobic training in 24 patients with HF and inspiratory muscle weakness improved inspiratory muscle performance, VO_2 peak and functional status compared to aerobic training without IMT (Winkelmann et al., 2009).

Regardless of the type of exercise performed, the beneficial effects do not persist long-term after completion of CR without a long-term maintenance program. Therefore, one of the principal objective of CR programmes is the preservation of its benefits over time by the identification of strategies aimed at motivating patients to continue with exercise at home.

Table 2.1. Indications and contraindications to participate in an exercise-based CR program. (Arena et al., 2007; Balady et al., 2007; Carrel and Mohacsi, 1998; Herdy et al., 2008; Lavie et al., 2009; Marwick et al., 2009; Myers et al., 2009; Naughton, 1992; Wenger, 2008)

Indications	Contraindications
• Recent myocardial infarction • Coronary bypass • Valvular heart disease • Valve surgery • Coronary angioplasty • Cardiac transplantation • Stable angina	• Unstable angina • Uncompensated heart failure • Uncontrolled arrhythmias • Severe ischemia, LV dysfunction during exercise testing • Poorly controlled hypertension • Acute thrombophlebitis • Pulmonary or systemic embolism • Myocarditis • Severe psychological disorders • Severe mobility limitations

2.2 Molecular mechanisms of Cardiac Rehabilitation: the role of oxidative stress

The hemodynamic and biochemical beneficial effects of exercise are widely known, but the mechanisms responsible for such benefits have not been fully elucidated. Exercise does not only reduce cardiovascular risk factors, such as diabetes, arteriosclerosis and hypertension, but it also improves the functional status and clinical outcomes in patients with heart disease (McMullen et al., 2007; Naughton, 1992).

The cardiovascular benefits of exercise are multifactorial and occur both at local and systemic level. Many studies have demonstrated that physical activity reduces adipocyte mass and body mass index (BMI), affects insulin sensitivity and glucose uptake by skeletal muscle and regulates cholesterol profiles (Thompson et al., 2012). Physical activity leads to beneficial changes in the systemic and coronary vasculature, improving arterial compliance and reducing systolic and diastolic pressure (Nualnim et al., 2012; Pescatello et al., 2004).

Normally, during exercise, the increase in skeletal muscle blood flow and demand for more oxygen is satisfied by arterial dilatation. In patients with CHF, blood flow is decreased because of a reduction in arteriolar dilatation and a reduction in cardiac output due to an increase in neurohormonal mediated vasoconstriction. Activation of the sympathetic nervous system and activation of renin-angiotensin-aldosterone system (RAAS) have adverse hemodynamic consequences in HF because they enhance vasoconstriction, caused by release of angiotensin II (ANG II), and promote fluid retention.

High concentrations of ANG II induce necrosis of cardiomyocytes (Mann et al., 1992; Tan et al., 1991), and adversely influence matrix structure of myocardium (Weber, 1997). Previous studies have shown that pharmacologic suppression of neurohormonal activation as well as a regular endurance exercise improve symptoms and survival in CHF patients (Braith et al., 1999).

ANG II stimulates NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activity, which increases local ROS production, leading to endothelial dysfunction. This latter has an important role in CVDs and contributes to impair exercise capacity. During life, vascular endothelium is constantly exposed to "oxidative stress", which occurs when Reactive Oxygen Species (ROS), such as superoxide

(O_2^-), hydroxyl (OH) and many others (e.g., hydrogen peroxide [H_2O_2]) are produced in excess respect to endogenous antioxidants (Conti et al., 2015).

These molecules are continuously produced in the cell and are involved in physiological events such as primary immune defence, cell differentiation and signalling (Gülçin, 2012; Poli et al., 2004). Indeed, some ROS such as H_2O_2 are versatile players of the molecular signalling machinery because they are small, highly diffusible, and can be rapidly generated and degraded (Gough and Cotter, 2011).

High levels of ROS or exposure for a prolonged period to these molecules seem to produce deleterious effects in both cardiac and vascular myocytes, where they may contribute to contractile dysfunction and progression of maladaptive myocardial remodelling in advanced HF. Specifically, ROS can directly impair contractile function by modifying proteins involved in excitation-contraction coupling, activate a broad variety of hypertrophy signalling kinases and transcription factors and mediate apoptosis (Lee et al., 2012).

Although the presence of oxidative stress under pathophysiological conditions has been linked with elevated levels of ROS formation and lipid peroxidation, direct cause-effect relationship needs to be verified (Dhalla et al., 2000).

To counteract the negative effects derived by ROS accumulation, mammalian cells defend themselves by activating a variety of antioxidant enzymes, including SuperOxide Dismutases (SOD), Catalase (Cat), and Glutathione Peroxidases (GPx) (Harrison, 2014; Pisoschi and Pop, 2015).

In particular, SOD catalyses the dismutation of superoxide into oxygen and hydrogen peroxide during physiological and pathological conditions; conversely, Cat reduces hydrogen peroxide to water, while GSHPx catalyses the reduction of H_2O_2 and hydroperoxides, which results in prevention of the more toxic radicals formation.

Some recent studies have suggested that these enzymes might play an important role in the pathophysiology of HF (Matsushima et al., 2006; Qin et al., 2010).

HF caused by ischemic or non-ischemic heart disease induces endothelial dysfunction and contributes to muscular dysfunction and symptoms, such as fatigue and dyspnoea that may limit the motor capability of the patient.

Exercise training in HF improves vascular shear stress and direct mechanical stimulation of muscle. In particular, the effect of exercise on redox balance is extremely complex depending on age, sex, and training level, as well as intensity, duration and workload of exercise. Regular moderate training appears beneficial for oxidative stress and health, while acute and strenuous bouts of aerobic and anaerobic exercise can induce ROS overproduction (Pingitore et al., 2015).

ET generally enhances ROS production, but at the same time leads to the up-regulation of several antioxidant enzymes, including SOD, Cat and GPx. Therefore, low or moderate levels of ROS, generated by a regular and moderate ET, improve exercise health-promoting effects (theory of hormesis) (Nikolaidis and Jamurtas, 2009), whereas relatively large and/or chronic amounts of the same species cause cellular damage or death because they exceed the capacity of the oxidative stress response to maintain homeostasis (Merksamer et al., 2013).

Rinaldi et al. (2006) demonstrated that a physical training program induced high levels of SOD and increased HSP70 and of HSP27 expression in trained old rats compared to sedentary old and young rats. This finding was in accordance with other studies where training protocols were able to increase SOD activity in sedentary old rats.

Conti and colleagues compared the amount and quality of response to antioxidant/oxidant activity in sera of athletes practicing from at least five years different sports classified as "aerobic" (triathlon), "mixed aerobic–anaerobic" (soccer), and "anaerobic" (sprint running). Results indicated that the aerobic exercise was able to promote the release of circulating factors, which induce favourable adaptations and organism response to stressful stimuli. Functional and hemodynamic variables did not differ between groups of athletes, whereas there were dramatic changes in serum markers for oxidative stress (Conti et al., 2012).

Many of the pathways involved in the oxidative stress and age-related diseases are regulated by a family of NAD^+-dependent deacetylases, known as sirtuins, that are activated in conditions of nutrient depletion and cellular stress. In particular, Sirt1 can mediate the favourable antioxidant effects of ET.

Of note, a study of Ferrara and co-workers demonstrated that moderate and prolonged exercise stimulated antioxidant system and induced the activity of Sirt1 in the heart and adipose tissue of aged rats. Sirt1, through the deacetylation of

Foxo3a, promoted the increase of antioxidant scavengers such as MnSOD and Cat. These results explained that ET, which significantly increases Sirt1 activity, could counteract age-related systems impairment (Ferrara et al., 2008).

The role of sirtuins and their modulation will be discussed in detail in the next paragraph.

2.3 The Sirtuin family

Sirtuin or Sir2 proteins are a class of enzymes that possess either mono-ADP-ribosyltransferase, or deacylase activity, including desuccinylase, demalonylase, and defatty-acylase activity (Du et al., 2011; Jiang et al., 2013; North and Verdin, 2004). The name Sir2 comes from the yeast gene 'silent mating-type information regulation 2', the gene responsible for cellular regulation in yeast. Mammalian sirtuin family consisted of seven members (Sirt1-Sirt7) with different localization, tissue specificity, activity and molecular targets (Frye, 2000). On the basis of phylogenetic classification, the family is divided into five classes (I-IV and U); class I and class IV are further divided into three and two subgroups, respectively. The U-class sirtuins are found only in Gram-positive bacteria (Frye, 2000) (**Table 2.2**).

All sirtuins are characterized by a 250 amino acid highly conserved core domain and nicotinamide adenine dinucleotide (NAD^+)-binding site. They require the presence of NAD^+ as cofactor to deacetylate substrates ranging from histones to transcriptional regulators; consequently, their enzymatic activity directly depends on cellular energy status, which, in turn, depends on NAD^+/NADH ratio.

Sirt1 and Sirt2 can be localized both in the nucleus and cytosol and the translocation from nucleus to cytosol occurs in specific conditions. Sirt3, Sirt4 and Sirt5 are mitochondrial, while Sirt6 and Sirt7 are almost exclusively nuclear (D'Onofrio et al., 2015).

Thanks to their localization and enzymatic characteristics, sirtuins are involved in a wide range of cellular functions. With their chromatin modifying activity, sirtuins induce global transcriptional changes generally associated to variation in energy metabolism and/or aging. They are, in fact, involved in several metabolic processes (e.g. gluconeogenesis, fatty acid oxidation, oxidative phosphorylation, and urea cycle), in the responses to hypoxic, heat shock or genotoxic stresses as well as to chronic inflammation; they can also control circadian clocks, mitochondrial biogenesis and embryonic development in many tissues and organs (Nakagawa and Guarente, 2011).

Studies on sirtuins biology have gained much interest in the past two decades, emphasizing the critical importance of these enzymes in human biology and disease.

Among sirtuins, Sirt1 is undoubtless the best-studied enzyme. First detected in the nucleus, later it became clear that the protein could shuttle from the nucleus to the cytoplasm under certain conditions. Sirt1 deacetylates several lysine residues of histones (e.g., histone H4 lysine 16, H3 lysine 9, and H1 lysine 26), and targets also non-histone proteins and some transcription factors like tumour suppressor p53, nuclear factor kappa B (NF-κB), Forkhead box O transcription factors (FOXOs), peroxisome proliferator-activated receptors (PPAR), and p300 (Rahman and Islam, 2011). Sirt1 is involved in numerous essential processes and performs various biological functions, including regulation of metabolism, promotion or suppression of tumorigenesis, lifespan control, and counteracts aging and oxidative stress.

Differently from Sirt1, Sirt2 is mainly localized in the cytoplasm but can shuttle to the nucleus. It deacetylates a wide range of targets influencing diverse pathways implicated in cell-cycle progression, microtubule dynamics, inflammation and oligodendroglial differentiation (North et al., 2003).Sirt2 regulates adipocyte differentiation through FOXO1 acetylation and deacetylation, as suggested by Jing and co-workers (Jing et al., 2007). Moreover, it is highly expressed in heart, testis, skeletal muscle and brain where it seems to be involved in α-synuclein-mediated toxicity in a Parkinson's disease model (Outeiro et al., 2007). However, further studies are needed to evaluate the physiological and pathological functions of Sirt2.

Sirt3 is the best-characterized mitochondrial sirtuin. It has deacetylase activity towards many proteins that regulate diverse mitochondrial functions such as ATP production, ROS management, β-oxidation and cell death (Lombard and Zwaans, 2014). In particular, Sirt3 regulates the tricarboxylic acid cycle (TCA) isocitrate dehydrogenase 2 (IDH2) and proteins in the electron transfer chain. It also plays an important role in the metabolic adaptive response; low levels of Sirt3 cause an increase of oxidative stress and insulin-resistance, as well as impaired glucose oxidation in muscle, associated with decreased pyruvate dehydrogenase (PDH) activity, accumulation of pyruvate and lactate metabolites, and an inability of insulin to suppress fatty acid oxidation(Jing et al., 2013). Although its localization is in mitochondria, Sirt3 can influence nuclear DNA repair mechanism. Kim and colleagues demonstrated that Sirt3 depletion leads to an increased level of

superoxide and genomic instability under stress condition, promoting tumour development in mammary glands (Kim et al., 2010). Sirt3 also regulates glutathione-mediated redox balance and suppresses hypoxia inducible factor 1α (HIF-1α) and tumour growth by inhibiting mitochondrial ROS production (Bell et al., 2011).

