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# ACUTE AND PROLONGED EFFECT OF NEW TREATMENTS (LEVOSIMENDAN AND SACUBITRIL/VALSARTAN) IN HEART FAILURE: AN HOLISTIC EVALUATION (BIO/14)

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## ABSTRACT

Alveolar-capillary membrane evaluated by carbon monoxide diffusion (DLCO) plays an important role in heart failure (HF). Surfactant Proteins (SPs) have also been suggested as a worthwhile marker. In acute HF, Levosimendan improves pulmonary hemodynamics and reduces lung fluids but associated SPs and DLCO changes are unknown. Sixty-five acute HF patients underwent spirometry, cardiopulmonary exercise test (CPET) and SPs determination before and after Levosimendan. Levosimendan caused natriuretic peptide-B (BNP) reduction, peakVO2 increase and VE/VCO2 slope reduction. Spirometry improved but DLCO did not. SP-A, SP-D and immature SP-B reduced ($73.7 \pm 25.3$ vs. $66.3 \pm 22.7$ ng/mL*, $247 \pm 121$ vs. $223 \pm 110$ ng/mL*, $39.4 \pm 18.7$ vs. $34.4 \pm 17.9$AU*, respectively); while mature SP-B increased ($424 \pm 218$ vs. $461 \pm 243$ ng/mL, * = $p < 0.001$). Spirometry, BNP and CPET changes suggest hemodynamic improvement and lung fluid reduction. SP-A, SP-D and immature SP-B reduction indicates a reduction of inflammatory stress; conversely mature SP-B increase suggests alveolar cell function restoration. In conclusion, acute lung fluid reduction is associated with SPs but not DLCO changes. SPs are fast responders to alveolar-capillary membrane condition changes. On the other hand, regarding chronic heart failure, Sacubitril/Valsartan represents a novel therapy in the treatment of chronic heart failure with reduced ejection fraction (HFrEF), has recently proved efficacy in improving exercise tolerance and cardiac performance. We enrolled a cohort of HFrEF outpatients eligible for sacubitril/valsartan and performed serial cardiopulmonary exercise tests (CPET), pulmonary function tests, laboratory and echocardiographic assessments before and during the gradual titration of this treatment, in order to evaluate its effects on cardiopulmonary function and left ventricular remodeling. In this interim analysis, we examined twenty-five patients treated with sacubitril/valsartan for at three months. At a mean follow-up of 169±74 days, 92% of patients reached the maximum dose, without important safety concerns. Ejection fraction increased (31.0±5.4 vs. 37.2±9.6 %; p=0.009), while left ventricular end-diastolic and end-systolic volumes decreased (respectively, 116.8±31.4 vs. 90.5±21.3 ml, p=0.011; 80.9±24.5 vs. 58.2±21.4 ml, p=0.004). Peak oxygen consumption ($VO_2$) improved from 63.4±12.5 to 70.3±13.3 % of predicted (p=0.002), along with workload at maximal exercise (97.0±39.3 vs. 103.7±39.7 watt, p=0.001) and $VO_2$ at the anaerobic threshold (881±278 to 1056±350 ml, p=0.012). Minute ventilation/carbon dioxide production relationship ($VE/VCO_2$ slope) did

not reach statistical significance in this sub-population. New York Heart Association functional class improved (p=0.004), together with a significant decrease of MECKI (Metabolic Exercise test data combined with Cardiac and Kidney Indexes) score from 3.0 (IQR 1.7-6.3) to 1.8 (0.8-3.6) %, with a positive impact on two-year HF prognosis (p=0.009).In conclusion medium-term treatment with sacubitril/valsartan demonstrated beneficial effects on exercise tolerance, left ventricular remodelling and functional status, confirming the results from previous clinical trials in real-life. The longer follow-up and larger population of the finished study will further contribute to the assessment of its positive effects on HF patients.

## RIASSUNTO

La membrana alveolo-capillare valutata tramite la diffusione del monossido di carbonio (DLCO) gioca un ruolo importante in pazienti con scompenso cardiaco acuto. Le proteine del surfattante polmonare (SPs) stanno diventando un importante utile marker di questa patologia. Nello scompenso cardiaco acuto, Levosimendan migliora l'emodinamica polmonare e riduce i fluidi nei polmoni, tuttavia l'associazione tra i livelli di SPs e la DLCO ad oggi rimane ignota. Sessantacinque pazienti con scompenso cardiaco acuto sono stati sottoposti prima e dopo l'infusione di Levosimendan ad una valutazione tramite spirometria, a un test da sforzo cardiopolmonare (CPET) e a esami ematochimici per valutare i livelli plasmatici di SPs. Innanzitutto, Levosimendan ha determinato una riduzione del peptide natriuretico di tipo B (BNP). Alla valutazione del test cardiopolmonare si è osservato un aumento del consumo di ossigeno al picco dell'esercizio (VO2picco) ed una riduzione della VE/VCO2 slope. Dopo infusione di Levosimendan la spirometria è migliorata in modo statisticamente significativo, mentre la DLCo è rimasta invariata. Le proteine del surfattante polmonare nelle sue forme SP-A, SP-D e la forma immatura di SP-B si sono ridotte (rispettivamente $73.7 \pm 25.3$ vs. $66.3 \pm 22.7$ ng/mL*, $247 \pm 121$ vs. $223 \pm 110$ ng/mL*, $39.4 \pm 18.7$ vs. $34.4 \pm 17.9$AU*), mentre la forma matura di SP-B è aumentata ($424 \pm 218$ vs. $461 \pm 243$ ng/mL, * = $p < 0.001$). La spirometria, il BNB e il CPET hanno mostrato un miglioramento emodinamico ed una riduzione dei fluidi nei polmoni. In seguito a trattamento con Levosimendan, la riduzione di SP-A, SP-D e della forma immatura di SP-B hanno indicato una riduzione dello stress infiammatorio, mentre l'aumento dei livelli plasmatici della forma matura di SP-B suggerisce una riattivazione delle funzioni vitali della cellula alveolare.

In conclusione, in pazienti con scompenso cardiaco acuto la riduzione dei fluidi nei polmoni è correlata con i livelli plasmatici di SPs, mentre la DLCO non subisce cambiamenti. Le SPs rappresentano un marker facilmente rintracciabile perchè riflettono un cambiamento della condizione della membrana alveolo-capillare. Per quanto riguarda lo scompenso cardiaco cronico, l'utilizzo di Sacubitril/Valsartan rappresenta un nuovo trattamento per lo scompenso cardiaco cronico con ridotta frazione d'eiezione; infatti ne è, stata recentemente dimostrata l'efficacia nella performance cardiaca e sulla capacità d'esercizio. In questo studio, sono stati inclusi una coorte di pazienti con scompenso cardiaco cronico eleggibili al trattamento con Sacubitril/Valsartan. Tutti i pazienti sono stati sottoposti, prima e durante tritazione del

dosaggio del farmaco ad una valutazione tramite spirometria, CPET, esami ematochimici ed ecocardiogramma  al fine di valutare la funzione cardiopolmonare e il rimodellamento del ventricolo sinistro. In una analisi intermedia, abbiamo valutato 55 pazienti trattati con Sacubitril/Valsartan per tre mesi. Ad un follow up medio di 169±74 giorni, il 92% dei pazienti assumevano il dosaggio massimo di farmaco, senza importati problemi di sicurezza. La frazione d'eiezione è aumentata (31.0±5.4 vs. 37.2±9.6 %; p=0.009), mentre il volume ventricolare tele-diastolico e il volume ventricolare tele-sistolico sono diminuiti (rispettivamente 116.8±31.4 vs. 90.5±21.3 ml, p=0.011; 80.9±24.5 vs. 58.2±21.4 ml, p=0.004). Il VO2picco è aumentato da 63.4±12.5 a 70.3±13.3 % del predetto (p=0.002) con un carico di lavoro al picco dell'esercizio 97.0±39.3 vs. 103.7±39.7 watt, p=0.001, il consumo di ossigeno alla soglia anaerobica è migliorato da 881±278 a 1056±350 ml, p=0.012. La VE/VCO2 non ha mostrato un cambiamento significativo dopo trattamento con Sacubitril/Valsartan. La classificazione NYHA (New York Heart Association) è migliorata, mentre si è osservata una riduzione del MECKI (Metabolic Exercise test data combined with Cardiac and Kidney Indexes) score da 3.0 (IQR 1.7-6.3) a 1.8 (0.8-3.6) %, con un impatto positivo sulla prognosi a due anni dello scompenso cardiaco (p=0.009). In conclusione, in pazienti con scompenso cardiaco cronico il trattamento con Sacubitril/Valsartan ha dimostrato un effetto positivo sulla tolleranza all'esercizio, sulla funzione e sul rimodellamento del ventricolo sinistro, confermando quindi il risutlato del precedente studio clinico. Certamente un follow up più lungo aiuterà a confermare l'effetto positivo dell'utilizzo di Sacubitril/alsartan nei pazienti con scompenso cardiaco cronico.

# CHAPTER I

## INTRODUCTION

### 1.1. Definition, etiology and epidemiology of heart failure

Heart Failure is a clinical syndrome characterized by typical symptoms (e.g. breathlessness at rest or on exercise, ankle swelling and fatigue) that may be accompanied by signs (e.g. elevated jugular venous pressure, pulmonary crackles and peripheral edema) caused by a structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress [1]. This is only one of the many definitions given to this complex syndrome that is characterized by the inability of the heart to pump blood with normal efficiency, so that it is unable to provide adequate blood flow to other organs such as the brain, liver, and kidneys. Therefore, heart failure is a chronic, progressive condition in which the heart cannot keep up with its workload.

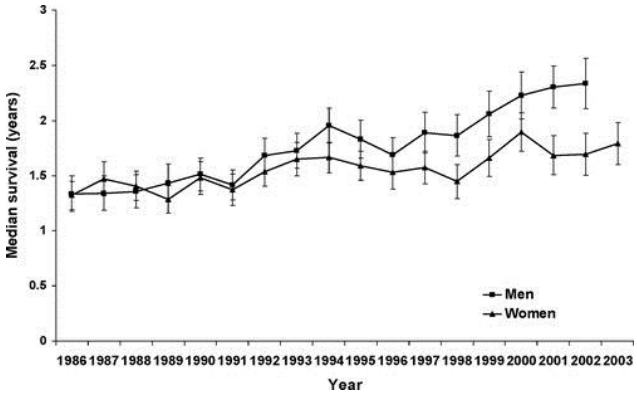
It is important to find out the underlying cardiac problems leading to heart failure, for therapeutic reasons too, however the aetiology of this syndrome can be different. The most common causes of heart dysfunction can be divided into three categories [1]:

1) Myocardial disease caused by coronary heart disease, acute or chronic ischaemia, cardiotoxic substances, cardiomyopathies, immune-mediated and inflammatory damage (myocarditis), infiltration and hormonal or nutritional derangements;
2) Abnormal loading conditions caused by hypertension, valvular disease and pericardial or endomyocardial pathologies;
3) Arrhythmias in the form of tachyarrhythmias such as atrial fibrillation and ventricular arrhythmias or bradyarrhytmias due to conduction disorders.

However, it is often difficult to discover the primary aetiology of heart failure in a patient with multiple potential causes.

HF affects approximately 1–2% of the adult population in developed countries and both incidence and prevalence increase progressively with age [2]. The number of patients with HF is increasing, not only because of the greater life expectancy, but also as a result of interventions that prolong survival after damaging cardiac insults. Moreover, over the last few

years, improvements in treatments and their implementation have increased survival and reduced the hospitalization rate in these patients (Fig. 1 and 2) [3].



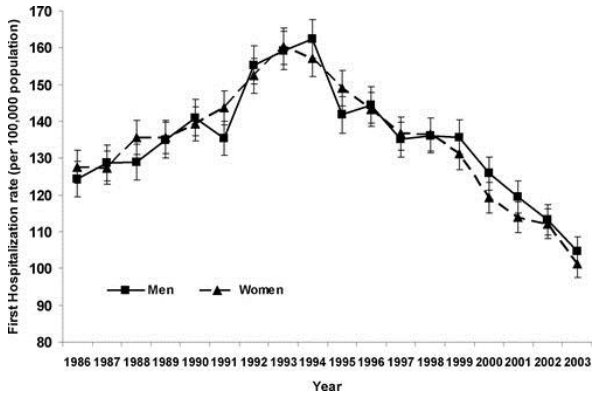




**Figure 1.** Trends in median HF survival according to sex and year of admission

**Figure 2.** Age-adjusted trends in first hospitalization for HF according to sex

Nevertheless, HF remains a major public health problem associated with a high mortality rate, frequent hospitalizations and poor quality of life. HF is a chronic condition characterized by an acute exacerbation and followed by increasing symptoms that lead to repeated hospitalizations and a significantly greater risk of premature death. Each year, a total of 1.0 million patients are hospitalized due to HF in the US [4] and in Europe approximately 5% of all acute hospital admissions are HF-related [5]. Approximately 40% of HF patients admitted to hospital will either die or be readmitted within 1 year and nearly 50% of HF patients die within 4 years of diagnosis [6]. The overall 5-year survival rate for HF is as poor as, or worse than, that for advanced cancer or stroke [4].