Like Sirt3, also Sirt4 localizes in mitochondria and it is broadly expressed in all tissues except in the foetal thymus and adult leukocytes. In contrast to Sirt3, Sirt4 has only ADP-ribosylation but no deacetylation activity. It has been shown that Sirt4 can ribosylate glutamate dehydrogenase (GDH) and control insulin secretion in pancreatic β-cells in response to caloric restriction (Haigis et al., 2006), but it is mostly known for its role in the glutamine metabolism. Sirt4 is also implicated in fatty acid oxidation where it acts as a repressor of malonyl-CoA decarboxylase (MCD), an enzyme that provides the carbon skeleton for lipogenesis. Through deacetylation and inhibition of MCD activity, Sirt4 favours fatty acid synthesis over fatty acid oxidation in fed condition and deletion of Sirt4 is protective against diet-induced obesity (Laurent et al., 2013). Recently, Jeong et al. demonstrated that Sirt4 loss leads to both increased glutamine-dependent proliferation and stress-induced genomic instability, resulting in tumorigenic phenotypes (Jeong et al., 2013).

Sirt5 acts as a histone deacetylase, lysine desuccinylase, demalonynase, primarily involved in the urea cycle (Wagner and Hirschey, 2014). It regulates nitrogen balance and some aspects of mitochondrial metabolism, but further studies will have to be conducted to clarify its biological role.

Sirt6 is a stress responsive protein deacetylase and mono-ADP ribosyltransferase enzyme, implicated in multiple molecular pathways related to aging and metabolic homeostasis, including DNA repair, telomere maintenance and genomic stability, glycolysis and inflammation (Cardus et al., 2013; Michishita et al., 2008). Sirt6 knockout mice develop severe hypoglycaemia that leads to death 4 weeks after birth (Mostoslavsky et al., 2006). In addition, it protects against several age-related diseases, including cancer.

As for Sirt1 and Sirt3, existing evidence support both oncogenic and tumour suppressor properties for Sirt6 (Liu et al., 2013; Van Meter et al., 2011). It is implicated in numerous DNA repair pathways by protecting telomeric chromatin

from DNA damage and genomic instability. Chua and her group first illustrated that Sirt6 is necessary for efficient DNA Double-Strand Breaks (DSB) repair as well, mainly by stabilizing DNA-PK (DNA-dependent protein kinase) at DSB sites which in turn promotes NHEJ (Non-homologous end joining) repair (McCord et al., 2009; Michishita et al., 2008).

Moreover, a recent work on Sirt6 has shown how favourable modification of cellular processes through deacetylation slows HF progression. *In vitro* studies have suggested that this sirtuin suppresses cardiomyocyte hypertrophy via its interaction with NF-kB (Yu et al., 2013). Overall appears that, through its effects on histone deacetylation, Sirt6 plays an important role in maintaining both lifespan and health span.

Among sirtuins, Sirt7 is the least studied member. It is a nuclear protein highly expressed in the spleen, ovary and thyroid. In the nucleus, Sirt7 interacts with chromatin remodelling complexes to silence gene expression. In particular, it has been shown to be a positive regulator of RNA polymerase I (Ford et al., 2006) and a key mediator of many cellular activities (Kiran et al., 2015).

Recent studies highlighted that Sirt7 is an important stress adaptor molecule for supporting cell survival in different stress conditions. Additionally, high expression of Sirt7 has been reported in few cancers, where it might play an important role as oncogene (Kiran et al., 2015).

Although all seven sirtuins are probably equally important, most of the studies conducted to understand the mechanisms of action and biological relevance of these proteins have involved only Sirt1. Thus, in recent years, a large body of data have contributed to show that Sirt1 represents a critical upstream enzyme able to regulate fundamental biological processes and pathophysiology of age-associated diseases.

Table 2.2. Classification, prevalent activity and localization of sirtuins. (Frye, 2000)

SIRT	Class	Enzymatic activity	Localization
1	I	NAD$^+$-dependent deacetylase	nucleus and cytoplasm
2	I	NAD$^+$-dependent deacetylase	cytoplasm
3	I	NAD$^+$-dependent deacetylase	nucleus and mitochondria
4	II	ADP-ribosyltransferase	mitochondria
5	III	NAD$^+$-dependent deacetylase Desuccinylase Demalonylase	mitochondria
6	IV	NAD$^+$-dependent deacetylase ADP-ribosyltransferase Defatty-acylase	nucleus
7	IV	NAD$^+$-dependent deacetylase	nucleolus

Class I shows high homology to yeast sirtuins, has no equivalents among prokaryotes and presents strong deacetylase activity; class II exerts mostly ADP-ribosyltransferase activity and is also present in some bacteria; class III is abundantly spread among prokaryotes and little is known about its enzymatic activities; class IV is present only in eukaryotes and exerts different enzymatic activities, such as demyristoylase, depalmitoylase, ADP-ribosyltransferase and deacetylase; lastly, class U is present in gram positive prokaryotes as well as in gram negative hyperthermophilic bacterium Thermotoga maritima and regulates acetyl-CoA synthetase.

2.4 Role of Sirt1 in Heart Failure

Several studies suggest that sirtuins modulation may have beneficial effects in the treatment of a number of human diseases, including HF (Hewitson et al., 2013; Villalba and Alcaín, 2012).

Sirt1 and Sirt3 have been most extensively investigated in the cardiovascular system, with emerging evidence suggesting that they play a protective role in contrasting the onset and progression of HF. Sirtuin functions include regulation of energy and metabolism of cardiomyocyte, oxidative stress response, intracellular signalling and Ca^{2+} handling, angiogenesis and cellular survival (Shi et al., 2010; Tanno et al., 2012).

As previously reported, Sirt1 exerts its function through deacetylation of important substrates, such as peroxisome proliferators-activated receptor-γ (PPAR-γ) and its coactivator-1α (PGC-1α), FoxOs, AMP-activated protein kinase (AMPK), NF-κB, and many others to modulate cellular pathways implicated in age-related diseases (Zeng et al., 2009).

In particular, Sirt1 promotes vessel growth and function by interacting with FoxOs, which have unexpected and diverse roles in countering stress, determining cell fate, and regulating energy availability. As the heart constantly adapts itself in response to different stressors and metabolic conditions, FoxOs also appear to play an important role in cardiac physiology (Evans-Anderson et al., 2008; Ronnebaum and Patterson, 2010).

Several studies revealed that FOXO1 loss of function leads to embryonic lethality in mice, and mice lacking FOXO3 are apparently normal at birth but develop cardiac hypertrophy and HF in adult life (Hosaka et al., 2004; Ni et al., 2006).

By deacetylation of FoxOs, Sirt1 stimulates the expression and activity of antioxidant enzymes, e.g., Cat and MnSOD, and also potentiates its expression via an auto-feedback loop (Brunet et al., 2004; Sengupta et al., 2011; Xiong et al., 2011). The Sirt1/FoxO axis is an evolutionarily conserved survival pathway, which regulates cellular responses to both metabolic changes and many insults including oxidative stress.

Moreover, Sirt1 has been shown to delay atherosclerosis progression influencing vascular endothelial homeostasis. It maintains the balance between vasodilation and vasoconstriction by controlling endothelial Nitric Oxide Synthase (eNOS), the

enzyme that catalyses NO synthesis (Hung et al., 2004). NO has important anti-atherosclerotic properties and acts as a major mediator of endothelial function, contributing to the regulation of vasomotor tone and vessel wall permeability, suppression of leucocyte adhesion to the endothelial surface, inhibition of platelet aggregation, vascular smooth muscle cell migration and proliferation (Qian and Fulton, 2013).

It has been demonstrated that inhibition of Sirt1 blocks endothelium-dependent vasodilatation and NO bioavailability (Mattagajasingh et al., 2007), causing endothelial dysfunction, which is a main characteristic of vascular aging (Collins and Tzima, 2011). Conversely, resveratrol, a Sirt1 activator, activates eNOS, improves endothelial function, and restores vascular eNOS activity, thus preventing the increase of blood pressure and consequent hypertension (Xia et al., 2013). On one hand, resveratrol increases eNOS gene transcription by activating Sirt1, FOXO factors (Xia et al., 2013) and Krüppel-like factor 2 (KLF2), a transcription factor with anti-thrombotic, anti-inflammatory and vasodilatory properties (Gracia-Sancho et al., 2010); on the other hand, resveratrol improves eNOS activity via promoting Sirt1 deacetylation at lysines 496 and 506 in the calmodulin-binding domain of eNOS.

Several studies have investigated the protective role of Sirt1 against cardiac apoptosis, and its modulation on cardiomyocyte is accentuated under stress condition. There is a substantial evidence showing that cardioprotective Sirt1 effects are strongly dependent on its expression levels. Indeed, Alcendor and co-workers demonstrated in transgenic mice that a moderate expression of Sirt1 (up to 7.5-fold) protected the heart from oxidative stress induced by paraquat, with increased expression of antioxidants, such as Cat, while a higher level (12.5-fold) of Sirt1 increased oxidative stress and apoptosis and evoked pathological changes in the heart (hypertrophy and decreased cardiac function) (Alcendor et al., 2004, 2007).

Similarly, Kawashima et al. demonstrated that cardiac-specific overexpression of Sirt1 at a high level (20-fold) caused dilated cardiomyopathy and also moderate (6.8-fold) overexpression impaired cardiac diastolic function (Kawashima et al., 2011).

Taken together, these results suggest that Sirt1 could retard aging and confer stress resistance to the heart *in vivo*, but, importantly, these beneficial effects occur only at low to moderate levels (up to 7.5-fold). Probably, high levels of this sirtuin induce mitochondrial dysfunction through consumption of NAD^+, which is required for mitochondrial respiration; in this way, depletion of NAD^+ could lead to deficiency in ATP and, consequently, cell death (Alcendor et al., 2007).

Cardiac-specific expression of Sirt1 has also shown protection against oxidative stress in the heart by hampering myocytes cell death induced by Poly (ADP-ribose) polymerase-1 (PARP) activation.

As demonstrated by Pillai and colleagues, in both failing hearts and cultured cardiac myocytes, the increased activity of PARP was associated with depletion of cellular NAD^+ levels and reduced Sirt1 deacetylase activity. However, myocyte cell death induced by PARP activation was prevented by repletion of cellular NAD^+ levels either by adding NAD^+ directly to the culture medium or by overexpressing NAD^+ biosynthetic enzymes. In addition, the beneficial effect of NAD^+ repletion was seen only in presence of Sirt1. Knocking down Sirt1 levels by small interfering RNA eliminated this benefit, indicating that Sirt1 is a downstream target of NAD^+ replenishment leading to cell protection (Pillai et al., 2005).

Sirt1 is also involved in energy homeostasis that has undoubtedly a critical role in development and progression of HF (Tanno et al., 2012). Energy depletion characterizes the failing heart and results from its decreased ability to produce ATP from the available substrates and the extent of the deficit may vary depending on the stage of disease. With regard to substrate utilization, fatty acids are preferred substrates; their oxidation inhibits glucose uptake, whereas glucose together with insulin inhibits fatty acid oxidation. This reciprocal metabolic control, known as the Randle cycle, is perturbed in HF (Beauloye et al., 2011).

In physiological conditions, the heart predominantly uses free fatty acid (FFA) for ATP production (i.e., approximately 70% of total ATP) (An and Rodrigues, 2006), while in the early stage of HF, the heart switches the substrate to glucose, which produces more ATP per molecule of oxygen consumed than FFA, at the expense of low energy yield compared with the yield in FFA oxidation. In advanced HF, inevitably, insulin resistance develops in the myocardium and glucose utilization

declines, limiting ATP production and leading to myocardial contractile dysfunction (Witteles and Fowler, 2008).

ATP deficiency leads to AMPK activation, a serine/threonine kinase that senses the energy status of the cell and coordinates a global metabolic response to energy deprivation. Once activated, AMPK switches on catabolic pathways to produce ATP while simultaneously shutting down energy-consuming anabolic processes. Thus, biosynthetic processes, such as gluconeogenesis, glycogen synthesis, lipogenesis, cholesterol synthesis, and protein synthesis are inhibited, whereas glucose utilization, fatty acid oxidation, and mitochondrial biogenesis are stimulated (Beauloye et al., 2011).

In order to perform these actions, AMPK can quickly regulate metabolic enzymes through direct phosphorylation, but, additionally, it also has long-term effects at the transcriptional level in order to adapt gene expression to energy demands.

AMPK enhances Sirt1 activity by increasing cellular NAD^+ levels, resulting in the modulation of downstream Sirt1 targets, including PGC-1α, a master switch of mitochondrial biogenesis. It has been showed that PGC-1α–deficient mice developed early signs of HF resulting from an inability of the heart to meet energy demands, thus emphasizing the importance of metabolic and energy homeostasis in cardiac health (Arany et al., 2005).

AMPK and Sirt1 directly affect PGC-1α activity through phosphorylation and deacetylation, respectively. While the physiology relevance of these modification and their molecular consequences are still unknown, recent insight from different *in vivo* transgenic models clearly suggest that axis AMPK-Sirt1-PGC-1α might act as a coordinated network to improve metabolic fitness (Cantó and Auwerx, 2009).

In conclusion, the mechanisms underlying the development of HF are multiple, complex, and not well understood. Accumulating data suggest that sirtuins, especially Sirt1, are potential targets for natural and pharmaceutical interventions particularly in aging-associated diseases. Among natural interventions, it seems that caloric restriction, regular physical exercise and resveratrol and other polyphenolic compounds could improve human health through activation of sirtuin-mediated processes (Radak et al., 2013).

In particular, studies on animal models have shown benefits of resveratrol including increased longevity, amelioration of cardiovascular disease, improved

sensitivity to insulin, reduced aging-related neurocognitive decline and neuropathies through mechanisms largely centred on pathways related to sirtuin gene activation (Baur et al., 2006; Pearson et al., 2008). Recently, multiple human clinical trials exploring the health impact of resveratrol treatment in pathologies like obesity, diabetes, and CVD have been performed (Smoliga et al., 2013); unfortunately, the results of these trials are not always consistent with laboratory animal data. Indeed, when resveratrol clinical trials have been directly translated to healthy populations, the results have often been disappointing (e.g. Poulsen et al., 2013; Yoshino et al., 2012).

In conclusion, the presence of Sirt1 activators and/or modulators into human trials is exciting, but there is a clear need to perform more investigations aimed at better defining the molecular pathways mediating their effects *in vivo*.

AIMS OF THE STUDY

Cardiac rehabilitation (CR), based on exercise training, has been recognized as a fundamental component in the continuum of care for patients with CVDs, including HF. It is a cost-effective tool as it improves prognosis by reducing recurrent hospitalization and health care expenditures, while prolonging life span.

The benefits of exercise as an essential component of CR programmes are associated with a significant improvement in hemodynamic parameters; although the functional and hemodynamic effects of CR are well known, few data explaining the molecular mechanisms underlying exercise-based CR are available.

Exercise training counteracts toxic oxygen metabolite accumulation and stimulates natural antioxidant defences. Recently, it has been discovered that the NAD^+-dependent deacetylase Sirtuin1 (Sirt1) is effective against oxidative stress and endothelial dysfunction, phenomena strictly correlated with CVDs.

The main aim of my PhD project was to evaluate whether a 4-week CR program was able to induce a ROS-dependent cellular adaptation in patients with stable HF, focusing attention to the role played by Sirt1 in the response to oxidative stress and in the control of cellular senescence.

CHAPTER 3

Materials and Methods

3.1 Study design and population

Male subjects with post-ischemic HF in clinically stable conditions, classified as in NYHA II and III class and with a preserved EF [10 patients with HF preserved borderline EF and 40 with HFpEF], consecutively admitted to the Cardiac Rehabilitation Unit of "San Gennaro dei Poveri" Hospital in Naples, were enrolled. All definitions were based on the ESC and ACCF/AHA criteria, in which the term "stable" defines treated patients with symptoms and signs that have remained generally unchanged for at least a month. (McMurray et al., 2012; WRITING COMMITTEE MEMBERS et al., 2013)

Patient information and consent forms were approved by the local Medical Research Ethics Committee.

Exclusion criteria included unstable angina pectoris, uncompensated HF, complex ventricular arrhythmias, use of nitrates, pacemaker implantation and orthopaedic or neurological limitations to exercise. None of the patients had experienced a MI in the 12 months preceding the study and, on the basis of BMI, none were cachectic.

All enrolled patients underwent a physical examination, collection of demographic and routine blood chemistry tests, chest X-ray, blood pressure measurement, electrocardiographic and echocardiographic examinations, cardiopulmonary stress test and a 6-minute walking test with Borg index evaluation.

3.2 Training protocol

The rehabilitation program consisted of 30-minute sessions of aerobic exercise, 5 days a week. A daily training session comprised a warm-up (10 min), endurance training (15 min) and a cool-down (5 min) on a cycle ergometer at 50% of the VO_2 max achieved on the cardiopulmonary stress test. For interval training, low muscle commitment calisthenics and respiratory exercises were performed.

3.3 Blood sample collection

Overnight fasting blood samples were obtained from patients before starting and at the end of the rehabilitation program. After centrifugation at 1,500 × g for 10 min, serum samples were transferred to clean tubes and stored at -80°C until analysis. Lymphocytes were isolated from whole blood by Ficoll-Paque PLUS (GE Healthcare, Munich, Germany), according to manufacturer's procedures.

Samples isolated from patients before CR were indicated as P, while those collected from patients after CR were designated as RP.

3.4 Animal experiments

All animal procedures and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of University of Salerno. The procedures were the same used by Leosco et al. and Rengo et al. (Leosco et al., 2008; Rengo et al., 2012)

Fifty Wistar male rats (3 months old) were randomly assigned to one of the 3 experimental groups as follows: Sham-operated (Sh, n = 10), Sedentary post-MI HF rats (S, n=25) and Trained post-MI HF rats (T, n= 15).

Sh and S were housed sedentary for 14 weeks, while T were assigned to a 10-week (2 weeks of acclimation and 8 weeks of exercise) treadmill exercise protocol.

3.5 Experimental myocardial infarction

Rats were placed in an induction chamber and anaesthesia was induced with 4% isoflurane, animals were intubated and ventilated with a mixture of O_2 and 1% isoflurane with a pressure controlled ventilator (Harvard Instruments). The ventilation rate was set at 45-50 strokes/min. A heating pad was used to maintain rats body temperature at 37°C. A thoracotomy was performed through the third left intercostal space, and the pericardium opened. Afterwards, in rats randomized to Sedentary post-MI HF rats (S) and Trained post-MI HF rats (T) groups, MI was induced by permanent ligation of the left anterior descending coronary artery (LAD) near its origin with a 7-0 silk suture (BBraun; Aesculap AG&CO. KG,

Germany). Subsequently, the chest and the skin were closed with a 2-0 silk and a 4-0 silk suture, respectively.

The rats were observed until they awakened, and then they were returned to the Animal Care Unit.

Standard postoperative care, including analgesics and antibiotics, was provided. The mortality rate was ~44%, with most deaths occurring within the first 24 h.

In rats randomized to Sham-operated (Sh) group, the suture was pulled through the myocardium, but the LAD was not ligated.

To measure global cardiac function, echocardiography was performed in anaesthetized animals (2% isoflurane, v/v) at 24 h, 4 and 10 weeks post-MI by use of the VisualSonics VeVo 770 imaging system with a 714 scanhead. Only rats with a confirmed large ischemic area (≥40% of entire left ventricle [LV] circumference) were included into MI study groups. The internal diameter of the LV was measured in the short-axis view from M-mode recordings in end diastole and end systole. VisualSonics analysis software was used to calculate EF and fractional shortening (FS). The 10-week echocardiographic data includes those animals which completed the whole study.

3.6 Animal training protocol

Four weeks after MI, rats assigned to the trained group were acclimated to a mild treadmill exercise protocol optimized to obtain an exercise relative intensity at 40% to 50% of maximal oxygen uptake (VO$_2$ max). This acclimation period consisted of walking at a speed of 10 m/min, 30 min/day (15° inclination), for 2 weeks on a 2-lane treadmill (type 50190 TAKRAF, Schmalkalden, Germany). After this period, exercise intensity was increased and rats underwent an aerobic training program of 8 weeks, consisting in 5 days/week, 45 min/day, treadmill exercise with a speed running of 17 m/min, with an exercise relative intensity calculated at 75% to 80% of VO$_2$ max (Armstrong et al., 1983; Lawler et al., 1993). Due to the poor compliance of rats to run spontaneously, our protocol could not provide a voluntary treadmill running and animals were encouraged to run by means of a shock bar positioned at the bottom of the treadmill lane. In order to limit the stress, when rats failed to stay off the shock bar, the system was switched-off and exercise was

stopped for 5 min. After this resting period, animals restarted to run till they reached an overall daily exercising time of 45 min. Exercise resistance was evaluated immediately before and after ET period. Each evaluation was performed twice in each rat, in separated tests, and the average score was considered for analysis. The protocol for exercise resistance test consisted of walking at 10 m/min for 5 min followed by 2 m/min increase in speed every 2 min until the rat reached exhaustion. Rats were considered exhausted when they failed to stay off of the shock bar. The grade of the treadmill was set at 15°. Rats followed a 10 weeks sedentary protocol consisting in walking 10 min/day once a week to maintain treadmill familiarity.

The animals were killed by cervical dislocation under deep anaesthesia. Blood samples were collected by cardiac puncture, and serum was isolated by centrifugation at 1500 x g for 10 min. Serum was transferred to clean tubes and stored at -80°C until analysis.

3.7 Cell culture and treatments

Human Umbilical Vein Endothelial Cells (HUVECs, ECs) were purchased from Clonetics (Walkersville, MD). ECs were cultured in an endothelial growth medium, containing FBS at a concentration of 2% and bovine brain extract (with FGF-2 at a concentration of 100-500 pg/mL). The cells were subcultured by trypsinization, seeded on cell culture dishes coated with 0.1% gelatin and growth in an atmosphere of 5% CO_2 at 37°C.

Pilot experiments to identify the concentration of hydrogen peroxide (H_2O_2 = 100-750 µM) that effectively induced a significant decrease in the survival of control cells, were conducted. Therefore, a concentration of 500 µM was chosen. Moreover, the effect of oxidative stress 12, 24, 48, and 72 h after a treatment with 500 µM H_2O_2 was evaluated. Finally, the time of 48 h as representative of the most relevant changes was chosen.

The cells were seeded and cultured for 48 h in a medium supplemented with either the patient's serum (10%) at time 0 (Patient serum-conditioned ECs, P-ECs) and after CR (Rehabilitated Patient serum-conditioned ECs, RP-ECs), or FBS (10%) as a control and were exposed or not to oxidative stress induced by 500 µM H_2O_2.

Four hours after H_2O_2 exposure, the growth medium was replaced with fresh medium containing FBS.

Similar experiments were conducted by conditioning the ECs in media supplemented with the rats' sera, and the cells were classified as follows: Sham-operated rats' serum-conditioned ECs, Sh-ECs; Sedentary post-MI HF rats' serum-conditioned ECs, S-ECs; and Trained post-MI HF rats' serum-conditioned ECs, T-ECs.

All experiments were performed at a population doubling level (PDL) of 8 to 12.

3.8 Sirt1 activity

Sirt1 activity was determined in nuclei extracted from patients' lymphocytes and in ECs using a SIRT1/Sir2 Deacetylase Fluorometric Assay (CycLex, Ina, Nagano, Japan).

Crude nuclear samples were extracted by suspending the cells into 1 mL of lysis buffer (10 mM Tris HCl at pH 7.5, 10 mM NaCl, 15 mM $MgCl_2$, 250 mM sucrose, 0.5% NP-40, 0.1 mM EGTA). Cells were spun through 4 mL of sucrose cushion (30% sucrose, 10 mM Tris HCl at pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$) at 1,300 x g for 10 min at 4°C. The isolated nuclei were suspended in 50-100 µL of extraction buffer (50 mM HEPES KOH at pH 7.5, 420 mM NaCl, 0.5 mM EDTA Na_2, 0.1 mM EGTA, 10% glycerol). After centrifugation at 15,000 rpm for 10 min, the protein concentration of the crude nuclear extract without protease inhibitor was determined by the Bradford method. Sirt1 activity in the nuclei was determined using the CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit (Ina, Nagano, Japan). The reaction was carried out by simultaneously mixing fluorescent-labeled acetylated peptide as substrate, 10 µL of the sample, trichostatin A, NAD, and lysyl endopeptidase. The intensity of the fluorescence at 440 nm was measured 60 min after the onset of the reaction. Values are reported as relative fluorescence/µg of protein (AU). All data are the means ± S.D. of three independent experiments.