## 1.2. Classification of heart failure

A first classification of heart failure is based on measurement of the Left Ventricular Ejection Fraction (LVEF), which is the fraction of blood ejected from the left ventricle of the heart with each heartbeat and it is calculated dividing the stroke volume by the end-diastolic volume. LVEF is measured using echocardiography or magnetic resonance and its value is an index of the pumping efficiency of the heart. Based on LVEF, heart failure is divided into three main categories:

1) Heart Failure with reduced Ejection Fraction (HFrEF): patients with a LVEF < 40%;
2) Heart Failure with preserved Ejection Fraction (HFpEF): patients with a LVEF ≥ 50%;
3) Heart Failure with mid-range Ejection Fraction (HFmrEF): patients with a LVEF between 40% and 49%.

Obviously, the diagnosis of HFpEF is more difficult than the diagnosis of HFrEF because patients with HFpEF generally do not have a dilated left ventricle, but often has an increase in left ventricle wall thickness and increased left atrial size as a sign of increased filling pressures.The recent ESC guidelines have finally provided diagnostic criteria for a newly defined group of HF patients with a mild systolic dysfunction and a LVEF in the range of 40–49%, known as HF with mid-range LVEF (HFmrEF). A classification of patients with HF based on LVEF is important due to the different underlying etiologies, demographics, co-morbidities and response to therapies [7].

Another approach is to classify patients according to the severity of symptoms, as per the New York Heart Association (NYHA) functional classification, based on the patient's symptomatology and exercise intolerance, and the American College of Cardiology Foundation/American Heart Association (ACCF/AHA) classification, which describes stages of HF development based on structural changes and symptoms. The two classification systems are compared in Table 1.

<table>
<tr><th colspan="2">NYHA functional classification</th><th colspan="2">ACCF/AHA stages of heart failure</th></tr>
<tr><td colspan="2">None</td><td>A</td><td>At high risk of HF but without structural heart disease or symptoms</td></tr>
<tr><td>I</td><td>No limitation of physical activity. Ordinary physical activity does not cause undue breathlessness, fatigue, or palpitations.</td><td>B</td><td>Structural heart disease but without signs or symptoms of HF.</td></tr>
<tr><td>I</td><td>No limitation of physical activity. Ordinary physical activity does not cause undue breathlessness, fatigue, or palpitations.</td><td rowspan="4">C</td><td rowspan="4">Structural heart disease with prior or current symptoms of HF</td></tr>
<tr><td>II</td><td>Slight limitation of physical activity. Comfortable at rest, but ordinary physical activity results in undue breathlessness, fatigue, or palpitations.</td></tr>
<tr><td>III</td><td>Marked limitation of physical activity. Comfortable at rest, but less than ordinary physical activities results in undue breathlessness, fatigue, or palpitations.</td></tr>
<tr><td>IV</td><td>Unable to carry on a physical activity without discomfort. Symptoms at rest</td></tr>
<tr><td>IV</td><td>Unable to carry on a physical activity without discomfort. Symptoms at rest can be present.</td><td>D</td><td>Refractory HF requiring specialized interventions.</td></tr>
</table>

**Table 1.** Comparison of NYHA functional class and ACCF/AHA stages of HF

Finally, according to its clinical presentation, heart failure can be classified in:

1) Acute Heart Failure (AHF): it refers to rapid onset or worsening of symptoms and signs of heart failure. AHF can be a single episode, which may be the patient's first presentation of HF (*de novo* heart failure), or a consequence of acute decompensation of chronic HF. Acute myocardial dysfunction, acute valve insufficiency or pericardial tamponade are among the most frequent acute primary cardiac causes of AHF.

2) Chronic Heart Failure (CHF): it refers to a long-term clinical condition with a progressive reduction of the myocardial function often leading to congestive heart failure. If symptoms and signs remain generally unchanged for at least 1 month and are kept under control with therapy the patient is considered "stable". However, patients with CHF could have episodes of deterioration leading to hospital admission, known as "acute decompensation" or they could get worse and develop Advanced Chronic Heart Failure (ACHF)

## 1.3. Pathophysiological modifications in heart failure

Heart failure is a syndrome in which the heart cannot provide an adequate amount of blood (cardiac output) to fulfill the demand of the organism. Cardiac output is the product between heart rate and stroke volume that depends on three factors: preload, afterload and contractility [8].

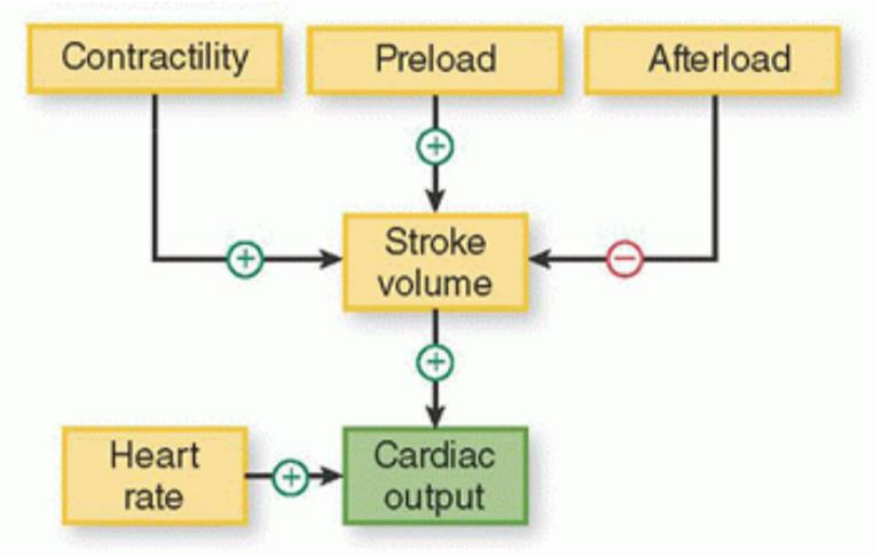



**Figure 3.** Key mediators of cardiac output = heart rate × stroke volume

- **<u>Preload:</u>** it is defined as the ventricular myocardial stretch at the end of diastole, caused by the blood filling the ventricle immediately before the contraction. Therefore, the preload corresponds to the end-diastolic volume. Within a physiologic range, the larger the ventricular volume during diastole, the more the fibers are stretched and the greater the force of the next contraction is. This is the basis of the Frank-Starling relationship, the observation that cardiac output increases in relation to the preload because stretching the muscle before stimulation optimizes the interaction of myosin and actin filaments and it also increases the sensitivity of the myofilaments to calcium, which further augments force development.

  The Frank-Starling curve, also known as the ventricular function curve, is shown in the graph that relates preload, measured as left ventricular end-diastolic volume or pressure, to cardiac performance, measured as ventricular stroke volume or cardiac output (Fig. 4).

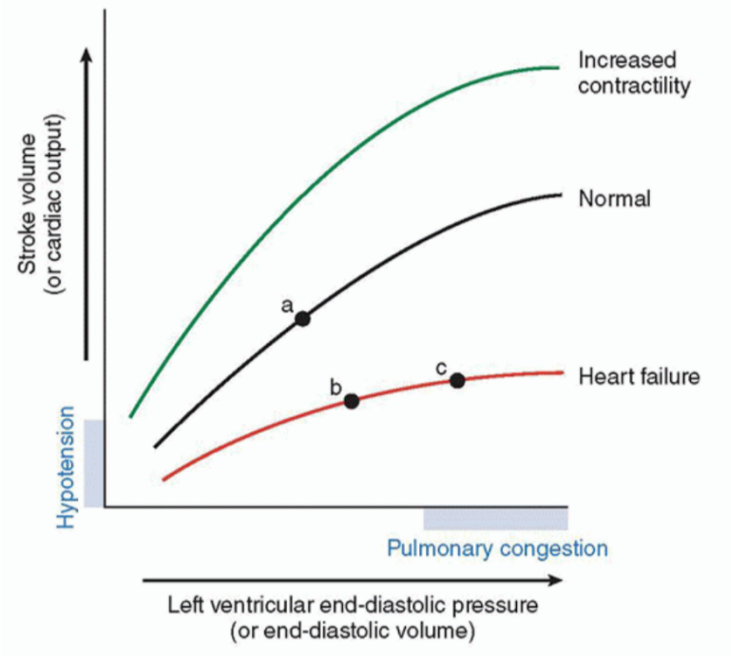



**Figure 4.** Frank-Starling curves. Point *a* is a normal subject at rest, point *b* is a HF patient with LV systolic dysfunction, point *c* is a patient with severe HF.

In Figure 4 point **a** represents a healthy person at rest, while point **b** shows a person with systolic dysfunction and heart failure. In fact, stroke volume has fallen, and the point is on the ascending curve where the EDV is slightly increased compared to point **a** as a compensatory response to dysfunction and it allows an increase in the stroke volume. Point **c** indicates further augmentation of the left ventricle filling and the point is on the flat part of the curve where stroke volume is only slightly augmented and the increased EDV leads to pulmonary congestion.

- **Afterload**: it is the ventricular wall tension during contraction, reflecting the resistance that the ventricle must overcome to empty its contents. According to the Laplace relationship, ventricular wall stress rises in response to a higher-pressure load (e.g. hypertension) or an increased chamber size (e.g. a dilated left ventricle). Conversely, hypertrophy increases wall thickness, serving as a compensatory mechanism in reducing wall stress, because in this way the force is distributed over a greater mass of ventricular muscle. Just like preload, an increase in afterload shifts the Frank-Starling curve down and to the right (Fig. 4), decreasing stroke volume and, at the same time, increasing left ventricular end-diastolic pressure. The reason is a decrease in fiber shortening velocity that reduces the rate of volume ejection and consequently increases end-systolic volume. This residual volume is added to the venous return into the ventricle and this augments end-diastolic volume. This increase in preload activates the Frank-Starling mechanism to partially compensate for the reduction in stroke volume caused by the increase in afterload.
- **Contractility:** it is the innate ability of the heart muscle to contract and it is enhanced by the activity of the sympathetic nervous system through the release of noradrenalin and its action on the β-adrenergic receptors. Other factors that influence contractility are inotropic drugs, circulating catecholamine and hypoxia. In the Frank-Starling curve, a change in contractility shifts the entire curve in an upward or downward direction. Thus, when contractility is enhanced, the ventricular performance curve is displaced upward so that at any given preload, the stroke volume is increased. Conversely, when the ventricle's contractile function is impaired (as in certain types of HF), the curve shifts downward, leading to reductions in stroke volume and cardiac output at any given preload.

The alteration of one of these elements leads to heart failure that is characterized by a reduced stroke volume and cardiac output, generally due to a dysfunction in the myocardial muscle. [9]. In order to maintain cardiac output between normal ranges, the organism carry out a series of compensatory mechanisms, but in the long run these compensatory responses cause a worsening on the heart function and contribute to the aggravation of heart failure.

## 1.4. Compensatory mechanisms

Several natural compensatory mechanisms are called into action in patients with HF to balance the fall in cardiac output and help preserve a sufficient blood pressure to perfuse the vital organs. These compensations include: the Frank-Starling mechanism (see paragraph 2.3), neurohormonal mechanisms, and myocardial hypertrophy with ventricular remodeling.