3.9 Markers of oxidative stress

Lipid peroxidation was determined with the thiobarbituric acid-reactive substances (TBARS) method. The assay was performed with 10 µL of serum or cell lysate. The chromogen (TBARS) was quantified using a spectrophotometer at a wavelength of 532 nm, with 1,1,3,3-tetramethoxypropane as the standard. The amount of TBARS is expressed as nmol/µg of protein. All data are the means ± S.D. of three independent experiments.

8-Hydroxy-2-deoxyguanosine (8-OH-dG), an index of oxidative damage to DNA, was determined in serum samples using an 8-OH-dG EIA kit (StressMarq Biosciences Inc., Victoria, Canada), according to the manufacturer's instructions. The assay was conducted with 50 µL of diluted serum (1:10). The absorbance was read at a wavelength of 420 nm. 8-OH-dG levels (pg/mL) were calculated using a computer spreadsheet available for data analysis (StressMarq Biosciences Inc.).

3.10 Catalase and Superoxide Dismutase antioxidant activities

Catalase (Cat) activity was determined using the Cayman Catalase Assay Kit (Cayman Chemical, USA). Samples were previously diluted with buffer (1:10 for serum; 1:2 for cell lysate). All samples (10 µL) were incubated for 20 min in the presence of 100 µL diluted Assay Buffer, 30 µL methanol, and 20 µL H_2O_2 (3.5mM) at room temperature. This method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The reaction was quenched by the addition of 30 µL potassium hydroxide. The formaldehyde produced was measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald, Cayman Chemical, USA) as the chromogen. The absorbance was read at 540 nm using a plate reader. One unit of Cat activity is defined as the amount of enzyme that leads to the formation of 1.0 nmol of formaldehyde per minute at 25°C.

Superoxide dismutase (SOD) activity was determined using the Cayman Superoxide Dismutase Assay Kit (Cayman Chemical, USA). Samples were previously diluted with buffer (1:10 for serum; 1:2 for cell lysate). The kit utilized a

tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The reaction was quenched by adding 20 μL of diluted xanthine oxidase. After 20 min of incubation at room temperature, the absorbance was read at 460 nm using a plate reader. One unit of SOD activity was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The SOD assay measured all three types of SOD (Cu/Zn, Mn, and FeSOD).

The values were reported as U/μg of protein. All data are the means ± S.D. of three independent experiments.

3.11 NO bioavailability

NO bioavailability was determined by the Griess method. Samples of serum (diluted 1:2) were incubated at room temperature with 500 μL of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 5% hydrochloric acid). Optical density was measured at 550 nm with a spectrophotometer. The amount of nitrite (NO_2^-) was calculated from a standard curve generated using serial dilutions of sodium nitrite. The results are reported as nmol/mg of protein.

3.12 Senescence-associated β-galactosidase (SA-β-gal) activity

Cultured cells were washed in PBS and fixed with 2% formaldehyde and 2% glutaraldehyde for 10 min at room temperature. After being washed twice, the cells were incubated at 37°C in staining buffer with the following components: 40 mM citric acid/sodium phosphate (pH 6.0), 0.15 M NaCl, 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, and 1 mg/mL X-gal (5-bromo-4 chloro-3-indolyl β-D-galactoside). After 4h incubation, the SA-β-gal rate was obtained by counting four random fields per dish and assessing the percentage of SA-β-gal-positive cells from 100 cells per field. Senescence values are shown as a percentage of the reference condition (FBS-conditioned ECs) which is 100%.

3.13 Sirt1 and Catalase activities inhibition

To investigate if Sirt1 or Cat activity influenced the senescence of the conditioned cells either exposed or not to H_2O_2, their respective activities were inhibited using EX-527 (Sigma, Milan-Italy) at a concentration of 5 µM for 1 hour and 3-amino-1,2,4-triazole (ATZ) (Sigma, Milan-Italy) at a concentration of 10 mM for 3 hours.

3.14 Statistical analysis

Continuous variables are expressed as mean ± standard deviation (SD) and compared by paired or unpaired Student's t test (normally distributed variables), or as median ± interquartile range value and compared by the Mann-Whitney U test (not normally distributed). Normality of data distribution was evaluated using the Kolmogorov-Smirnov test. Non-normally distributed continuous variables were converted to their natural log functions. Categorical variables are expressed as a proportion and compared by the χ^2 test, with risk ratios and 95% confidence intervals quoted.

Correlation between variables was assessed by linear regression analysis, and variables that demonstrated statistical significance in a univariate model were then included in a multivariate analysis.

All data were analysed using SPSS version 19.0 (SPSS, Inc., Chicago, Illinois-USA). Statistical significance was accepted at $p < 0.05$.

CHAPTER 4:

Results

4.1 Study population

Fifty-nine consecutive patients affected by post-ischemic HF were recruited from the Cardiac Rehabilitation Unit of "San Gennaro dei Poveri" Hospital in Naples, Italy. As only three patients were women, they were excluded from the analysis. The data coming from the analysis of six patients were not considered because they underwent nitrate therapy that may have biased the NO bioavailability assay. Ultimately, the study population consisted of 50 Caucasians male patients (mean age 68.6±6.3 years), and all of them completed the CR program.

Tables 4.1 and **4.2** show patients' characteristics and drug therapies, respectively. No sex-based or racial/ethnic-based differences were observed. At baseline, no differences in medical therapy were found, and no changes occurred during the study period. Changes in biochemical, echocardiographic and cardiopulmonary stress tests induced by CR are listed in **Tables 4.3**.

As showed, CR is able to improve biochemical levels of cholesterol and creatinine, and overall hemodynamic and respiratory parameters, indicating enhancement in exercise tolerance, one of the most crucial target in the HF treatment.

Table 4.1. Study population characteristics.

Age (y.o.) ± mean (SD)	68.6±6.3
Gender (M/F)	50/0
BMI (kg/m^2) ± mean (SD)	28.03±3.17
SBP (mmHg) ± mean (SD)	122±6
DBP (mmHg) ± mean (SD)	80±9
HR (bpm) ± mean (SD)	84±8
CAD, n (%)	49 (98)
Ischemic	47 (94)
Hypertrophic	1 (2)
Dilatative	1 (2)
PTCA, n (%)	33 (66)
CABG, n (%)	9 (18)
Valvular substitution, n (%)	1 (2)
Smoking, n (%)	35 (70)
Familiarity, n (%)	15 (30)
Hypertension, n (%)	29 (58)
Dislipidemia, n (%)	28 (56)
Diabetes, n (%)	13 (26)
COPD , n (%)	6 (12)
Obesity, n (%)	4 (8)
Peripheral Artery Disease, n (%)	2 (4)
Arrhythmias, n (%)	2 (4)
Distyroidism, n (%)	1 (2)
Other diseases, n (%)	1 (2)

Data are expressed as mean±SD or number of subjects (%). BMI= Body Mass Index; SBP= Systolic Blood Pressure; DPB= Diastolic Blood Pressure; HR= Heart Rate; bpm= beat/minutes; CAD= Coronary Artery Disease; PTCA= Percutaneous Transluminal Coronary Angioplasty; CABG= Coronary Artery Bypass Graft; COPD= Chronic Obstructive Pulmonary Disease.

Table 4.2. Study population medication use.

	N (%)
β-blockers	46 (92)
ACE-inhibitors	29 (58)
ARBs	9 (18)
Diuretics	8 (16)
Ca^{2+}-antagonists	4 (8)
α-antagonists	2 (4)
Aspirin	46 (92)
Anticoagulants	31 (62)
Other cardiac drugs	3 (6)
Antiarrhythmics	2 (4)
Statins	49 (98)
Gastro-protective drugs	44 (88)
Polyunsaturate fats	13 (26)
Oral hypoglycemics	9 (18)
Insulin	5 (10)

Data are expressed as number of subjects (%). ARBs= Angiotensin II Receptor Blockers.

Table 4.3.Changes in biochemical, echocardiographic and cardiopulmonary stress test parameters induced by CR.

	P	RP	pvalue
Biochemistry			
Cholesterol (mmol/L)	157.61 ± 37.11	150.67 ± 30.37	**<0.0001**
Creatininemia (mmol/L)	0.94 ± 0.24	0.97 ± 0.20	**<0.0001**
Hemoglobin (g/dL)	13.58 ± 1.35	13.42 ± 0.53	0.494
Echocardiographic parameters			
EF (%)	53.33 ± 8.97	55.10 ± 6.79	**0.011**
LVEDD (mm)	51.37 ± 3.77	51.31 ± 3.13	0.804
Cardiopulmonary stress test			
SBP max (mmHg)	169.02 ± 15.54	165.51 ± 19.29	**0.002**
DBP max (mmHg)	81.46 ± 5.62	80.64 ± 5.15	0.077
HR max (bpm)	117.56 ± 21.55	123.13 ± 13.20	**0.034**
Rate-pressure product (mmHg x bpm)	19892.69 ± 3841.64	20261.54 ± 3743.32	**<0.0001**
Test duration (sec)	359.41 ± 112.57	451.89 ± 109.78	**<0.0001**
VO_2 max (ml/kg/min)	20.30 ± 4.61	24.51 ± 5.81	**<0.0001**

Data are expressed as mean ± S.D. EF= Ejection Fraction; LVEDD= Left Ventricle End Diastolic Diameter; SBP= Systolic Blood Pressure; DPB= Diastolic Blood Pressure; HR= Heart Rate; bpm= beat/minutes. A *p* value <0.05 was considered significant.

4.2 Cardiac Rehabilitation on Sirt1 activity in lymphocytes, and NO bioavailability, oxidants and antioxidants in serum

Figure 4.1 shows Sirt1 activity in patients' lymphocytes, and oxidants (TBARS and 8-OH-dG), antioxidants (Cat and SOD activities) and NO bioavailability measured in patients' sera.

Surprisingly, TBARS and 8-OH-dG levels were greater in Rehabilitated Patients (RP) sera compared with Patients (P) sera before CR (both, $p<0.05$) (**Figure 4.1, Panel A and B**).

Similarly, Cat and SOD activities increased in RP relative to P sera ($p<0.005$ and $p<0.05$, respectively) (**Figure 4.1, Panel C and D**).
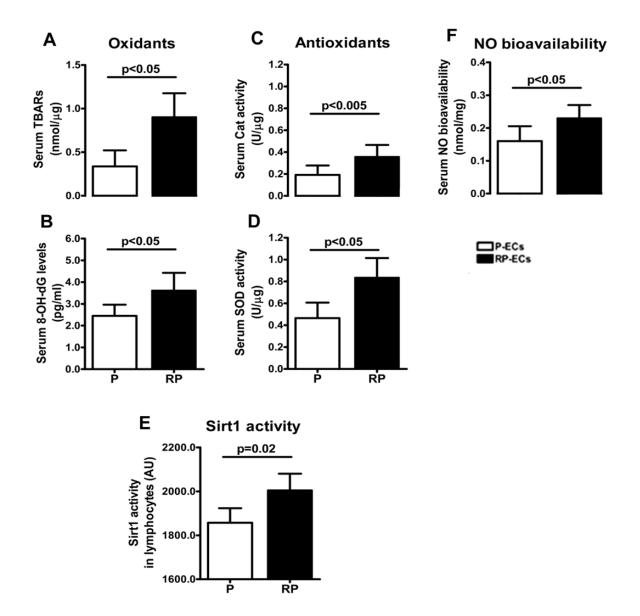
Given the pivotal role of Sirt1 in modulating stress responses in mammalian cells, its activity in patients' lymphocytes was evaluated.

Sirt1 activity in lymphocytes of RP was greater than in P ($p=0.02$) (**Figure 4.1, Panel E**).

It is conceivable that the increase of oxidants results in a higher antioxidant response, and Sirt1 is the mediator of such response.

In addition, NO bioavailability was measured. As expected, NO bioavailability in RP was higher than P sera (both, $p<0.05$) (**Figure 4.1, Panel F**).

Figure 4.1. Cardiac rehabilitation increases oxidants and antioxidants levels in sera with a concomitant rise of Sirt1 activity in lymphocytes and enhances NO bioavailability.