### 1.4.1. Neurohormonal activation

As we can see in the figure 5, the reduced cardiac output leads to neuroendocrine reactions to restore a normal cardiac output and perfusion. The first mechanism that takes place thanks to baroreceptors and chemoreceptors is the activation of the sympathetic nervous system, which causes an increase in heart rate (positive chronotropic effect) and cardiac contractility (positive inotropic effect). However, when heart rate exceeds 160 bpm, diastolic time is too short to permit a relevant filling, thus the stroke volume decreases. In addition to this, the sympathetic activation determines arteriolar vasoconstriction in the most expendable districts of the body, encouraging blood flow to the vital organs like heart and brain [10]. Another compensatory mechanism derives from the reduced cardiac output and renal hypoperfusion that lead to a greater sodium and water retention with the activation of the renin-angiotensin-aldosterone (RAA) system that is also responsible for arteriolar vasoconstriction. The RAA system regulates blood pressure and fluid balance, activating in case of renal hypoperfusion thanks to the juxtaglomerular cells in the kidneys that convert pro-renin into renin and secrete it into the circulation. Then renin converts angiotensinogen into angiotensin I that in turn is converted into angiotensin II by the angiotensin-converting enzyme (ACE) in the lungs. Angiotensin II is a vasoconstrictor that causes an increase in blood pressure and it stimulates the secretion of aldosterone, which is a hormone that increases the reabsorption of sodium and water into the blood, while promoting potassium excretion to maintain electrolytes balance acting on the distal convoluted tube. Because of the higher retention, the venous

return to the heart increases together with blood volume and this increases preload improving cardiac function [11, 12]. Other neuroendocrine responses are the release of vasoconstrictor substances like endothelin that increases peripheral resistances and distribution of cardiac output and vasopressin, which is also an antidiuretic peptide. Finally, there is an increase in the release of cytokines like the tumor necrosis factor leading to cachexia in the last stages of heart failure[8].

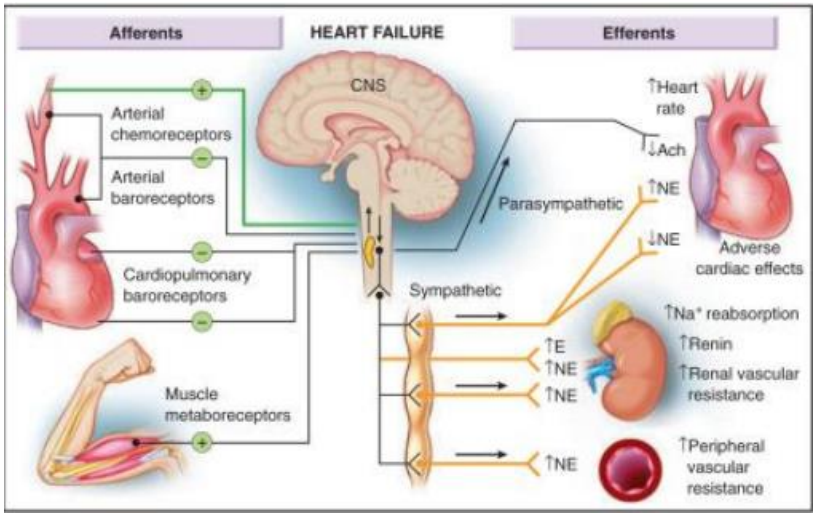



**Figure 5:** Neuroendocrine mechanisms in heart failure

## 1.4.2. Myocardial hypertrophy with ventricular remodeling

Ventricular hypertrophy and remodelling are important compensatory processes that develop over time in response to hemodynamic burdens in order to improve contractility. A sustained wall stress, along with neurohormonal and cytokine alterations, stimulates the development of myocardial hypertrophy and the deposition of extracellular matrix. This increased mass of muscle fibers serves as a compensatory mechanism to help maintain contractile force and, therefore, cardiac function. However, when hypertrophy together with fibrosis are excessive, there is an impairment in both systolic and diastolic functions. Finally, as the disease progresses there is the expansion of the cardiac chamber with thinning of the walls and fibrosis.

All these compensations are useful at short term, but they can be counterproductive at long term. In fact, vasoconstriction increases the afterload by increasing peripheral resistances and this leads to a reduced cardiac output that in turn stimulates vasoconstriction again, giving

life to a vicious cycle (see Figure 6). Farther, the greater sodium and water retention improves stroke volume increasing venous return, but only until the myocardial fibers do not reach their maximum stretching beyond which the expansion of total blood volume is related to a reduced stroke volume, according to the Frank-Starling law. To cope with the massive vasoconstriction and the high sodium and water retention, the organism carries out counter-regulation mechanisms. The most important of them consists in natriuretic peptides A and B that increase urinary excretion of sodium and water thus reducing blood volume and preload [13].

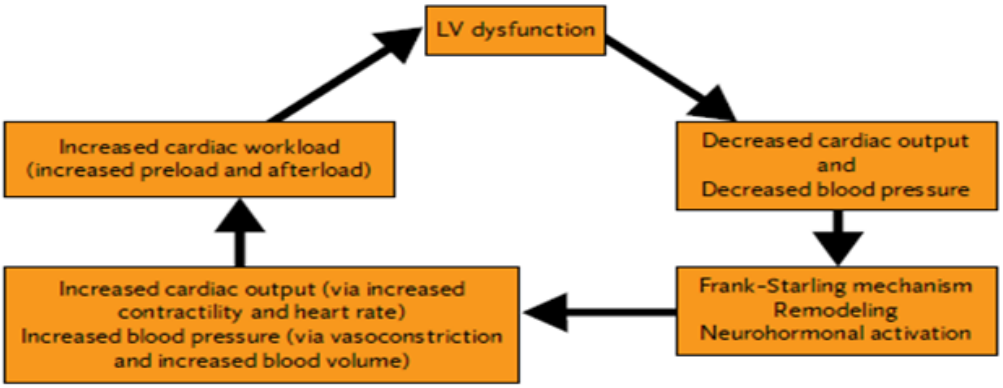



**Figure 6:** Vicious cycle of heart failure

### 1.4.3. Downsides of the compensatory mechanisms

Although the acute effects of these compensatory mechanisms are beneficial, their chronic activation often ultimately proves detrimental to the failing heart and contributes to a progressive downhill course [14]. In fact, vasoconstriction increases afterload, which may then impede cardiac output, while the excess fluid retention contributes to peripheral oedema and pulmonary congestion. In addition, the increased heart rate augments the metabolic demand on the failing heart. Continuous sympathetic activation results in down-regulation of cardiac β-adrenergic receptors, contributing to a decrease in the myocardial sensitivity to circulating catecholamines and a reduced inotropic response. Moreover, chronically elevated levels of angiotensin II lead to the production of cytokines and stimulate fibroblasts, resulting in fibrosis and adverse remodelling of the failing heart.

Because the undesired consequences of chronic neurohormonal activation eventually outweigh the benefits, much of the current pharmacological therapy of HF aims to moderate these compensatory mechanisms.

## 1.5. Clinical manifestations of heart failure

The main symptoms of heart failure concern the respiratory function, muscle activity, and diuresis [8].

- Respiratory function: dyspnea is the most characteristic symptom of heart failure due to pulmonary congestion that leads to oedema reducing lung distension and blood oxygenation. This causes more work for the respiratory muscles and determines the sensation of lack of air. The appearance of oedema is the consequence of an imbalance in the hydrostatic and osmotic pressures between capillaries and alveoli: the hydrostatic forces push fluid out of the capillaries whilst osmotic forces keep fluid in. The fluid leaving the capillaries enters the interstitium and the excess is drained by the lymphatic system; indeed, when there is a higher leak of fluids into the interstitum causing interstitial oedema, the lymphatic activity increases. Interstitial oedema can evolve into pulmonary oedema when the leak is massive and fluids go into the alveoli resulting in impaired gas exchange and problems in breathing. In chronic heart failure the increased hydrostatic capillary pressure caused by ventricular dysfunction, can lead to pulmonary oedema, but there are some pulmonary vascular changes to counteract the oedema formation. These changes are the increased capillary membrane thickness and capillary dilatation, intimal thickening of the arteries and veins, circumferential fibrosis of both veins and arteries, thickening of the alveolar wall and compression of the peripheral airways by increased connective tissue. All these structural changes allow the resistance to pulmonary oedema, but on the other hand, they encourage the pulmonary restrictive syndrome typical of chronic heart failure, which takes part in the reduced exercise capacity [15].

- Muscle activity: symptoms linked to the muscle activity are very frequent, but also nonspecific and can be summarized in asthenia during physical activity. The reduced stroke volume that leads to muscle hypoperfusion with increased anaerobic metabolism and lactic acid production causes these symptoms [8].

- Renal function: diuresis impairment is very common in heart failure and generally, it is worse during the day, while it gets better at night because the supine position increases the

venous return to the heart as well as kidney perfusion. However, in advanced chronic heart failure renal hypoperfusion becomes steady with oliguria [8].

- Peripheral oedema: especially up to the ankles, also reflects increased hydrostatic venous pressures. Because of the effects of gravity, it tends to worsen during the day and is often improved by the morning after lying supine at night. Even before peripheral oedema develops, the patient may note an unexpected weight gain resulting from the accumulation of interstitial fluid.

- Asthenia and weakness: Reduced perfusion also affect skeletal muscles where anaerobic metabolism and lactic acid production result increased. Therefore, HF patients often present symptoms like asthenia and weakness during physical activity.

## 1.6. Heart failure prognosis and diagnosis

Determining prognosis in heart failure is complex, but it is of great importance because it can help patients, their families and clinicians decide on the appropriate type and timing of therapies[1]. Numerous prognostic markers have been identified in patients with HF, but their clinical applicability is limited. All these prognostic markers are of various kinds:

- Demographic data: age and socio-economic status;
- Severity of heart failure: NYHA class, peak oxygen consumption, VE/VCO2 slope;
- Clinical status: resting heart rate, blood pressure, fluid overload, peripheral hypoperfusion;
- Myocardial remodeling and severity of heart dysfunction: LVEF, filling pressures, arrhythmias, LV hypertrophy, wide QRS;
- Biomarkers of neurohormonal activation: sodium, natriuretic peptides;
- Other biomarkers: inflammatory markers, markers of renal function, cardiac stress markers;
- Cardiovascular co-morbidities: coronary artery disease, stroke/TIA;

- Non-cardiovascular co-morbidities: diabetes, anemia, iron levels, COPD, renal failure, sleep apnea.

In same way, there is no single diagnostic test for HF; therefore, diagnosis is based on a clinical evaluation requiring a history, physical examination, and laboratory testing confirming HF, determining its potential causes and identifying comorbid illnesses.

Whereas HF signs and symptoms may be typical (decreased exercise tolerance with dyspnea, fatigue, generalized weakness and fluid retention, with peripheral or abdominal swelling and possibly orthopnea), they are often non-specific and, as such, do not help discriminate between HF and other problems. As a result, clinical history and physical examination are useful to evaluate for alternative or reversible causes of these symptoms. More objective tests can help confirm the suspicion of HF [1]:

1. echocardiography is the most widely accepted and available method to identify systolic and diastolic dysfunction and therefore assist in the diagnosis of HF. In fact, echocardiography can assess LVEF, left ventricular size, wall thickness, valve function, and left atrial pressure [16];
2. electrocardiography (ECG) is useful to identify abnormalities such as left bundle branch block, left ventricular hypertrophy, acute or previous myocardial infarction, or atrial fibrillation, which could provide further information on the etiology of HF [17];
3. chest radiography can identify pulmonary causes of dyspnea other than HF and allows the assessment of pulmonary congestion and interstitial oedema. Other findings, such as pleural effusion or cardiomegaly, may also increase the likelihood of HF.

Besides these instrumental diagnostic tests, there are other parameters that make the diagnosis more accurate and also have a prognostic value:

1. plasma concentrations of natriuretic peptides, BNP and NT-proBNP in particular, can be used to evaluate patients with dyspnea for HF since, as previously assessed, they are secreted in response to stretching or increased wall tension.
2. exercise testing helps evaluate exercise tolerance and symptoms that appear on exertion, like dyspnea and asthenia.
3. pulmonary function tests can confirm or exclude other respiratory causes of breathlessness and for assessing concomitant pulmonary diseases.

4. The diffusing capacity of the lung for CO (DLCO) is evaluated and precisely the diffusion through the alveolar-capillary membrane.

### 1.6.1. Natriuretic peptides

Natriuretic peptides are important tools to establish both prognosis and diagnosis in heart failure. In humans, there are three types of natriuretic peptides: Atrial Natriuretic Peptide (APN) predominantly expressed in the cardiac atria, B-type Natriuretic Peptide (BNP) secreted mainly in the cardiac ventricles, and C-type Natriuretic Peptide (CNP) expressed in the central nervous system, reproductive tract, bone, and endothelium of blood vessels. These peptides bind to three different receptors: NPR-A, which is the high affinity receptor for both ANP and BNP, NPR-B that binds selectively to CNP and NPR-C, the so-called clearance receptor, which binds with high affinity to all three natriuretic peptides capturing and degrading them. NPR-A and NPR-B with their ligands alter intracellular cGMP levels because of the activation of the particulate guanylyl cyclase domain in these receptors and the increased cGMP leads to a major activation of the protein kinase C that is able to phosphorylate its substrates [18, 19].

Among all the natriuretic peptides, BNP has been shown to be a powerful marker for prognosis and risk stratification in the setting of heart failure [20]. Changes in plasma BNP levels were significantly related to changes in limitations of physical activities and were a powerful predictor of the functional status deterioration. BNP and its mRNA concentrations are much higher in the human cardiac atrium than in the ventricles, but given the greater ventricular mass, most cardiac BNP and its mRNA derive from the ventricle. The BNP gene is located on chromosome 1 and after transcription, its mRNA is translated to 134-amino acid pre-proBNP; then the 26-amino acid signal peptide is removed, yielding 108-amino acid proBNP. After that, proBNP-108 is cleaved into the active form 32-amino acid BNP and the inactive form 76-amino acid N-terminal (NT)-proBNP that are both secreted in the blood. BNP is released in response to ventricular stretches determined by left ventricular pressure and volume overload [21, 22]. BNP binds to NPR-A and has multiple effects depending on the location of the receptor:

1) Cardiovascular system: BNP acts on the vascular smooth muscle bringing both venous and arterial vasodilation, reducing peripheral resistance and lowering blood pressure. Moreover, it increases the permeability of the endothelium promoting the flow of fluids towards the extravascular compartment. Doing all this, BNP reduces both the preload and the afterload [23, 24];

2) Kidneys: it has a diuretic and natriuretic effect that allows the increase of electrolytes and water excretion by functionally antagonizing the renin–angiotensin–aldosterone system; finally it limits water reabsorption thanks to the inhibition of vasopressin [25];

3) Neuroendocrine system: besides interfering with the renin-angiotensin-aldosterone system, BNP stops the release of endothelin-1, which is a potent vasoconstrictor, and acts on the sympathetic nervous system reducing catecholamine release from the nerve endings [20];

4) Immune system: it stops the production of TNF-α in the macrophages and blocks the synthesis of cytokines and chemokines. This action blocks both the recruitment of leucocytes and the activation of the inflammatory response[20].

Plasma concentrations of BNP and NT-proBNP are also useful biomarkers for the diagnosis of heart failure because patients with this syndrome have higher BNP levels as an adaptive response to the myocardial dysfunction and to the changes in the left ventricle filling pressures. However, plasma levels of BNP can be altered by other factors such as the treatment of heart failure or obesity that bring a decrease in BNP levels, while age and renal failure cause an increase that can weaken the diagnostic power of this peptide [22, 26]. Concerning prognosis of heart failure, it has been shown that high levels of BNP and NT-proBNP despite optimal treatment indicate a poor prognosis. Indeed, patients with high circulating levels of BNP have a higher probability of deterioration of their functional status and hospital readmission or death, as compared with those with only moderately increased levels. (see Figure 7) [27] Therefore, determination of plasma BNP can improve the current approach to patients with CHF by helping to identify those patients who need more extensive risk stratification: low BNP levels could exclude the need for further risk stratification, while high BNP levels necessitate further investigation [20].

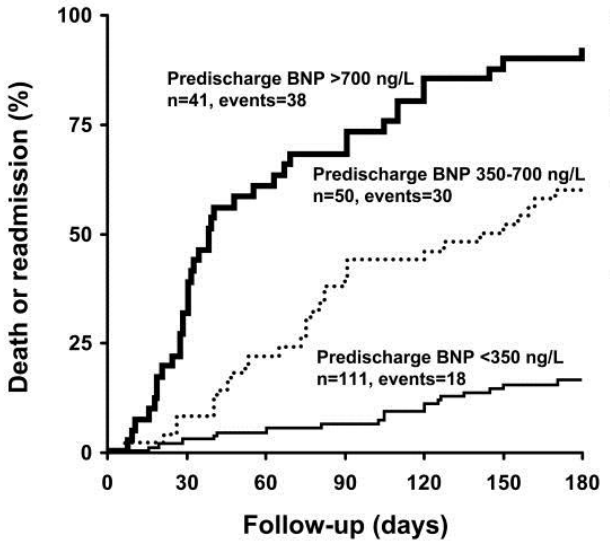



**Figure 7:** BNP levels correlated to re-hospedalization/death in patients with HF [24]

Finally, some recent studies reported the use of BNP or NT-proBNP to guide the effectiveness of therapy in patients with heart failure, but to date there is no therapeutic algorithm that allows managing the treatment of heart failure based on BNP or NT-proBNP levels [28-30].

### 1.6.2. Cardiopulmonary exercise test

Cardiopulmonary exercise test (CPET) is an important clinical tool to evaluate exercise capacity and predict outcome in patients with heart failure. It provides assessment of the integrative exercise responses involving the pulmonary, cardiovascular, and skeletal muscle systems thanks to the evaluation of both cardiac parameters, such as heart rhythm and blood pressure and respiratory parameters like gas exchanges (see Figure 8) [31]. Because of its importance in both prognosis and diagnosis of heart failure, the use of CPET is rising and its results have direct impact on treatment decisions [32]. CPET offers various parameters that help understanding if the cause of the limited exercise capacity is mainly pulmonary (ventilation or diffusion problem) or cardiogenic or peripheral (oxygen transport or its peripheral extraction).

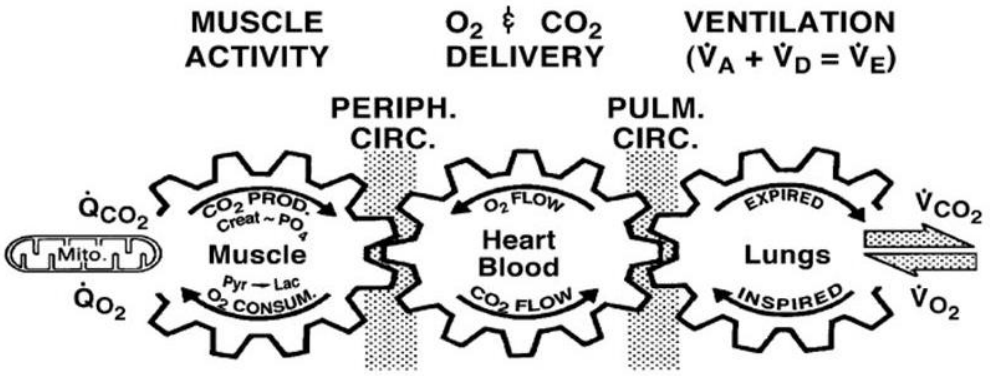



**Figure 8:** Integrative exercise responses involving pulmonary, cardiovascular and muscle system

CPET evaluates peak exercise capacity that is defined as the maximum ability of the cardiovascular system to deliver oxygen to exercising skeletal muscle and of the exercising muscle to extract oxygen from the blood [33]. Therefore, exercise tolerance is determined by three factors: pulmonary gas exchange, cardiovascular performance, and skeletal muscle metabolism.

At rest, the organism needs energy to allow all the organs to carry out their functions and most of this energy derives from the oxidation reaction between oxygen and the compounds in our nutrition (carbohydrates, proteins, lipids). The final product of this reaction is $CO_2$ that is eliminated through the lungs. As much oxygen our organism can use, even more it is efficient because the aerobic metabolism is the most inexpensive way to produce energy. Hence, measurement of oxygen consumption permits to quantify the functional capacity of an individual. During exercise, more energy is required to sustain the muscle activity so more oxygen is needed and the organism tries to tackle this request by some adaptive responses such as increasing tidal volume and respiratory rate, followed by an increase in cardiac output and heart rate. These reactions lead to higher levels of $CO_2$ that lungs have to remove and this causes an increased ventilation. In patients with heart failure, exercise capacity is reduced and cardiac output does not increase adequately with the increased oxygen uptake. This inability to increase appropriately cardiac output results in insufficient increase in perfusion to exercising muscles, which can cause early anaerobic metabolism and muscle fatigue. Oxygen uptake ($VO_2$) is measured throughout the test and can be defined as:

$VO_2 = Q \times (\Delta_{a-v}) O_2$

Q=cardiac output, $(\Delta_{a-v}) O_2$= arteriovenous difference for oxygen

The key measurement of CPET is peak oxygen uptake (peak$VO_2$) that is the highest rate of oxygen uptake achieved during the incremental exercise. This value is matched with predicted peak$VO_2$ that is normalized per age, sex and height, but not per weight; indeed, for obese patients an adjustment in the formula is needed because oxygen uptake from adipose tissue is one third of that of the muscle tissue [34]. Peak$VO_2$ has been identified as a predictor of heart failure prognosis and more specifically, low values are known to identify patients at a higher mortality risk. [35, 36]. In a study, Mancini et al. aimed to determine whether measurement of peak$VO_2$ during maximal exercise testing could be used to identify patients with chronic heart failure in whom transplantation can be safely deferred; the results showed that cardiac transplantation can be safely deferred in patients with severe left ventricular dysfunction and peak$VO_2$ of more than 14 ml/min/kg [37].

Another important parameter derived from CPET is the anaerobic threshold (AT) that marks the passage from an aerobic metabolism to one with an addicted anaerobic component. This occurs during the latter half of exercise, because oxygen supply cannot keep up with the increasing metabolic requirements of exercising muscles. At this time, there is a significant increase in lactic acid production in the muscles and in the blood lactate concentration deriving from anaerobic glycolysis. To avoid acidosis, bicarbonates buffer blood lactates and this reaction leads to $CO_2$ production:

$HCO_3^- + H+ \leftrightarrow H_2CO_3 \leftrightarrow CO_2 + H2O$

The higher production of $CO_2$ leads to an increase in ventilation after the stimulation of chemoreceptors operated by $CO_2$. Therefore, the anaerobic threshold is the point at which ventilation increases disproportionately relative to $VO_2$ and it is lower in patients with heart disease because oxygen consumption at the AT depends on factors that affect oxygen delivery to the tissues (i.e. cardiac output that is reduced in patients with heart failure). The ability to achieve the AT can help distinguish cardiac and non-cardiac (pulmonary or musculoskeletal) causes of exercise limitation: a failure to reach this threshold strongly suggests poor motivation or non-cardiovascular problems [31, 38]. However, this is not universally true

because patients with CHF could not reach the AT, while patients with chronic obstructive pulmonary disease could pass the threshold. The predictive value of this parameter is based on whether the AT has been reached or if it is determinable and its correlation with peak$VO_2$ [39]. Indeed, there are some cases in which patients with chronic heart failure performed a maximal effort and reached anaerobic metabolism, but the anaerobic threshold was not determinable; an indeterminable AT is inversely related to peak$VO_2$ and prognosis: these patients belong to a high-risk category with a very poor prognosis [40]. In the cardiopulmonary exercise test, there are three methods to determine the anaerobic threshold [31]:

1) AT is the point at which the ventilatory equivalent for $O_2$ ($VE/VO_2$) begin to increase systematically without an immediate increase in the ventilatory equivalent for $CO_2$ ($VE/VCO_2$);

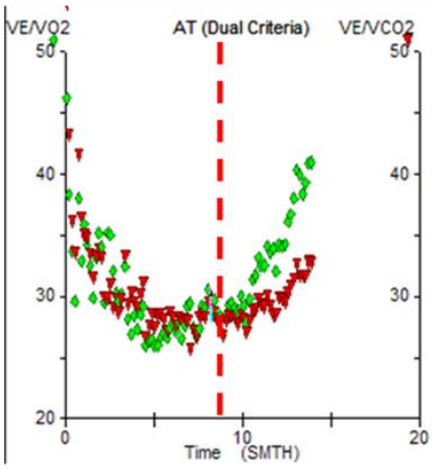



**Figure 9:** Anaerobic threshold $VE/VO_2$ – $VE/VCO_2$

2) AT corresponds to the point at which end-tidal oxygen tension ($PETO_2$) begin to increase systematically without an immediate increase in the end-tidal $CO_2$ tension ($PETCO_2$);

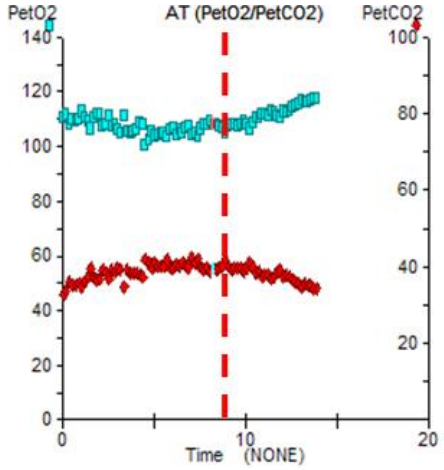



**Figure 10:** Anaerobic threshold: $PetO_2/PetCO_2$

3) AT is the point in the plot $VO_2/VCO_2$ at which $VCO_2$ undergoes an increase not equally followed by $VO_2$