Lipid peroxidation levels (TBARS) **(A)**, 8-hydroxy-2-deoxyguanosine (8-OH-dG) **(B)**, Catalase (Cat) **(C)** and SuperOxide Dismutase (SOD) activities **(D)**, and NO bioavailability **(F)** were determined in serum samples.
Conversely, Sirt1 activity **(E)** was determined in the nuclei extracted from the patients' lymphocytes before (P) and after 4 weeks of cardiac rehabilitation (RP).
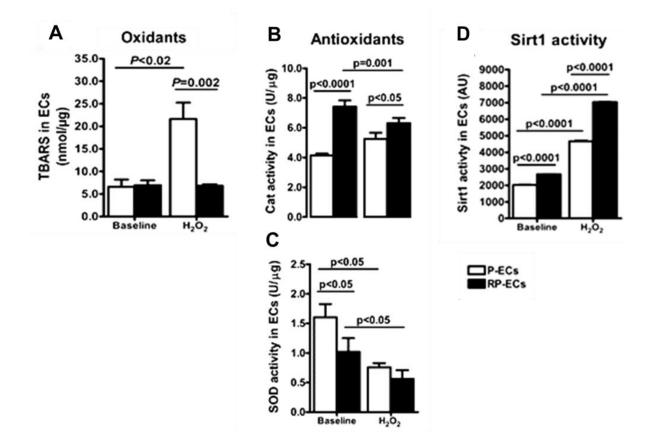
4.3 TBARS levels, Cat, SOD and Sirt1 activities in RP-ECs stressed with H_2O_2

After H_2O_2-stress induction, endothelial cells conditioned with P sera (P-ECs) showed a drastic increase of TBARS levels ($P<0.02$), while those conditioned with RP sera (RP-ECs) exhibited the same levels of TBARS when compared with basal values **(Figure 2, Panel A)**.

Cat activity increased in RP-ECs, in the absence ($p<0.0001$) and in the presence of H_2O_2 ($p<0.05$), compared with P-ECs. Conversely, SOD activity decreased in RP-ECs ($p<0.05$) compared with P-ECs but did not change in the presence of H_2O_2 **(Figure 4.2, Panels B and C)**. These findings suggest a crucial role played by Cat in determining the antioxidant response.

As shown in **Figure 4.2 Panel D**, Sirt1 activity increased in RP-ECs both in the absence and in the presence of H_2O_2 compared with P-ECs (both, $p<0.0001$).

Figure 4.2. TBARS levels, Cat, SOD and Sirt1 activities in conditioned endothelial cells.

TBARS levels **(A)**, Cat **(B)**, SOD **(C)** and Sirt1 **(D)** activities were determined in endothelial cells (ECs) conditioned with patients' sera before (P) and after (RP) 4 weeks of CR in either the presence or absence of H_2O_2-induced oxidative stress.

4.4 Sirt1 and Cat activities inhibition on the endothelial cell senescence

To investigate the role played by Sirt1 and its molecular target, Cat, in the modulation of cell senescence, P-ECs or RP-ECs were treated with Sirt1 inhibitor (EX-527) or Cat inhibitor (ATZ) and exposed or not to oxidative stress.

As shown in **Figure 4.3 panel A**, senescence decreased in RP-ECs both in the absence and in the presence of oxidative stress induction (both, $p<0.0001$), compared to levels recorded in P-ECs. The inhibition of Sirt1 activity by EX-527 caused an increase of senescence only in RP-ECs compared with baseline ($p=0.001$). Interestingly, in the ECs exposed to H_2O_2 oxidative stress, EX-527 precipitated a rise in senescence, both in P-ECs ($p<0.05$) and in RP-ECs ($p=0.001$), compared to ECs treated with H_2O_2.
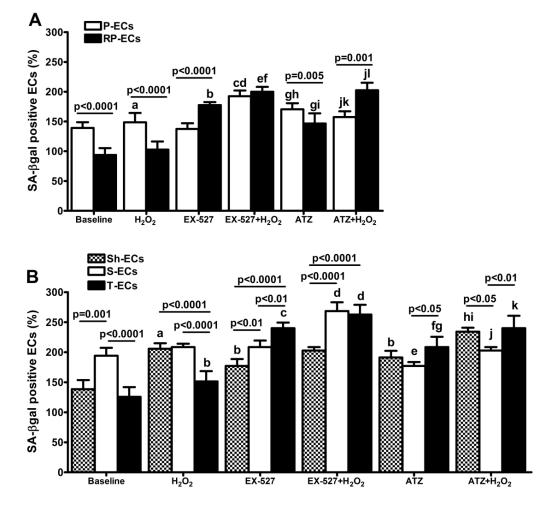
The inhibition of Cat activity by ATZ resulted in an increased senescence, both in P-ECs and in RP-ECs (both, $P<0.0001$), compared with baseline. In the RP-ECs exposed to H_2O_2, ATZ caused an increase in senescence ($P<0.0001$) compared to ECs treated with H_2O_2.

These data suggest that Sirt1 and Cat play a role in the control of endothelial cell senescence, roles confirmed by *in vivo* experiments performed in a rat model of post-ischemic HF.

Indeed, as shown in **Figure 4.3 panel B**, senescence decreased in endothelial cells conditioned with sera of trained rats (T-ECs), both in the absence and in the presence of H_2O_2 (both, $p<0.0001$), compared to levels recorded in ECs conditioned with sera of sedentary rats (S-ECs). In the absence of H_2O_2, the senescence of T-ECs was comparable to levels measured in ECs conditioned with sham-operated rats (Sh-ECs), whereas it was lower ($p<0.0001$) in the presence of H_2O_2. The inhibition of Sirt1 activity induced an increase in senescence in T-ECs compared with S-ECs ($p<0.01$) and with baseline ($p<0.005$), eliminating the protective effect of exercise. In the presence of H_2O_2 and EX-527, senescence increased both in S-ECs and in T-ECs (both, $p=0.002$).

The inhibition of Cat activity likewise eliminated the protective effect of exercise, as demonstrated by an increase in the senescence of T-ECs in the absence of oxidative stress ($p<0.01$) compared with baseline, and in the presence of oxidative stress ($p<0.005$) compared with stressed ECs.

Figure 4.3. Effects of inhibition of Sirt1 and Cat activities on endothelial cell senescence.

SA-β-gal staining of ECs conditioned with patients' sera (A) or rats' sera (B) in either the presence or absence of oxidative stress. Sirt1 and Cat activities were inhibited by EX-527 and 3-amino-1,2,3-triazole (ATZ), respectively. Senescence values are shown as a percentage of the reference condition (ECs conditioned with FBS) which is 100%.

Panel A: a) $p<0.05$ vs baseline; b) $p=0.001$ vs baseline; c) $p<0.05$ vs H_2O_2; d) $p=0.01$ vs EX-527; e) $p=0.001$ vs H_2O_2; f) $p<0.02$ vs EX-527; g) $p<0.0001$ vs baseline; h) $p<0.01$ vs EX-527; i) $p<0.05$ vs EX-527; j) $p<0.05$ vs ATZ; k) $p<0.02$ vs EX-527+H_2O_2; l) $p<0.0001$ vs H_2O_2.

Panel B: a) $p=0.001$ vs baseline; b) $p<0.02$ vs baseline; c) $p<0.005$ vs baseline; d) $p=0.002$ vs H_2O_2; e) $p=0.01$ vs EX-527; f) $p<0.01$ vs baseline; g) $p<0.05$ vs EX-527; h) $p<0.05$ vs H_2O_2; i) $p=0.002$ vs EX-527+H_2O_2; j) $p=0.001$ vs EX-527+H_2O_2; k) $p<0.005$ vs H_2O_2.
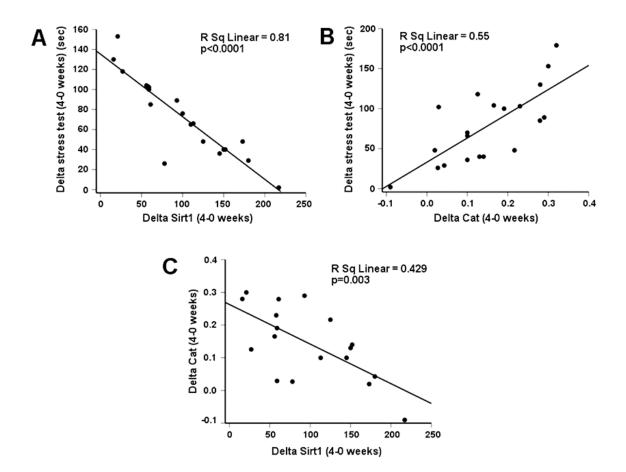
4.5 Association among delta Sirt1, delta Cat and delta stress test duration

As showed in **table 4.3**, patients underwent CR demonstrated an improvement in hemodynamic and respiratory parameters, measured during cardiopulmonary stress test. Of note, a significant association between delta Sirt1, delta Cat and delta stress test duration was found.

Based on univariate analysis, predictors of delta stress test duration were delta Sirt1 ([Sirt1 activity at 4-week] minus [Sirt1 activity at time 0]) (P<0.0001) **(Figure 4.4, panel A)** and delta catalase ([catalase activity at 4-week] minus [catalase activity at time 0]) (P<0.0001) **(Figure 4.4, panel B)**. Upon multivariate analysis, delta Sirt1 remained a predictor of delta stress test duration (P<0.0001) **(Table 4.4)**.

A strict inverse correlation was also observed between delta Sirt1 and delta Cat (P=0.003) **(Figure 4.4, Panel C)**.

Figure 4.4. Linear correlation among delta Sirt1, delta Cat and delta stress test duration.

(A) A linear correlation was observed between delta stress test duration and delta Sirt1 levels ($r^2=0.81$; $p<0.0001$). **(B)** A linear correlation was found between delta stress test duration and delta Cat levels ($r^2=0.55$; $p<0.0001$). **(C)** A linear correlation was found between delta Sirt1 and delta Cat ($r^2=0.43$; $p<0.003$). Delta indicates the difference between values at 4 and value at 0 weeks.

Table 4.4. Linear regression analysis for delta stress test duration.

	Univariate analysis				Multivariate analysis			
	B	95% CI Lower	95% CI Upper	p value	β	95% CI Lower	95% CI Upper	p value
Age	-0.044	-3.842	2.988	0.801	---	---	---	---
BMI	-0.077	-10.398	6.659	0.658	---	---	---	---
Delta azotemia	0.059	-2.948	3.956	0.766	---	---	---	---
Delta glycaemia	0.141	-1.581	3.308	0.474	---	---	---	---
Delta creatininemia	0.297	-56.012	344.540	0.150	---	---	---	---
Delta bilirubinemia	0.278	-51.538	220.333	0.210	---	---	---	---
Delta AST	0.225	-3.361	7.567	0.421	---	---	---	---
Delta ALT	-0.130	-2.196	1.410	0.645	---	---	---	---
Delta CK	0.166	-0.882	1.484	0.587	---	---	---	---
Delta LDH	0.148	-0.747	1.215	0.612	---	---	---	---
Delta LDL	0.157	-1.588	2.697	0.577	---	---	---	---
Delta HDL	-0.304	-7.456	2.126	0.253	---	---	---	---
Delta total cholesterol	0.158	-0.785	1.859	0.412	---	---	---	---
Delta triglycerides	0.055	-0.546	0.725	0.776	---	---	---	---
Delta Na^+	0.180	-11.340	19.291	0.576	---	---	---	---
Delta Kaliemia	0.225	-40.962	80.864	0.482	---	---	---	---
Delta Cl^-	0.463	-2.599	17.536	0.129	---	---	---	---
PTCA	0.087	-742	67.331	0.619	---	---	---	---
CABG	-0.150	-88.826	35.612	0.391	---	---	---	---
Smoking	0.049	-47.871	63.431	0.778	---	---	---	---
Dyslipidaemia	0.126	-32.636	69.303	0.469	---	---	---	---
Diabetes	-0.173	84.805	28.651	0.321	---	---	---	---
Previous AMI	0.071	-31.848	47.877	0.685	---	---	---	---
Familiarity	-0.172	-81.987	27.787	0.322	---	---	---	---
Obesity	-0.235	-147.037	27.766	0.174	---	---	---	---
Peripheral Arteriopathy	43	121.451	95.238	0.807	---	---	---	---
Arrhythmias	0.219	-38.849	172.788	0.207	---	---	---	---
Dysthyroidisms	-0.053	-173.380	128.380	0.763	---	---	---	---
Blood Hypertension	-0.115	-66.576	33.813	0.511	---	---	---	---
COPD	0.181	-33.943	107.543	0.298	---	---	---	---
β-blockers	-0.113	-117.939	60.751	0.519	---	---	---	---
Ca^{2+}-antagonists	0.134	-55.041	123.166	0.442	---	---	---	---
ACE-Inhibitors	0.127	-32.272	68.639	0.469	---	---	---	---
ARBs	-0.243	-87.125	14.895	0.159	---	---	---	---
Diuretics	0.116	-47.605	93.025	0.515	---	---	---	---
Other cardiac drugs	0.063	-87.779	124.904	0.725	---	---	---	---
ASA	-0.027	-162.507	139.565	0.878	---	---	---	---
Anticoagulants	0.063	-42.112	60.445	0.718	---	---	---	---
Statins	0.260	-34.855	256.913	0.131	---	---	---	---

Gastroprotectors	-0.038	-99.488	80.217	0.829	---	---	---	---
Hypoglycemic drugs	-0.086	-134.405	81.678	0.623	---	---	---	---
Insulin	-0.102	-139.018	76.745	0.561	---	---	---	---
α-antagonists	0.253	-27.330	182.482	0.142	---	---	---	---
Polyunsatured fats	0.047	-49.858	65.200	0.788	---	---	---	---
Antiarrhytmics	0.167	-55.865	157.986	0.338	---	---	---	---
N° drugs	0.104	-13.346	24.232	0.559	---	---	---	---
Delta SBP	0.269	-0.279	2.344	0.119	---	---	---	---
Delta DBP	0.059	-2.580	3.623	0.734	---	---	---	---
Delta EF	0.079	-7.508	10.390	0.739	---	---	---	---
Delta LVEDD	-0.234	-43.231	16.231	0.350	---	---	---	---
Delta TBARS	-0.327	-173.543	84.155	0.429	---	---	---	---
Delta NO	0.343	-210.378	551.487	0.332	---	---	---	---
Delta SOD	0.511	-27.405	175.604	0.131	---	---	---	---
Delta 8-OH-dG	-0.519	-90.926	45.431	0.370	---	---	---	---
Delta Sirt1	-0.900	-0.776	-0.476	**0.000**	-0.775	-0.747	-0.330	**0.000**
Delta Cat	0.741	166.830	437.827	**0.000**	0.191	-40.710	184.156	0.194

CI, Confidence Interval; BMI, Body Mass Index; AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase; CK, Creatine Kinase; LDH, Lactate Dehydrogenase; LDL, Low Density Lipoprotein; HDL, High Density Lipoprotein; PTCA, Percutaneous Transluminal Coronary Angioplasty; CABG, Coronary Artery Bypass Graft; AMI, Acute Myocardial Infarction; COPD, Chronic obstructive pulmonary disease; ARBs, Angiotensin II Receptor Blockers; ASA, Acetyl Salicylic Acid; EF, Ejection Fraction; LVEDD, Left Ventricle and Diastolic Diameter; TBARS, Thiobarbituric Acid Reactive Substances; NO, Nitric Oxide; SOD, Superoxide Dismutase; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; Cat, Catalase; Sirt1, Sirtuin1. p value is referred to univariate and multivariate analysis against delta stress test duration. Variables are continuous natural log transformed.

CHAPTER 5

5.1 Discussion

Most of the studies on exercise training (ET) in both young and older adults were planned after considering functional and hemodynamic outcomes, without clarifying the effects from a molecular point of view and without delineating a correlation between molecular and functional induced changes.

The present study demonstrated that an exercise-based CR of 4 weeks caused an increase of oxidant species in sera of patients with stable post-ischemic HF undergoing CR with concomitant stimulation of Sirt1 activity and antioxidant capability. Of note, it was suggested that CR stimulated an oxidative stress response in a hormetic way.

Hormesis was originally defined in the field of toxicology as a phenomenon in which small amounts of a harmful substance (e.g., ionizing radiation, heavy metals, and toxins) have positive stimulatory effects on living organisms (Luckey, 1982). Otherwise, in the biomedical field, hormesis refers to an adaptive response of cells and organisms to moderate or intermittent stress (Calabrese et al., 2007).

Generally, it is characterized by a dose-response relationship in which low-dose stimulation induces various positive physiological responses, but high-dose stimulation has harmful consequences. Therefore, according to the principle of hormesis, mild to moderate oxidative stress (i.e., increased ROS generation) induced by exercise could be a necessary stimulus for exercise-related responses (Koyama, 2014).

Although the antioxidant defence system may be temporarily inhibited by ROS production through acute exercise of low to moderate intensity, it may be adversely enhanced during the recovery phase as a result of the initial oxidant stimulation. Furthermore, repeated exposure to increased ROS through chronic physical exercise induces adaptive responses that up-regulate the antioxidant defence system (Fisher-Wellman and Bloomer, 2009; Koyama, 2014; Powers et al., 2011). Thus, in order to obtain health-promoting adaptation (e.g., enhanced antioxidant defence capacity), the physiological stimulus (in this case, exercise-induced oxidative stress) is essential; conversely, high levels of ROS can lead to impaired cellular function, macromolecule damage, apoptosis, and necrosis (Koyama,

2014). Intriguingly, the effects of oral antioxidant supplementation (e.g., vitamin C or E) on physical performance, health promotion, and disease prevention in humans is still under debate. Most RCTs including different types of patients have concluded that antioxidant supplementation has a strong deleterious influence on morbidity and mortality associated with a variety of diseases (Bjelakovic et al., 2014; Lonn et al., 2005; Vivekananthan et al., 2003). Moreover, there is no conclusive evidence to support the use of antioxidant supplements as primary and secondary prevention, mostly in healthy people. Interestingly, Ristow et al. showed that ROS might mediate some health promoting effects of physical exercise in healthy subjects and that supplementation with antioxidants may abrogate these benefits (Ristow et al., 2009). Basically, exercise-induced oxidative stress itself is not necessarily harmful; instead, it is a fundamental ubiquitous biological response to exercise-induced changes in redox homeostasis and functions as a valuable mechanism driving hormetic adaptive responses.

Moreover, it is well known that the beneficial effects of ET are also associated with an increase in NO bioavailability; indeed, the results here presented show higher NO levels in the sera of Rehabilitated Patients (RP) when compared with those isolated from Patients before CR (P).

Yang et al. showed that training induces a significant enlargement of collateral vessels in striated ischemic muscle and that these effects were lost upon inhibition of NO production (Yang et al., 2008). Eksakulkla and colleagues suggested that physical activity could improve endothelial dysfunction in aged rats by increasing NO bioavailability (Eksakulkla et al., 2009).

NO plays a crucial role in the cardiovascular system, and the reduction of its bioavailability leads to diminished endothelium-dependent vasodilation, as well as impairment in vascular vessel homeostasis, which affects the onset and progression of cardiovascular pathologies (Toblli et al., 2012).

Sirt1 influences the NO pathway, and its decreased activity with advancing age may alter eNOS regulation. Likewise, Sirt1 promotes endothelial-dependent vasodilatation by targeting eNOS, leading to the enhancement of NO production (Mattagajasingh et al., 2007). A recent survey about the mechanism by which eNOS modulates Sirt1 suggested that Sirt1-activating compounds may be useful in treating coronary heart disease (Shinmura et al., 2008). In addition, through an

in vivo-in vitro technique used in the present study, it was demonstrated that CR protected against endothelial dysfunction by enhancing antioxidant efficacy and inhibited senescence in endothelial cells via Sirt1 and Cat activation.

Cellular senescence is a hallmark of aging and a process in which competent cells are brought into a permanent form of growth arrest. If and how senescence is correlated with age-associated frailty and diseases is still one of the major unanswered questions in aging physiology and clinical geriatrics (Campisi, 2011; López-Otín et al., 2013). An increase in senescence can be dangerous to endothelial cells, resulting in impairment of endothelial structure and function. Some authors showed that cellular senescence is involved in endothelial dysfunction and atherogenesis, and this was confirmed by a histological study on atherosclerotic human plaques that demonstrated morphological features of senescence (Minamino and Komuro, 2007). As oxidative stress-induced endothelial dysfunction is strictly connected to HF, developing methods able to modify this condition is certainly of clinical interest.

Recently, it has been demonstrated that ROS up-regulation represents an early event contributing to the onset and progression of HF phenotype, while improved antioxidant effectiveness protected heart against these abnormalities (Xu et al., 2011). Because Sirt1 and its target Cat seem to be strictly involved in mediating CR effects in both patients' sera and in conditioned ECs, their activities were specifically inhibited and it was found that the activated forms of these enzymes play important roles in the modulation of endothelial cell senescence. The anti-senescent effects of Sirt1 and Cat were confirmed using a rat model of post-ischemic HF. As in humans, a decrease in senescence also in ECs conditioned with sera from trained rats compared to ECs growth in sedentary rats sera was observed. This effect was present both in the absence and the presence of H_2O_2, and the protective role of exercise was eliminated via Sirt1 or Cat activity inhibition.

The role played by Sirt1 in the regulation of aging, endothelial homeostasis and cellular senescence is well recognized. Moreover, our data are in accordance with recent studies focused on the involvement of catalase in aging and senescence control (Schriner et al., 2005).

Previous studies have demonstrated that Cat is a key regulator of endothelial senescence's mediation of the effects of aerobic ET (Conti et al., 2012, 2013). According to this, only an aerobic training program (like that used in the CR program) was able to stimulate an increase in Sirt1 activity in the presence of an exogenous source of oxidative stress.

Some recent studies in animal models showed that over-expression of Cat in heart and vessels has a beneficial impact on HF. In particular, Cat may prevent adverse myocardial remodelling (Qin et al., 2010) and contribute to the preservation of geometric and functional changes by alleviating stress in the endoplasmic reticulum (Ge et al., 2010). The quality of life in patients with HF mainly depends on their tolerance to exercise, which is why CR has an important role in the management of HF. In this study, it has been found that changes in Cat activity were directly related to modifications in cardiopulmonary stress test duration, whereas changes in Sirt1 were inversely associated with this parameter. The predictive effect of delta Sirt1 on stress test duration was also confirmed in the multivariate analysis, suggesting that circulating levels of Sirt1 and Cat activity could represent good markers for assessing CR program efficacy, one of the most important problems in the assessment of ET in HF patients. In particular, the inverse correlation between delta Sirt1 and delta Cat may explain the consecutive roles of these molecules in regulating the beneficial cellular effects of CR. CR is responsible for the initial Sirt1 activation via an oxidative stimulus, which is, in turn, responsible for the increased antioxidant response by Cat. We suggest that at this stage, the cellular activation of Sirt1 is no longer necessary, as the antioxidant system is adequately effective in cells. Finally, the increased stress test duration could be viewed as a functional expression of these phenomena. However, molecular mechanisms underlying exercise-based CR are showed in **Figure 5.1**.

The ability of ET to regulate vascular endothelial function, NO bioavailability, and oxidative stress response is an example of how lifestyle and/or tools such as exercise-based CR can complement both clinical and pharmacological means of managing CVDs. This finding is important because comorbidity and polypharmacy and, consequentially, drug interactions and adverse events represent the norm especially in HF old patients (Del Sindaco et al., 2010). Therefore, exercise may be a valid resource, particularly in the elderly population.
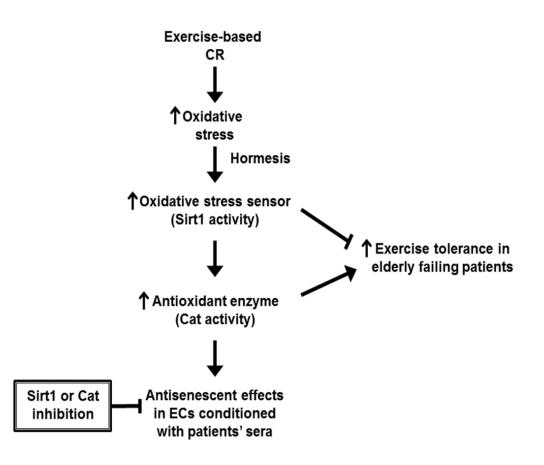
Figure 5.1. Scheme showing molecular mechanisms underlying exercise-based CR.

Hormesis links exercise-based CR and subsequent formation of reactive oxygen species to antioxidant efficiency in patients with HF. CR stimulates the activity of a well-known molecular sensor of oxidative stress (Sirt1) and, as a consequence, leads to an induction of Cat activity, a key enzyme of ROS defence, in the sera of HF patients.