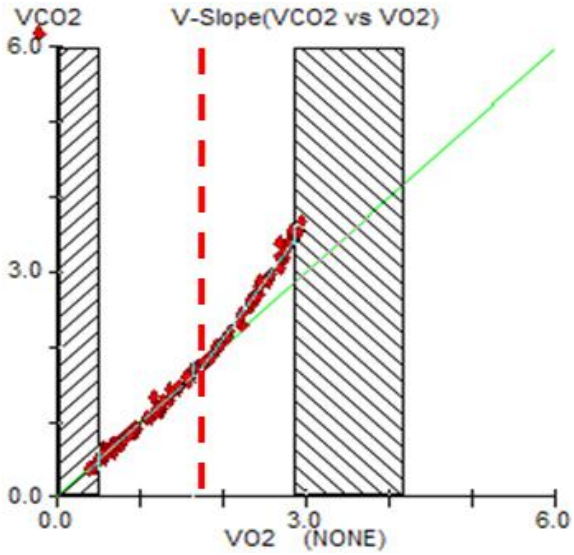



**Figure 11:** Anaerobic threshold $VCO_2$ vs $VO_2$

Finally, there is another parameter derived from CPET with a very important prognostic role in patients with chronic heart failure: the relationship between minute ventilation (VE) and $CO_2$ production ($VCO_2$), known as $VE/VCO_2$ Slope. This value represents the ventilatory efficiency of the organism and it shows the increase of ventilation required to remove a certain amount of $CO_2$. Normally $VE/VCO_2$ slope is < 30 independently from age and gender, but it can be higher in particular disease states, for example in patients with chronic heart failure or with pulmonary hypertension it can be >

60. High $VE/VCO_2$ slope during exercise is associated with ventilatory inefficiency and it is a prognostic marker in chronic heart failure, with the degree of the slope elevation reflecting disease severity [41, 42]. However multiple factors lead to a high $VE/VCO_2$ slope in heart failure like ventilation-perfusion mismatching in the lungs, chemoreceptor and ergoreceptor heightened sensitivities due to the increased activity of the sympathetic nervous system, increased anatomical and physiological dead space, reduced cardiac output and alveolar-capillary conductance and pulmonary oedema. Therefore, patients with chronic heart failure with a higher $VE/VCO_2$ slope have worse prognosis. Lastly, $VE/VCO_2$ slope is a prognostic marker independent from symptoms and age as opposed to peak$VO_2$ [36, 43].

Although CPET can give many information on the prognosis of heart failure, information about risk-stratification need to be integrated into clinical practice, so parameters derived from CPET have been combined with demographic data, medical history, and laboratory values in multivariable prognostic risk scores. In particular, Agostoni et al. developed a new risk score for systolic heart failure: the Metabolic Exercise test data combined with Cardiac and Kidney Indexes (MECKI) score. This score has been suggested to identify the risk of cardiovascular mortality and urgent heart transplantation and it relies on six simple variables: hemoglobin, sodium, kidney function as the Modification of Diet in Renal Disease (MDRD), left ventricle ejection fraction, percentage of predicted peak oxygen consumption ($ppVO_2$) and $VE/VCO_2$ slope [44, 45]. Recently, it has been demonstrated that the prognostic accuracy of the MECKI score is superior to that of other prognostic scores in chronic heart failure with reduced ejection fraction [46].

### 1.6.3. Pulmonary function testing

As previously exposed (see paragraph 3.5), patients with HF may develop pulmonary function abnormalities such as restrictive, obstructive or diffusive defects [47]. The most common pulmonary function testing that could help identify lung defects is spirometry, the measurement of the lung volumes mobilized with inspiratory or expiratory manoeuvers.

A restrictive ventilatory defect is expressed by a reduction of total lung capacity (TLC) and forced vital capacity (FVC), the maximal volume of gas exhaled as forcefully as possible after full inhalation. This reduction of lung volumes is the result of accumulation of liquid in the

interstitial and alveolar space, cardiomegaly and pleural effusion. This evidence is often combined with an increase in the residual volume (RV), the air remaining in the lungs at the end of maximal expiration, because not all portions of the lungs are affected by HF [48, 49].

An obstructive defect in HF is caused by airway narrowing, evidenced by a reduced forced expiratory volume in 1 second to FVC ratio (FEV1/FVC). The main reason for the airway caliber reduction is the compression caused by the alveolar fluid accumulation and the increase in bronchial wall thickness induced by the congestion. An increase in the RV is also common in case of an obstructive defect, because the airway closure during expiration causes air trapping [50, 51].

HF patients may also show a reduction of respiratory muscle strength, caused by the hypoperfusion of respiratory muscles due to the impaired cardiac output. The increased work of breathing of these underperfused and weak respiratory muscles is one of the main reasons for the development of dyspnea on exertion [52, 53].

Moreover, in patients with HF, the alveolar-capillary membrane undergoes a remodelling process characterized by fibrosis, deposition of connective tissue and microthrombosis. These structural changes negatively affect the physiological functions of the membrane, such as gas exchange or pulmonary fluids homeostasis. These biological functions are possible because of the peculiar anatomic configuration of the blood-gas barrier, composed of three layers: the alveolar epithelium, the interstitial space and the capillary endothelium [54].

### 1.6.4. Diffusing capacity of the Lung for Carbon Monoxide (DLCO)

In patients with chronic heart failure, the alveolar-capillary membrane undergoes a remodeling process characterized by an increase in fibrosis, deposition of connective tissue and microthrombosis. This remodeling affects negatively the physiological functions of the membrane, such as gas exchange or pulmonary fluids homeostasis. These biological functions are possible thanks to the peculiar anatomic configuration of the blood-gas barrier, which is composed of the following three layers: the alveolar epithelium, the capillary endothelium, and the interstitial space, which is interposed between the first two layers [54].

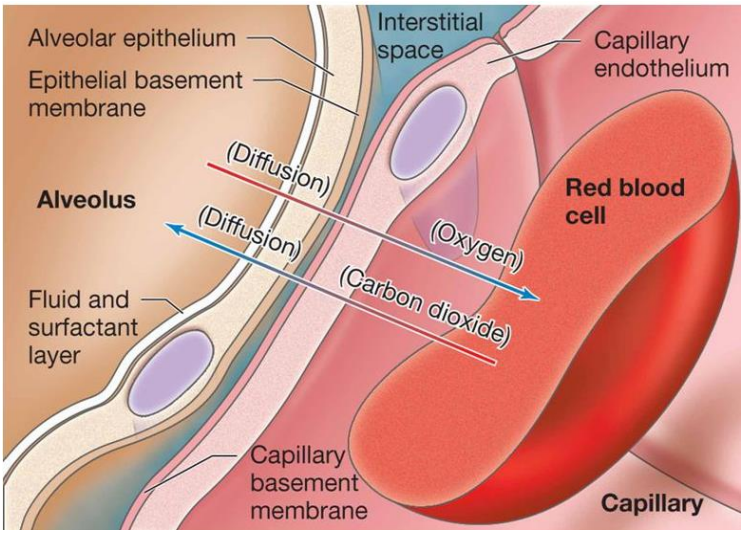



**Figure 12:** Alveolar-capillary membrane

Gas exchange through the alveolar-capillary membrane is commonly assessed using inert gases such as carbon monoxide (CO) and nitric oxide (NO). Among these gases, CO is the most used because diffuses across the alveoli and binds to hemoglobin with a 240-fold greater affinity than $O_2$. After inhalation, CO transits through the airways towards the alveoli, then it crosses the alveolar-capillary membrane reaching the blood, where it binds to hemoglobin. In clinical practice, the diffusing capacity of the lung for CO (DLCO) is evaluated and precisely the diffusion through the alveolar-capillary membrane [55]. DLCO may be partitioned into its two subcomponents using the Roughton and Forster method: Dm that is the conductance of carbon monoxide across the alveolar–capillary membrane and the chemical reaction of CO with pulmonary capillary blood expressed by the product between the rate of reaction of CO

with hemoglobin (θ) and the pulmonary capillary blood volume (Vc). The Roughton and Forster equation expresses these components in terms of resistances:

$1/DLCO = (1/Dm) + (1/\theta Vc)$

where 1/Dm is the diffusive resistance of the alveolar–capillary membrane and 1/ θVc is the reactive resistance due to pulmonary capillary blood [56].

Physiological changes can affect Dm and/or θVc leading to changes in DLCO. For example, an increase in DLCO can derive both from an increase in Dm due to an extension of the exchange area, or from a rise in Vc through capillary dilation after exercise.

Besides these physiological changes, pathological conditions can also alter DLCO, meaning that it can be used to confirm a suspected pathological status and to follow the course of the disease. In chronic heart failure, DLCO is impaired because of the remodeling of the alveolar-capillary membrane that causes a decrease in Dm and Vc and, in particular, it has been shown that the greater the severity of CHF, the lower are DLCO and Dm [54, 57, 58]. Indeed, Guazzi et al. presented Dm as an independent predictor of prognosis in stable chronic heart failure and they showed that Dm and DLCO may be improved by ACE-inhibitors, but there is no evidence for reversal after fluid removal or heart transplantation. On the contrary, airway dysfunction could be improved by tailored therapy, fluid withdrawal, and heart transplantation [59]. Furthermore, Vc decreases in parallel with DLCO and Dm and this reduction may be related to pulmonary vascular resistance increase, local thrombosis, microembolism, low blood flow due to the reduced cardiac output and low circulatory volume present in some patients because of the chronic high-dose diuretic therapy [58]. It is well known that the alveolar-capillary membrane is a target of some treatments for heart failure because lung diffusion abnormalities are related to exercise performance and chronic heart failure severity, like mentioned before. Specifically, ACE-inhibitors [60] and aldosterone antagonists [61] improve gas diffusion through the alveolar-capillary membrane, while other categories of drugs like AT1-antagonists [62, 63] have no effect, likely because the improvement seems to be dependent on the over-expression of the bradykinin-prostaglandin pathway.

## 1.7. Surfactant Proteins

In the last few years, more and more attention was given to the pursuit of new biomarkers of lung damage in heart failure. Indeed, in this syndrome there are abnormalities in gas exchange associated with anatomic changes of the alveolar-capillary membrane, as mentioned before, so the finding of a new marker as an index of the membrane function is of great importance in terms of prognosis of heart failure. About that, different surfactant proteins isoforms were evaluated as possible biomarkers of lung injury. In particular, SP-A was proposed as a predictor of lung damage caused by smoke or high altitude [64, 65], SP-D as a predictor of cardiovascular morbidity and mortality and as a prognostic marker of chronic kidney disease and lung disease, while SP-B was suggested as a biomarker of alveolar-capillary membrane damage [66-68].

Pulmonary surfactant is a complex macromolecular system composed of a mixture of lipids and proteins that is secreted into the alveolar space by type II epithelial cells. The main function of surfactant is to lower the surface tension at the air/liquid interface within the alveoli of the lung and in this way it reduces the work of breathing, prevents the collapse of the lung at end of expiration and increases lung compliance, which is the ability of the lung to alter its volume after the application of a certain pressure. Pulmonary surfactant composition includes phospholipids (about 80%), neutral lipids (about 10%), and proteins. Among phospholipids, dipalmitoylphosphatidylcholine (DPPC) is the most present in a lipid monolayer that covers the interface and is responsible for the low surface tension [69, 70]. Proteins are involved in defense mechanisms and in the stabilization of the surfactant and half of them are plasma proteins, while the other half is composed of apolipoproteins like surfactant proteins SP-A, SP-B, SP-C, and SP-D that are essential for the surfactant to exert its function.

- SP-A and SP-D: hydrophilic and multimeric proteins that regulate host immune defense and inflammatory response opsonizing bacterial cells in the alveoli and marking them for phagocytosis operated by the macrophages. They are also present in extra pulmonary tissues [71].

- SP-C: hydrophobic protein that facilitates the absorption and stabilization of the surfactant film. Its deficiency is associated with chronic lung diseases, but it does not affect survival [72].
- SP-B: hydrophobic protein belonging to the saposin-like family that is involved in the assembly of pulmonary surfactant and in its extracellular development to form stable surface-active films at air/liquid alveolar interface. It has been demonstrated that the lack of SP-B is incompatible with life because it leads to a lethal respiratory distress syndrome [73].

### 1.7.1 Surfactant Protein type B

Surfactant protein type B turned out to be the best prognostic marker of alveolar-capillary membrane damage because it is synthesized exclusively by alveolar epithelial cells unlike SP-A and SP-D, it is fundamental for the assembly of pulmonary surfactant and it undergoes a proteolytic processing in a pulmonary-cell-specific manner [74]. SP-B is encoded in a gene located on chromosome 2 where ten exons are transcribed into one mRNA that leads to the synthesis of the 381 amino-acid preproprotein (40kDa) and this prepro-SP-B is processed in the endoplasmic reticulum generating the intermediate immature forms of SP-B weighing 24 kDa and 17 kDa. After that, it is transported to the Golgi apparatus where sequential proteolytic cleavages by proteases yield the 8 kDa active mature SP-B composed of 79 amino-acids and 52% of them are hydrophobic [75-77]. The now mature SPB is stored in lamellar bodies and then secreted into the alveolar spaces and for this reason, the mature form of SP-B and even more its immature forms are undetectable in the blood in physiological conditions. On the contrary, various forms of SPB are present in the blood in pathological conditions characterized by lung damage such as acute pulmonary oedema, adult respiratory stress syndrome, and chronic heart failure. Although the various forms of SP-B are the most reliable biological marker of alveolar-capillary membrane dysfunction, the alveolar cells can be damaged in more than one clinical condition, as mentioned before, so SP-Bs cannot be used to differentiate between different causes of alveolar cell damage [66, 68, 78]. However, it has been demonstrated that patients with severe heart failure have higher plasma levels of immature SPB isoforms than those with less severe heart failure. (See Figure 13) [77]. Specifically, in chronic heart failure, this continuous SP-B flow into the circulation derives

from the chronic dynamic remodeling of the alveolar-capillary membrane caused by the reduction in number of the alveolar-capillary units, the increase in interstitial fibrosis and in cellularity and local thrombosis [66, 74]. In addition to this, both the mature and immature forms of SPB are sensitive and rapid markers of lung distress because it has been reported that they significantly increase whenever the lung is acutely stressed, as during positive pressure ventilation [67, 79].

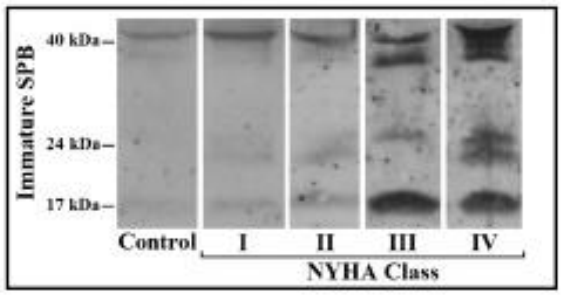



**Figure 13:** Immature forms of SP-B derived from plasma samples in controls and in patients with heart failure according to their NYHA class [75].

Dosing Method: quantitative and qualitative SP-B analysis is carried out on a plasma sample obtained from fresh blood (5 mL) centrifuged at 1500 x g for 10 minutes at 4°C, divided into aliquots and frozen at -80°C until assayed. The analysis of the three predominant immature forms of SP-B with molecular masses of 17, 24, and 42 kDa is generally performed by Western blotting on plasma samples, while the quantitative analysis of the mature form of SP-B weighting 8 kDa is performed by ELISA [68, 77].

1) Western blot or immunoblot is a widely used analytical technique that allows the detection of specific proteins in a biological sample. In this particular case, a polyacrylamide gel electrophoresis (PAGE) is used to separate proteins on the base of their molecular weight, while the following transfer to nitrocellulose serves to detect them through specific antibodies [80].

    - Sample preparation: after dosing total proteins, the samples that need to be analyzed are divided so that each one of them has the same amount of proteins;

- Polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate (SDS-PAGE) (see Figure 14): samples are loaded on a polyacrylamide gel (15% of polyacrylamide) and undergo to electrophoresis. The latter is a separation technique that exploits the differential migration of charged particles under the influence of an electric field in order to separate them. The ability of an analyte to migrate in a gel in presence of an electric potential difference is called electrophoretic mobility. This ability can depend on different factors such as the properties of the analyte (shape, dimension, and charge), the intensity of the electric field and the gel properties. The presence of anion detergents like SDS causes the denaturation of all the proteins in the samples. Indeed, at the pH at which gel electrophoresis is carried out, the SDS molecules are negatively charged and bind to proteins, approximately one molecule of SDS for every 2 amino acids, and doing so, SDS denatures the proteins, turning them into negatively charged linear polypeptide chains. When subjected to an electric field during electrophoresis, the negatively charged polypeptide chains travel toward the anode with different mobility depending only on their molecular size: small molecules move faster through the gel than the larger ones.

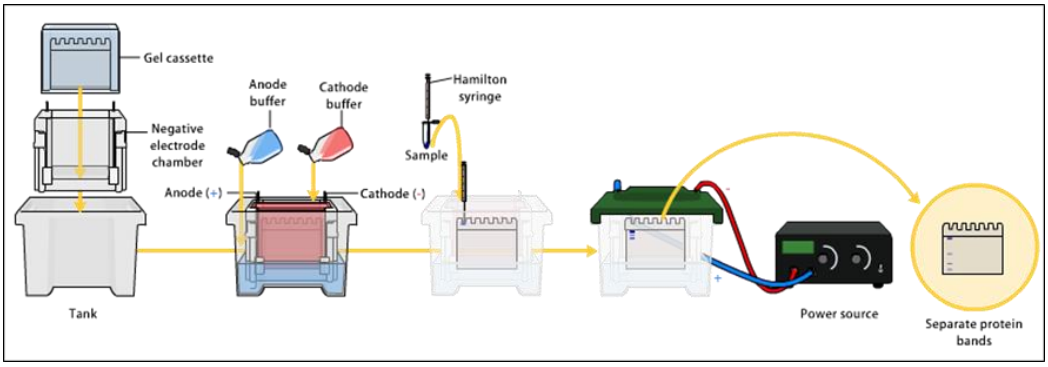



**Figure 14:** Polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate (SDS-PAGE)

- Transfer: after electrophoresis, proteins are transferred on a membrane of nitrocellulose through electro blotting that uses an electric current to pull proteins from the gel into the membrane at 60 V for 2 hours. The electric current direction is perpendicular to the gel and makes proteins move from within the

gel onto the membrane while maintaining the organization they have within the gel. The uniformity of the transfer is visible because nitrocellulose membrane is colored with red Ponceau.

- Detection of proteins: before the addition of specific antibodies to detect proteins, it is necessary to prevent the interactions between the membrane and the antibody. Therefore, the nitrocellulose membranes are placed in a dilute solution of 5% non-fat dry milk in tris-buffered saline (100mmol/L Tris-HCl, 150mmol/L NaCl, pH 7.5) containing 0.1% Tween 20 (TBS-T) for 1 hour at room temperature. The protein in the dilute solution attaches to the membrane in all places where there are not the target proteins, so that antibodies cannot bind to the membrane. After that, the membranes are incubated overnight at 4°C with primary antibody against the immature SPB; then the membranes are washed again with TBS-T to remove unbound antibodies and incubated for 1 hour with secondary antibody conjugated with horseradish peroxidase. These secondary antibodies generally derive from rabbits and are directed to a species-specific portion of the primary antibody, while peroxidase serves to cleave a chemiluminescent agent producing luminescence in proportion to the amount of protein. After washing again with TBS-T, the membranes are ready to be visualized and the revelation of the bands corresponding to the immature SPB is done using a chemiluminescent system followed by densitometry to acquire the image of the bands.

2) Enzyme-linked immunosorbent assay (ELISA): is a test designed for detecting and quantifying various substances, peptides and proteins included. In this technique, an antigen is immobilized on a solid surface and then complexed with an antibody that is linked to an enzyme. ELISA is typically performed in 96-well polystyrene plates, which passively bind the proteins to detect; after that, antibodies are added and the detection enzyme can be linked directly to the primary antibody or introduced through a secondary antibody that recognizes the primary antibody, depending on the type of ELISA performed. Detection is accomplished by assessing the conjugated enzyme activity during incubation with a substrate to produce a detectable and measureable signal, generally a change of color in the substrate.

## 1.8. Pharmacological treatment of acute heart failure: inotropic agents

For many years, inotropic agents have been used to treat acute decompensated chronic heart failure in patients with a reduced left ventricular ejection fraction; however, in recent years, their use has become common for other indications. Indeed, inotropic agents are used in selected patients with advanced chronic heart failure as a last resource before planning a more invasive and special intervention, like mechanical circulatory support or heart transplantation, in a context where optimal standard heart failure therapy is not enough to stop the progression of the syndrome. On the other hand, they are used as palliative care in patients not selected for either transplantation or mechanical support [81]. They act improving myocardial contractility, affecting also heart rate and peripheral vascular resistances, with different mechanisms of action, in order to restore or to maintain hemodynamic stability in acute decompensation or in those waiting for transplant / LVADs, respectively [82-84]. Inotropic agents that improve myocardial contractility through the increase in intracellular calcium levels are Digoxin, Dobutamine and phosphodiesterase III inhibitors, which are currently used in heart failure (See Figure 15).

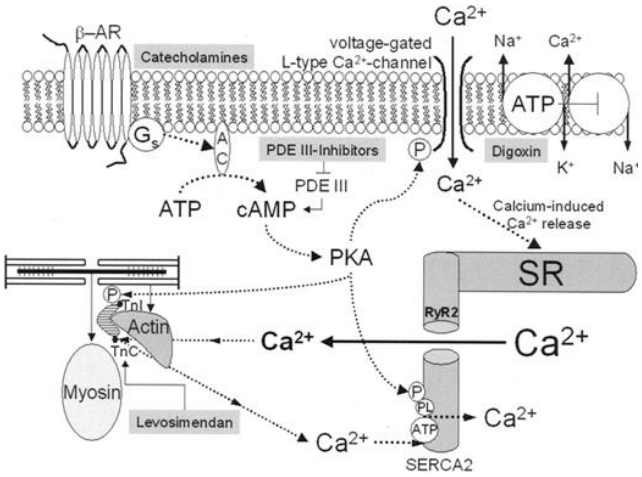



**Figure 15:** Mechanisms of action of the principals inotropic agents

- Digoxin: this drug belongs to the family of cardiac glycosides and it has been used for many years in long-term therapy of chronic heart failure because of its positive inotropic effect. Digoxin inhibits the $Na^+/K^+$ ATPase present in the myocardial fibers impeding the transport of sodium from the intracellular to the extracellular space and this affects the activity of the $Na^+/Ca^{2+}$ exchanger that promotes calcium efflux. In fact, the increased intracellular $Na^+$ levels reduce the exchanger ability to extrude calcium

outside of the cell, causing a rise in its intracellular levels with subsequent increase of myocardial contractility. All this leads to an improvement of left ventricular ejection fraction and cardiac output. Moreover, at low doses, digoxin has a neurohormonal effect that promotes vagal stimulation and reduces sympathetic activation, but the discovery of neurohormonal blockers that are capable, unlike digoxin, of reducing mortality in HFrEF has declined the use of this drug [85-87]. However, digoxin is useful in patients with heart failure and atrial fibrillation because it slows atrioventricular conduction thanks to the stimulation of the vagal tone; however, it is only recommended for the treatment of those patients when other therapeutic options cannot be pursued [88]. Another factor limiting its use is both cardiac and non-cardiac toxicity leading to bradycardia/tachycardia or nausea, vomit and delirium, respectively.

- Dobutamine: this molecule is a β-adrenergic agonist that interacts with both β1 and β2 receptors. Its primary activity derives from the stimulation of β1-receptors of the heart causing an increase in myocardial contractility and thus increasing stroke volume and cardiac output. This positive inotropic effect is due to the increase in cAMP levels that activate Protein Kinase A (PKA) that phosphorylates $Ca^{2+}$-dependent ATPase of the sarcoplasmic reticulum. This ATPase increases calcium re-uptake in the reticulum during diastole (lusitropic effect) and increases its release from the reticulum in systole (inotropic effect). The secondary effect of this drug is related to its activity on β2-receptors causing vasodilation and mild reduction of blood pressure in some patients [89]. As dobutamine acts on β-receptors, the use of β-blockers can mask its inotropic effect, in particular the use of Carvedilol [90]. Like other inotropic agents, the long-term use of dobutamine is associated with increased mortality and some patients have developed tolerance to this drug after prolonged administration, so it should be used only for short-term management of patients admitted with decompensated heart failure in order to improve their symptoms [91]. Furthermore, dobutamine has a half-life of few minutes and this is useful in critical situations where its hemodynamic effects are attainable within 10 to 15 minutes of dose administration, while most adverse events dissipate within short time of stopping the infusion [92]. In fact, it can cause

tachycardia and high blood pressure because of excessive stimulation of cardiac β1 receptors.