A conditioning of human endothelial cells with patients' sera suggest that CR may systemically arm human endothelial cells (ECs) against oxidative stress-dependent senescence. Notably, by inhibiting Sirt1 and Cat activity anti senescent effects of CR are abrogated.

The inverse correlation between delta Sirt1 and delta Cat with stress test duration in failing patients may explain the consecutive roles of these molecules in regulating the beneficial cellular effects of CR. CR is responsible for the initial Sirt1 activation via an oxidative stimulus, which, in turn, is responsible for the increased antioxidant response by Cat.

5.2 Conclusions

CR is a helpful medical practice in which several molecular factors mutually influence each other. The ET included in CR programmes acts as a non-pharmacological inductor of both antioxidant response and NO bioavailability.

The finding of a positive correlation between changes in Sirt1 and Cat activity and stress test duration, may indicate these molecules as markers of CR efficiency and potential parameters to control and monitor antioxidant effects, in order to develop the best program of exercise for each person.

To determine the molecular mechanisms underlying the beneficial effects of CR is an essential step in developing a strategy to facilitate the clinical practice of ET. Further studies should aim at reducing the number and the doses of drugs for HF by implementing exercise programs, especially in high-risk elderly patients.

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CHAPTER 6

INTERNATIONAL EXPERIENCE

From May to August 2015, I have been working under the supervision of Prof Munir Pirmohamed and Dr. Eunice Zhang at the Department of Molecular & Clinical Pharmacology, the Wolfson Centre for Personalised Medicine of University of Liverpool.

During this period, I have gained knowledge and skills in the fields of biostatistics, bioinformatics and pharmacogenomics. The statistical software package SPSS was used to perform statistical analyses and data management.

To research and analyze genetic polymorphisms involved in warfarin response, different bioinformatic programs such as HapMap, HaploReg, 1000 Genomes and Haploview were utilised. The HapMap is a catalog of common human genetic variants. It describes what the variants are, where they occur in the DNA, and how they are distributed among people within different ethnic populations in the world.

HaploReg is a tool for exploring annotations of the noncoding genome at variants on haplotype blocks, such as candidate regulatory SNPs at disease-associated loci. Using linkage disequilibrium (LD) information from the 1000 Genomes Project, linked SNPs and small indels can be visualized along with their predicted chromatin state, their sequence conservation across mammals, and their effect on regulatory motifs.

The 1000 Genomes Project is a community resource project that aims to discover genotype and provide accurate haplotype information on all forms of human DNA polymorphism in multiple human populations.

Haploview was used to analyse the presence of linkage disequilibrium between different SNPs in CYP2C9. Haploview is a bioinformatics software designed to visualize patterns of LD in genetic data. It generates marker quality statistics, haplotype blocks, population haplotype frequencies and single marker association statistics. Haploview can also perform association studies, choosing tagSNPs and estimating haplotype frequencies.

Primer design was undertaken using Primer3 Plus, and Custom TaqMan®Assay Design Tool.

In terms of my lab experience, I have worked on two different projects, the first on the pharmacogenetics of warfarin and the second about Acute Coronary Syndrome (ACS).

For the warfarin project, I have extracted and quantified DNA from 109 dried blood spots and genotyped the DNA for CYP2C9*2 (rs1799853), CYP2C9*3 (rs1057910) and VKORCI -1639 (rs9923231) SNPs.

In terms of the ACS project, I have measured the level of oxLDL in the serum of patients with history of acute coronary syndrome using indirect ELISA assay. All the techniques employed are described in detail below.

WARFARIN PROJECT

Introduction

Warfarin is one of the most widely used oral anticoagulants for the prevention and treatment of thromboembolic events in patients with mechanical heart valves, atrial fibrillation, deep venous thrombosis or pulmonary embolism (Kim et al., 2009). Its management is difficult due to the narrow therapeutic window and high inter- and intra-individual variability in dose requirements, where over-anticoagulation can lead to haemorrhagic complications while under-anticoagulation result in thrombosis. Several clinical factors have been found to be associated with warfarin requirements, including age, gender, compliance, nutritional status, presence of comorbid conditions and concomitant medications (Kimmel, 2008).

Variability in warfarin response also depends on polymorphisms at the level of drug-metabolising enzymes, drug targets/receptors and drug transport proteins involved in the drug pathway (Gage et al., 2004).

At present, VKORC1, CYP2C9 and CYP4F2 are the only genes showing a well-established relation with warfarin dose requirements, although many other candidates have been postulated (Wadelius et al., 2007). Vitamin K epoxide reductase complex subunit 1 (VKORC1) is the enzyme that controls the regeneration of reduced vitamin K in the vitamin K cycle and it is an essential cofactor in the formation of clotting factors. Warfarin works by non-competitively inhibiting VKORC1, thus blocking the clotting cascade. Warfarin is prescribed as a racemic mixture of R and S-enantiomers. The warfarin S-enantiomer, has much greater anticoagulant potency than the R-enantiomer and is metabolized mainly by CYP2C9 into inactive metabolites. Polymorphic variants in CYP2C9, in particular CYP2C9*2 (430 C>T) and CYP2C9*3 (1075A>C), lead to the synthesis of CYP2C9 isoforms with reduced enzymatic activity. Individuals carrying such variants therefore have greater sensitivity to warfarin and are prone to bleeding events if their warfarin dosage is not reduced.

Although clinical factors contribute to warfarin response, they account for only about 15 to 20% of the inter-patient variability in dose requirements and are usually insufficient in themselves to accurately predict the therapeutic dose (Gage et al., 2008; Roper et al., 2010).

Instead, at least 30-45% of warfarin dose variability is attributable to genetic polymorphisms in CYP2C9 and VKORC1 (Aithal et al., 1999; Rieder et al., 2005; Kim et al., 2009; Becquemont, 2008) and when they are combined with clinical factors, the percentage rises up to 55%-60%.

Warfarin maintenance dose so as to minimise the adverse effects associated with under- and over-dosing, numerous studies have developed pharmacogenetic algorithms utilising both clinical and genetic factors to predict warfarin dose requirements. (Gage et al., 2008; IWPC, 2009; Wadelius et al., 2009; Lenzini et al., 2010; Zambon et al., 2011; Horne et al., 2012; Wei et al., 2012)

Aims

As part of a large prospective study (n=1000), genotypes for CYP2C9*2, CYP2C9*3 and VKORC1 have previously been determined for all patients except for 109 patients. In order to evaluate the effects of CYP2C9 and VKORC1 genetic variants on warfarin dosing in the full cohort of 1000 patients, the aim of this project was to determine the genotypes of CYP2C9*2, CYP2C9*3 and VKORC1 in the remaining 109 patients.

Methods

Patient cohort

Patients were recruited prospectively as they were initiated onto warfarin at two hospitals in Liverpool, the Royal Liverpool and Broadgreen University Hospitals Trust and University Hospital Aintree. The main indications for warfarin therapy in patients were treatment of venous thromboembolism and prophylaxis against systemic emboli in patients with atrial fibrillation. All patients recruited were ≥ 18 years of age.

Blood samples (400 µL total, 80 µL per spot) were spotted onto Whatman 903 Protein saver cards (GE Healthcare Whatman, UK) and were dried for at least 4 hours at room temperature in a horizontal position. They were then kept in separate clean zipper bags at room temperature for long term storage.

DNA Extraction

DNA was extracted using the E.Z.N.A$^®$ Blood DNA Mini Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's recommendations with minor modifications as follows.

For each patient, one dried blood spots was cut into small pieces and transferred into a 1.5 mL nuclease-free microcentrifuge tube. To prevent cross contamination, careful cleaning of the pairs of scissors was carried out before handling samples from different patients. The small pieces of dried blood spots were soaked in 250 μL of phosphate-buffered saline (PBS) for 1 hour at 65°C, with a brief vortex every 20 min.

To digest contaminating proteins, 25 μL of OB Protease Solution was added and incubated at 65°C for 30 min, with brief vortexing every 10 min.

After centrifugation at 17,000 x g for 5 min, the supernatant was transferred to a nuclease-free microcentrifuge tube and 1 volume of BL Buffer and 1 volume of 100% ethanol were added.

The sample was subsequently pulse-vortexed and the mix was transferred to the HiBind$^®$ DNA Mini Column and centrifuged at 10,000 x g for 1 min.

Subsequently, 500μL of HBC Buffer was added to the column and centrifuged at 10,000 x g for 1 min.

The column was washed with 700 μL of DNA Wash Buffer twice, followed by centrifugation for 2 min at maximum speed (17,000 x g) to dry the column matrix.

DNA was eluted with 100 μL of Elution Buffer preheated to 65°C. After adding the Elution Buffer, the column was incubated for 5 min at room temperature and centrifuged at 17,000 x g for 1 min. Incubating the HiBind$^®$ DNA Mini Column at 65°C rather than room temperature will give a modest increase in DNA yield per elution.

To obtain higher DNA concentrations, the first eluate was used to perform a second elution.

A schematic workflow of the extraction process is outlined in **Figure 1**.
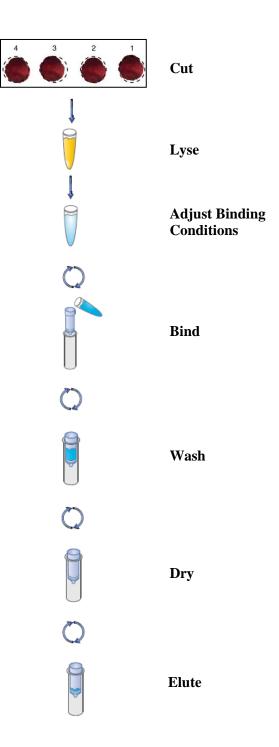
Figure 1. Schematic workflow of DNA extraction from dried blood spots using the E.Z.N.A.® Blood DNA Mini Kit (Omega Bio-Tek). The process of DNA extraction follows the usual workflow of column-based DNA clean-ups. Binding of DNA in high salt conditions to silica membranes, washing with Ethanol based wash buffers and elution in low salt conditions.

DNA quantification

The genomic DNA extracted was quantified using the NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 1.5 µL of the extracted DNA was used for measuring the DNA concentration and the absorbance ratio at 260/280 nm for evaluation of the purity of each sample. To quantify the amount of double-stranded DNA in each sample, the Quant-iT™ PicoGreen® dsDNA Assay kit and/or Qubit® dsDNA HS (High Sensitivity) Assay kit were used as described below.

1. DNA quantitation using Picogreen® dsDNA Reagent

The Quant-iT™ PicoGreen® dsDNA Assay (Invitrogen – Molecular Probes, Eugene, OR, USA), an ultrasensitive fluorescent nucleic acid stain, was used to quantify the amount of double-stranded DNA extracted from our dried blood spot samples.

The most commonly used technique for measuring nucleic acid concentration is the determination of absorbance at 260 nm (A_{260}). The major disadvantages of the absorbance method are the large relative contribution of nucleotides and single-stranded nucleic acids to the signal, the interference caused by contaminants commonly found in nucleic acid preparations, the inability to distinguish between DNA and RNA, and the relative insensitivity of the assay (an A_{260} of 0.1 corresponds to a 5 µg/mL dsDNA solution). Quant-iT™ PicoGreen® dsDNA reagent enables to quantitate as little as 25 pg/mL of dsDNA with a standard spectrofluorometer and fluorescein excitation and emission wavelengths.

The assay was performed using black coloured flat bottom 96-well microliter plates. First, a five-point standard curve ranging from 0 to 2,000 ng/mL in a volume of 100 µL was created by serially diluting the lambda DNA standard in 1X TE buffer. Lambda DNA was supplied by the manufacturer at a stock concentration of 100 ng/µL. 1 µL of DNA extracted from the dried blood spot was diluted in 99 µL of 1X TE buffer. A working solution of PicoGreen® dsDNA reagent was then prepared by making a 200-fold dilution of the concentrated stock in 1X TE. 100 µL of this reagent was then added to each DNA dilution to reach a final volume of 200 µL. The mix was incubated at room temperature for 5 min, protected from light to avoid photobleaching. After incubation, fluorescence intensity was measured. The

samples were excited at a wavelength of 485 nm and the fluorescence emission intensity was measured at a wavelength of 535 nm using a fluorescence microplate reader (Beckman Coulter DTX 880 Multimode Detector, Brea, CA, United States. The DNA concentration of blood spot samples was determined from the standard curve described above.