- Phosphodiesterase III (PDE) Inhibitors: The most used drugs belonging to this class are amrinone, milrinone and enoximone. They inhibit PDEIII, which is an enzyme responsible for cAMP degradation and because of this, cAMP levels increase leading to a major activity of PKA that promotes calcium influx in the myocardial cell. The augmented calcium in the myocardium causes an increase in cardiac contractility (inotropic effect), while on a vascular level it leads to peripheral vasodilation that reduces both preload and afterload. Unlike dobutamine, they have a greater vasodilating effect and their action on the heart is not affected by co-administration with β-blockers. They must be used with caution because they can cause arrhythmias and hypotension [82, 90].

Unfortunately, the use of these inotropic agents is limited because of a high risk/benefit ratio with an increase in mortality and morbidity mostly due to tachycardia, arrhythmias or myocardial ischemia and in addition they increase myocardial oxygen demand. In fact, these side effects together with the worse prognosis are mostly ascribable to the calcium overload that could be induced by these agents in the myocardial cell [83, 93]. Therefore, it would be useful to find drugs that increase myocardial contractility without causing calcium overload and this ability can be found in the new inotropic agents belonging to the class of $Ca^{2+}$ sensitizers, like Levosimendan.

## 1.9. Levosimendan

Levosimendan is a relatively new drug belonging to class III of $Ca^{2+}$ sensitizers that acts not only as an inotropic agent, but also as a vasodilator and it is able to modify pathways involved in the pathophysiology of heart failure. Today it is indicated for the short-term treatment of acutely decompensated congestive heart failure and in situations where conventional therapy is not sufficient, so that inotropic support is needed [94].

### 1.9.1 Levosimendan: pharmacokinetics

Levosimendan has been developed only for intravenous administration and its pharmacokinetics is linear at the therapeutic dose range of 0.05–0.2 μg/kg/minute. It has a half-life of about 1 hour that allows a fast onset of action, while the long-lasting effects of this drug are due to its metabolites OR-1855 and specially OR-1896, which has an elimination half-life of about 80 hours [95, 96]. During Levosimendan metabolism, intestinal bacteria acetylate about 5% of the parent drug generating the intermediate metabolite OR-1855, which in turn is acetylated in the liver forming the active metabolite OR-1896. Unlike the parent drug in which the 98% is bound to albumin, only the 40% of OR-1896 is bound to plasma proteins, so that low plasma levels of this metabolite can cause clinically significant effects [97]. As mentioned before, OR-1896 has a longer half-life than the parent drug and reaches its maximum concentrations 2 days after the end of a 24-hour infusion; this explains why this metabolite is responsible for the prolonged hemodynamic effects of Levosimendan that seem to last from 7 to 9 days after discontinuation of the 24-hour infusion. Moreover, it has been shown that renal dysfunction prolongs the elimination of OR-1896, while it has no effect on the plasma concentration of the parent drug, but on the contrary, liver cirrhosis prolongs the half-life of levosimendan, while its effect on OR-1896 is unknown [95, 98].

### 1.9.2. Levosimendan: mechanism of action

Levosimendan has different mechanisms of action that are responsible for the multiple effects of this drug in patients with decompensated heart failure.

*Positive Inotropic Effect:*

Levosimendan is primarily a $Ca^{2+}$ sensitizer that exerts its inotropic effect by increasing the affinity of troponin-C for $Ca^{2+}$, stabilizing the $Ca^{2+}$ induced conformation of troponin-C. This drug binds to troponin-C at the level of a hydrophobic region of its N-terminal domain thanks to hydrogen bonds with polar or charged amino acids and this bond enhances the opening of active sites of troponin-C, increasing its sensitivity to calcium [99] (See Figure 16). This is very important because it leads to positive inotropic effect without causing intracellular calcium overload; in fact, levosimendan enhances the use of $Ca^{2+}$ already present in the cell without promoting new calcium influx, unlike other inotropes [100, 101]. Another characteristic that distinguishes levosimendan from other inotropic agents is that the bond

with troponin-C is calcium dependent, so it is weaker during diastole when intracellular $Ca^{2+}$ levels are low, while it is stronger during systole when there is the maximum sensitization. On the contrary, other $Ca^{2+}$ sensitizers bind to troponin-C during both diastole and systole, impairing diastolic function; this impairment is not caused by levosimendan that helps maintaining a normal diastolic relaxation and enhances myocardial contractility with low arrhythmogenesis [102, 103]. Furthermore, the $Ca^{2+}$ sensitizing mechanism is energetically advantageous because extra energy is not needed in the myocardial cell, as there is not an increased $Ca^{2+}$ influx, so with levosimendan there is no rise in oxygen consumption, unlike with other inotropes, like dobutamine [104, 105].

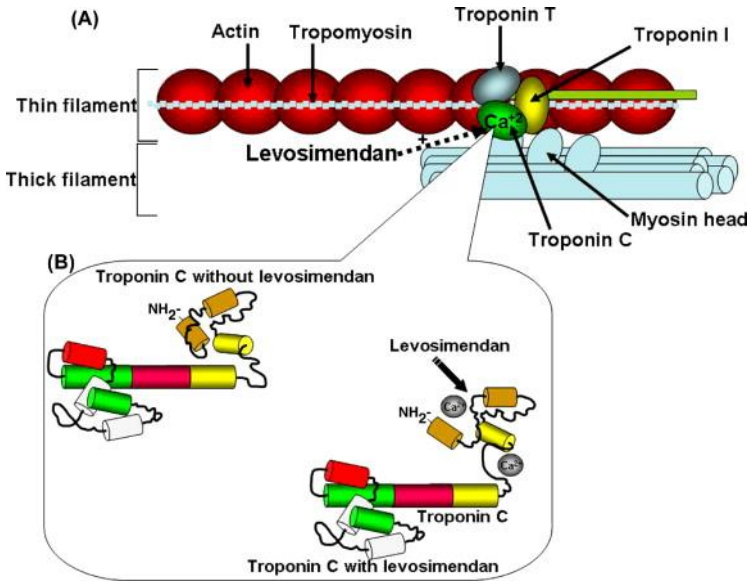



**Figure 16:** Positive inotropic effect of Levosimendan

*Vasodilation*:

Levosimendan causes vasodilation because of its ability to open ATP-dependent $K^+$ channels in vascular smooth muscle cells leading to hyperpolarization [106]. In particular, it opens ATP-dependent $K^+$ channels in resistance vessels, while in large conductance vessels it acts opening voltage-dependent ($K_v$) and $Ca^{2+}$-activated ($K_{ca}$) K+ channels; in both cases the generated hyperpolarization of the membranes inhibits the inward L-type $Ca^{2+}$ current and promotes the activity of the $Na^+/Ca^{2+}$ exchanger, which brings calcium to the outside of the cell [107]. The decreased intracellular calcium induces vasodilation in both veins and arteries leading to a reduction in both preload and afterload with decreased oxygen consumption,

which is helpful in the treatment of patients with decompensated heart failure. Moreover, coronary vasodilation together with the reduction in oxygen consumption may have anti-ischemic effects [108, 109] (See Figure 17).

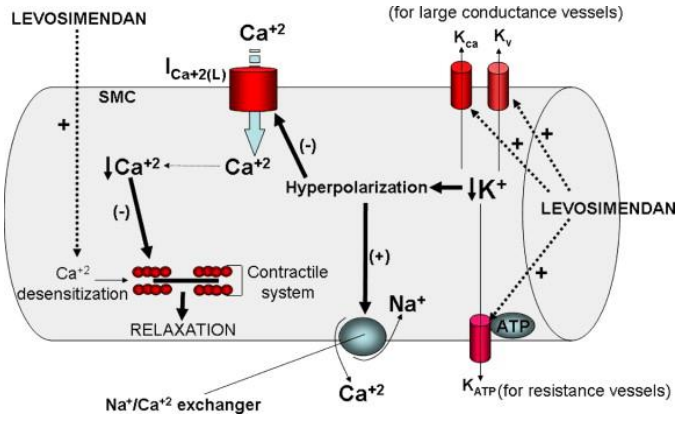



**Figure 17:** Vasodilating effect of Levosimendan

*Cardioprotection*:

It has been shown that levosimendan exerts also a cardioprotective effect linked to ischemic preconditioning, which is a phenomenon characterized by short episodes of ischemia that allows the myocardial tissue to develop a series of adaptive mechanisms in order to protect itself against subsequent ischemic insults, even if prolonged. Among these adaptive mechanisms, there is the opening of $K^+$ATP channels in both cardiac mitochondria and sarcolemma that seems to have a protective effect on the heart against ischemia-reperfusion damage [110, 111]. Levosimendan acts opening $K^+$ATP channels in mitochondria leading to the prevention of mitochondrial $Ca^{2+}$ overload, the improvement in the production of ATP by decreasing its consumption, the restoration of mitochondrial membrane potential and the regulation of mitochondrial matrix volume. On the other hand, the opening of $K^+$ATP channels in sarcolemma modulates the activity of the $Na^+/K^+$ ATPase pump and reduces the action potential duration [112, 113]. This kind of cardioprotection has suggested a possible application for levosimendan in clinical situations in which preconditioning would be beneficial such as in pre- and perioperative settings in cardiac surgery [114].

*Anti-inflammatory and Antiapoptotic Effects*:

In heart failure, proinflammatory cytokines contribute to the progression of the syndrome promoting cardiomyocyte apoptosis and cardiac remodeling; in fact, there are increased circulating levels of cytokines in patients with heart failure [115, 116]. It has been demonstrated that levosimendan administration causes a significant reduction of the circulating proinflammatory interleukin-6 and Tumor Necrosis Factor-α (TNF-α) in patients with decompensated heart failure [117]. These reductions may be the result of the improved hemodynamics caused by levosimendan that decreases left ventricle wall stress and improves peripheral perfusion through vasodilation, which leads to down-regulation of extra-cardiac cytokine production by transcriptional factors like nuclear factor-kB (NF-kB). These anti-inflammatory effects together with the absence of calcium overload in the myocardial cell lead to the down-regulation of apoptosis signaling pathways in the heart such as the one mediated by soluble FAS and Fas ligand, which are both reduced after levosimendan administration [118, 119]. In addition, it has been observed that small concentrations of levosimendan in vitro prevent oxidative cardiac myocytes apoptosis by the activation of mitochondrial $K^+$ATP channels [113, 118].

Levosimendan infusion is generally well tolerated by patients, but its vasodilatory effects can cause some side effects. The most common one is hypotension due to the peripheral vasodilation that leads to give levosimendan with caution to patients with low blood pressure and in these cases should be considered the use of lower infusion rates without the loading bolus [118, 120]. However, according to REVIVE and SURVIVE studies, despite hypotension was more frequent with levosimendan than with placebo, hypotension with dobutamine was even more frequent than with levosimendan [121, 122]. Other minor side effects caused by vasodilation are headache, dizziness and nausea [123]. Levosimendan may also affect cardiac rhythm and in particular, it was related to a higher incidence of atrial fibrillation compared with both dobutamine and placebo, but apart from this, most of clinical studies showed no evidence of increased life-threatening ventricular tachyarrhythmias after its administration [122, 124]. Another advantage of levosimendan compared to dobutamine is that it does not present any serious interactions with the standard therapy of heart failure; indeed, co-administration with β-blockers has beneficial or neutral effects on the inotropic efficacy of levosimendan, while it reduces dobutamine activity [125]

### 1.9.3. Clinical use and development of Levosimendan

According to ESC Guidelines [1], levosimendan is used to treat acute heart failure in patients that have a high degree of congestion with low perfusion ("wet and cold"), but without severe hypotension (systolic blood pressure < 85 mmHg). Levosimendan is also used in patients with acute worsening of chronic heart failure in order to restore their stable conditions. Over the years, many studies were performed in the clinical development of levosimendan and the first one [118] was the dose-finding study that aimed to identify the therapeutic dose range of this drug administered by 24-hours infusion. In this study, patients were treated with a bolus dose of 3-36 μg/Kg in 10 minutes, followed by infusion at doses ranging from 0.05 to 0.6 μg/Kg/min. The following dose escalation study assessed up-titration, maintenance, and withdrawal of levosimendan; here patients received an infusion at doses from 0.1 to 0.4 μg/Kg/min and the study drug was associated with dose-dependent increases in stroke volume and cardiac index [123]. To date, levosimendan is administered as a bolus of 12 μg/Kg over 10 minutes followed by an infusion at doses from 0.05 to 0.2 μg/Kg/min. However, in clinical practice it is preferable to start the infusion without any previous bolus administration because it has been seen that it is unnecessary and doing so, the patient is not submitted to a premature decrease in blood pressure. Another important study compared levosimendan with dobutamine (the LIDO study) revealing that in patients with severe, low-output heart failure levosimendan improved hemodynamic performance more effectively than dobutamine and this benefit was accompanied by lower mortality in the levosimendan group for up to 180 days. Furthermore, this study showed the difference between levosimendan and dobutamine in co-administration with β-blockers because there was a reduction in the hemodynamic effects only with dobutamine [125, 126]. Other two large studies on levosimendan are the REVIVE studies that aimed to evaluate its efficacy on symptoms of heart failure and they confirmed that levosimendan produces a significant dose-dependent increase in cardiac output and stroke volume, reducing pulmonary capillary wedge pressure and blood pressure. Moreover, levosimendan leads to a rapid and sustained decrease in natriuretic peptides and in the REVIVE II study, the effect on BNP was evident also 5 days after the administration [127, 128] (See Figure 18).