2. DNA quantitation using Qubit® dsDNA HS (High Sensitivity) Assay

The Qubit® dsDNA Assay uses fluorescent dyes to determine the concentration of nucleic acids and proteins in a sample. Each dye is specific for one type of molecule (DNA, RNA or protein) and these dyes have extremely low fluorescence until they bind to their targets. Upon binding, they become intensely fluorescent. The difference in fluorescence between bound and unbound dye is several orders of magnitude.

For example, the Qubit DNA dye used for the High Sensitivity Assay is highly selective for double-stranded DNA (dsDNA) and it has extremely low fluorescence until it binds to DNA. Upon binding to DNA, it assumes a more rigid shape and becomes intensely fluorescent. The amount of fluorescence signal from this mixture is directly proportional to the concentration of DNA in the solution. The Qubit fluorometer can pick up this fluorescence signal and convert it into a DNA concentration measurement using DNA standards of known concentration. The advantage of using specific dyes is that this measurement provides a much more accurate quantification number than UV absorbance methods, which are not highly selective for the molecule in question.

DNA extracted from dried blood spot samples were quantified using the Qubit® dsDNA HS (High Sensitivity) Kit (Life Technologies, Carlsbad, CA, USA) and the Qubit 3.0 Fluorometer (Life Technologies) following the manufacturer's instruction. Briefly, 1 µL of DNA sample was diluted 200-fold in Qubit® Working solution in clear plastic Qubit® assay tubes and measured on the fluorometer after 2 min of incubation at room temperature. Prior to taking the measurements, a two-point calibration curve was established using the supplied standards with the kit, at 0 ng/µL and 10 ng/µL. An assay workflow is shown in **Figure 2**.
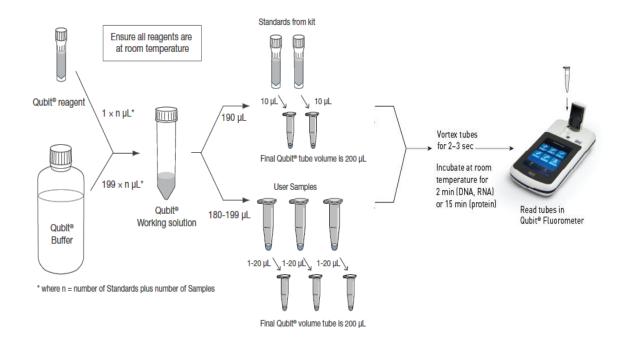
Figure 2. The Qubit$^{\circledR}$ quantitation assay workflow. Simply dilute the reagent using the buffer provided, add sample and read the concentration on the Qubit 3.0 Fluorometer. The assays are performed at room temperature, and the signal is stable for 3 hours.

CYP2C9*2, CYP2C9*3 and VKORC1 genotyping

Genotyping of CYP2C9*2 (rs1799853), CYP2C9*3 (rs1057910) and VKORCI - 1639 (rs9923231) were performed with TaqMan® SNP Genotyping assays (Applied Biosystems, Warrington, Ches., UK) by means of a procedure based on the the 5'-3' exonuclease activity of Taq DNA polymerase, using allele-specific TaqMan® minor groove binding (MGB) probes labelled with VIC® and FAM™, according to the manufacturer's instructions. The presence of two TaqMan® MGB probes in each reaction, both targeting the same single-nucleic polymorphism (SNP) site, one of them perfectly complementary to the wild type allele and the other to the mutated allele, allows genotyping of the two allelic variants of a SNP site in a target sequence (**Figure 3**).

During the extension cycle of the PCR, the 5' nuclease activity of Taq polymerase liberates the reporter dye, causing an increase in its fluorescence intensity (**Figure 4**). Genotyping with fluorogenic probes requires that fluorescence measurements be made after PCR is completed and detection system software automatically processes the fluorescence data to make genotype calls (**Figure 5**).

For this assay, approximately 20 ng genomic DNA was amplified in 5 µL reaction mixture in a 384-well plate containing 1X universal TaqMan® genotyping master mix and 1X assay mix containing a premix of the respective primers and fluorescent-labelled MGB probes. After initial denaturation and enzyme activation at 95°C for 10 min, the reaction mixture was subjected to 40 cycles of denaturation at 95°C for 15s and combined annealing and extension at 60°C for 1 min. The reactions were performed on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). As part of quality control, negative controls containing 1X TE instead of DNA and 10% duplicates were included in every run. End-point fluorescence and allelic discrimination were determined using the SDS version 2.3 software (Applied Biosystems). An example of the allelic discrimination plot is shown in **Figure 6**.
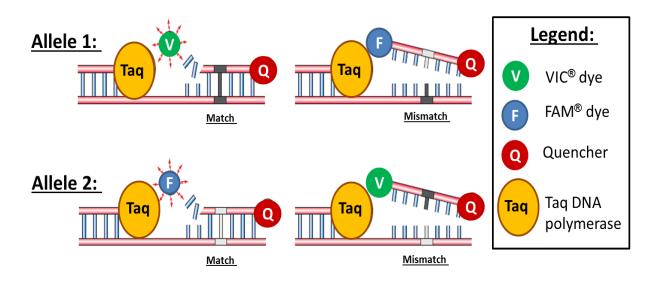
Figure 3. Design strategy for allelic discrimination assay with fluorogenic probes in the 5' nuclease assay. The presence of mismatch between probe and target destabilizes probe binding during strand displacement, reducing the efficiency of probe cleavage. (Livak et al., 1995)
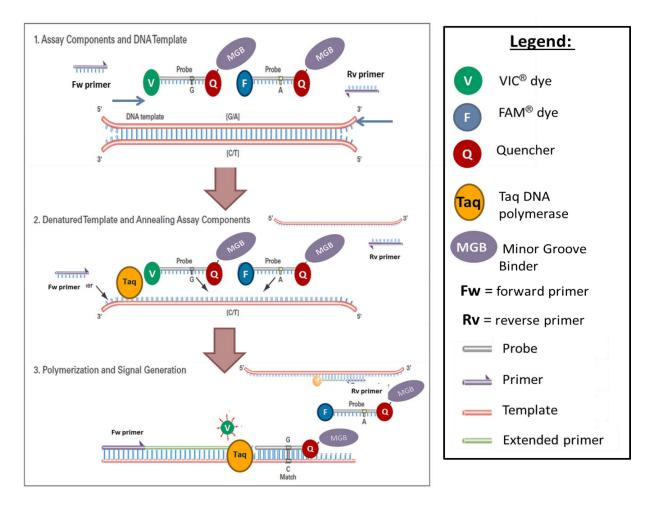
**Figure 4. PCR amplification and detection with fluorogenic probes in the 5'
nuclease assay.** Two dyes, a fluorescent reporter (R) and a quencher (Q), are
attached to the fluorogenic probe. When both dyes are attached to the probe,
reporter dye emission is quenched. During each extension cycle, the Taq DNA
polymerase cleaves the reporter dye from the probe. Once separated from the
quencher, the reporter dye emits its characteristic fluorescence.
(https://tools.thermofisher.com/content/sfs/brochures/cms_085696.pdf)

Fluorescence signals:	Results:
• VIC® dye fluorescence only • FAM® dye fluorecence only • Both fluorescence signals	• Homozygosity for allele 1 • Homozygosity for allele 2 • Heterozygosity for alleles 1 and 2

**Figure 5. Correlation between fluorescence signals and sequences in the
sample.** Each homozygous genotype will be detected by a specific probe and
show an increase in fluorescence for that particular reporter. A heterozygous
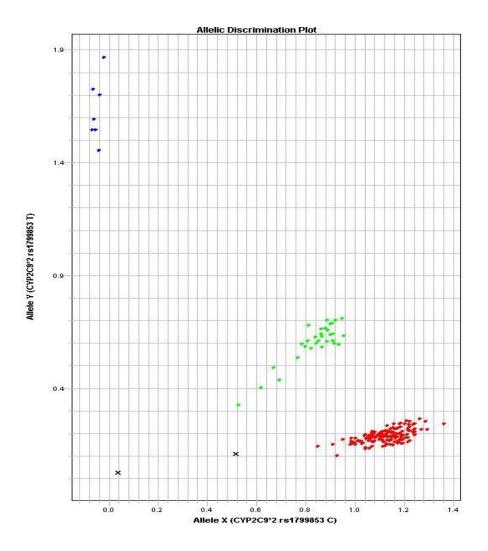genotype will show increased fluorescence with both reporters.

Figure 6. Example of Allelic Discrimination Plot of CYP2C9*2. Patients with homozygous T genotype (TT) are distributed along the vertical axis, heterozygous (CT) genotypes are distributed diagonally and patients homozygous for the C allele (CC) distributed on the horizontal axis. This variation is due to differences in the extent of reporter dye fluorescent intensity after PCR amplification. The negative control (NTC) is represented by X.

ACUTE CORONARY SYNDROME PROJECT

Acute coronary syndrome (ACS) is a major cause of mortality and morbidity among patients suffering from heart diseases. Many evidences suggest that inflammation-induced atherosclerotic plaque plays a decisive role in the pathophysiology of ACS (Hansson, 2005). For example, most cases of coronary thrombosis result from fractures in the protective fibrous cap of the atherosclerotic plaque, which lead to myocardial thrombosis or infarction (Libby, 2001). Oxidized low-density lipoprotein (oxLDL) is a factor that plays an important role in generating the inflammatory processes in atherosclerotic lesions and is a prerequisite of rapid LDL accumulation in macrophages and formation of foam cells (fat containing) (Inoue et al., 2001; Meisinger et al., 2005). Circulating oxLDL was found to be associated with all stages of atherosclerosis, from early atherogenesis to hypertension, coronary and peripheral arterial disease, acute coronary syndromes and ischemic cerebral infarction (Trpkovic et al., 2015).

Unlike native LDL, oxLDL binds to β_2GPI to form oxLDL-β_2GPI complexes. The interaction between oxLDL and β_2GPI is initially mediated by electrostatic forces. This initial interaction is followed by the formation of stable (non-dissociable) complexes mediated by covalent bonds. Stable complexes are regarded as pathogenic and more clinically relevant (Hasunuma et al., 1997; Kobayashi et al., 2001; Liu et al., 2002; Kobayashi et al., 2003).

Indeed, oxLDL-β_2GPI complexes act as a pro-inflammatory chemotactic factor for macrophages and T lymphocytes leading to the formation of foam cells and have been implicated as pro-atherogenic antigens. They may represent a serologic risk factor and/or a significant contributor for the development of thrombosis and atherosclerosis.

Aim

The aim of this part of the project was to evaluate the serum concentrations of oxLDL in patients with history of ACS.

Methods

Patient cohort

Patients were recruited prospectively at three hospitals in the UK: Blackpool Victoria Hospital, Liverpool Heart & Chest Hospital and Liverpool Royal Hospital. Subjects ≥ 18 years of age who have had acute coronary syndrome (as either a positive Troponin or ECG changes) as their main diagnosis in their index hospital admission were included in the study. All participants were followed up over a period of one year.

ELISA assay

Serum concentrations of ox-LDL were measured using AtherOx® Test Kit (oxLDL-β_2GPI Antigen Complex). Firstly, a six-point calibration curve ranging from 0 to 4.3 U/mL was created by serially diluting the hsAtherOx Serum Calibrator (human) with the Sample Diluent provided in the kit.

Diluted serum samples (either 1:100 or 1:1000), calibrators, and controls (1:100) were incubated for 1h at room temperature in microwells coated with purified anti-human monoclonal antibody (WB-CAL-1) directed only to complexed β_2GPI. Incubation allowed the ox-LDL-β2GPI antigen complex present in the samples to react with the immobilized antibody. After removal of unbound serum proteins by washing, 100 µL of anti-human apoB100 (LDL) monoclonal antibodies, conjugated to biotin, was added to form complexes with the bound antigen at room temperature for 30 min. Washing was then carried out to remove unbound LDL monocloncal antibodies, 100 µL Horseradish Peroxidase conjugated Streptavidin (HRP-SA) was added to form complexes with the bound biotin conjugated antibody at room temperature for 30 min. This is followed by another washing step to remove unbound HRP-SA. The bound HRP-SA conjugate was assayed by the addition of 100 µL of tetramethylbenzidine (TMB) / hydrogen peroxide (H_2O_2), a chromogenic substrate, to produce a coloured reaction that was measured at 450 nm. The intensity of colour development in the wells is proportional to the serum concentration of oxLDL-β_2GPI antigen complex. Results were calculated against a calibration curve prepared from the calibrator provided in the kit.

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