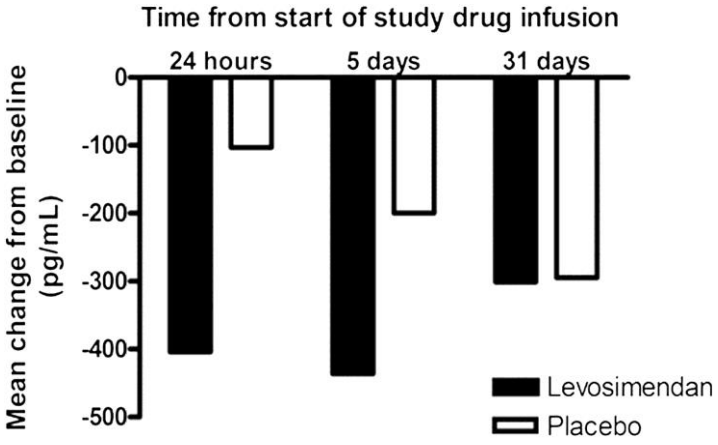



**Figure 18:** Effect of Levosimendan on BNP levels in the REVIVE-II study

In 2005 the SURVIVE [122] study was the first trial using mortality as the primary endpoint in evaluating the efficacy of levosimendan compared with dobutamine in high-risk patients with severe heart failure (LVEF < 30%); the result was that at 180 days, no differences in mortality have been observed between patients treated with levosimendan and dobutamine (See Figure 18). After this result many meta-analysis have been made and in particular, A. Delaney et al. [129] showed that there was no survival benefit when levosimendan was compared to placebo, while it was associated with an improvement in both hemodynamics and survival when compared to dobutamine. Because of all these different results regarding mortality, further studies are needed to demonstrate whether levosimendan has the ability to improve survival in patients with severe heart failure: mortality remains an issue yet to be resolved. On the other hand, levosimendan seems to reduce significantly hospitalizations; in fact in the LIDO study, patients with levosimendan spent significantly more days out of hospital than patients treated with dobutamine. [125] Furthermore, in the REVIVE II study levosimendan reduced the mean duration of the initial hospitalization and in a recent meta-analysis by Landoni et al. the mean length of stay in hospital was 1.59 days shorter in levosimendan-treated patients ($p < 0.0001$) [128, 130].

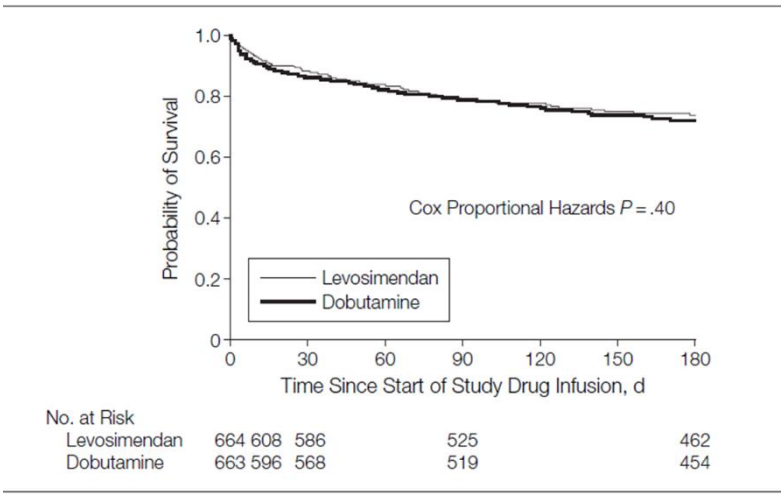



**Figure 19:** Mortality in patients treated with Levosimendan and Dobutamine after 180 days in the SURVIVE study

## 1.10. Pharmacological treatment of chronic heart failure

The main goals of heart failure therapy are the improvement of symptoms and the amelioration of prognosis. Furthermore, besides all the pharmacological treatments, there are all the medical and surgical interventions intended to correct or remove the primary cause of heart failure (for example coronary or valve disease). In addition to this, heart failure therapy includes treatments that prevent or remove all the factors that can make the syndrome worse like arrhythmias, infections, or embolism. However, patients with heart failure must take some general measures in any case; for example, they have to avoid imposing too much work to the heart, yet the total absence of physical activity can favor venous thromboembolism or muscle hypotonia, so a cautious physical activity, based on the specific patient, is recommended. Besides physical efforts, patients have to avoid emotional stress and food excesses, in fact, they have to follow a light diet and limit salt assumption because it promotes water retention increasing the preload (See Figure 20) [8].

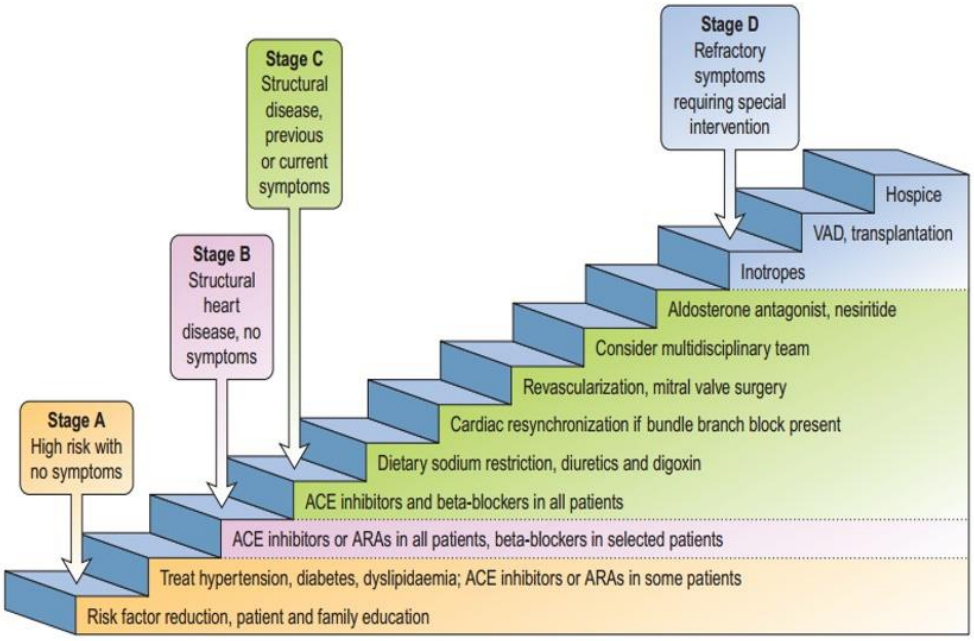



**Figure 20:** Heart failure therapy based on the different stages of the syndrome according to the ACCF/AHA classification

In chronic heart failure, there are three main categories of drugs recommended in all symptomatic patients with reduced ejection fraction: ACE-Inhibitors, β-blockers and aldosterone antagonists [1].

1) ACE-inhibitors: the use of these drugs in association with β-blockers is first line in heart failure treatment [1]. They inhibit the Angiotensin-Converting-Enzyme (ACE) blocking the conversion of angiotensin I into angiotensin II and preventing the inactivation of bradykinin. This causes peripheral vasodilation with reduced resistances that lead to a decreased afterload and an increased stroke volume together with cardiac output. In addition to this, they counteract the direct effect of angiotensin II on the heart that consists of increasing the activity of cardiac fibroblasts; in fact, ACE-inhibitors lead to an increase of bradykinin that restricts cardiac remodeling promoted by angiotensin II. Another effect of these drugs is the reduction of aldosterone secretion with decreased sodium and water retention, which leads to the reduction of preload and increased potassium retention [131]. The ACE-inhibitors mostly used in heart failure are Captopril, Enalapril, Ramipril, Lisinopril, Quinapril and Fosinopril and the treatment with these drugs should be started at low doses because in many patients a sudden pressure drop can occur. Indeed, the most frequent

side effects are hypotension, hyperkalemia, especially in patients with renal failure, and dry cough. The latter is due to the high bradykinin levels that cause a sensitization of airway sensory nerves stimulating the cough reflex [132].

2) β-Blockers: it has been shown that the use of these drugs reduces mortality and morbidity in heart failure, although at the beginning they were thought to be contraindicated in patients with heart failure because of their negative inotropic effect [133-135]. Actually, they counteract the hyperactivation of the sympathetic nervous system that has deleterious effects on the dysfunctional myocardium; in fact, they are competitive antagonists of the β-adrenergic receptors and prevent the bond with noradrenalin. In human, there are three types of β-receptors located in different areas: β1 receptors that are mostly present in the heart and in smaller quantities in kidneys and in the eyes, β2 that are mostly present in blood vessels, smooth muscle cells, heart, liver, skeletal muscles and pancreas, and β3 that are typical of the adipose tissue. Based on the receptor selectivity, β-blockers can be divided into two main groups: non-selective and β1-selective drugs. The former do not show any preference in receptors and display both β1 and β2 antagonism, while the latter mostly antagonize β1 receptors and are known to be cardioselective. Therefore, they are used in chronic heart failure because of their negative chronotropic effect that slows heart rate allowing the left ventricle to fill more completely reducing susceptibility to ventricular arrhythmias and thus reducing the risk of sudden death together with the risk of thromboembolic events. Furthermore, they bring down renin secretion decreasing sodium and water retention together with blood volume and preload, contributing in reducing cardiac work and oxygen demands. β-blockers are prescribed in patients that have mild to moderate symptoms and can be used together with ACE-inhibitors or angiotensin type 1 receptor blockers and diuretics based on the severity of the syndrome. The ones used in heart failure are Bisoprolol (β1-selective), Carvedilol (non-selective) and Metoprolol (β1 selective) that should be initiated in clinically stable patients at a low dose and gradually up-titrated to the maximum tolerated dose because a depression in ventricular function is always possible and they are contraindicated in patients with bradycardia, hypotension and asthma [131].

3) Aldosterone antagonists: they antagonize the aldosterone receptor preventing the bond with its substrate, doing so they inhibit sodium and water retention reducing potassium and uric acid excretion at the level of the distal convoluted tubule and the collecting duct of the nephron. Apart from this potassium-sparing diuretic effect, aldosterone antagonists are used in chronic heart failure because they reduce the fibrotic activity of aldosterone; in fact, this hormone worsens the patients' conditions increasing oxidative stress and promoting cardiac and vascular hypertrophy and fibrosis [136]. Another negative effect of aldosterone is the inhibition of adrenaline re-uptake in the myocardium with arrhythmogenic effect, thus aldosterone antagonists help reducing this activity by increasing adrenaline re-uptake. The active substances commonly used are Spironolactone, Eplerenone and Canrenone and they are administered in symptomatic patients with chronic heart failure, however regular checks on potassium levels and renal function should be performed because these drugs can cause hyperkalemia as a side effect [131].

Besides these drugs, there are other pharmacological treatments recommended for selected symptomatic patients with heart failure with reduced ejection fraction such as angiotensin II type I receptor blockers, diuretics, angiotensin receptor neprilysin inhibitor, If-channel inhibitor and nitrates [1].

- Angiotensin II type I receptor blockers (ARBs): these drugs are mostly used as an alternative in patients intolerant of ACE-inhibitors and they block the activation of angiotensin II type I receptors on the resistance vessels causing direct vasodilation and preventing the synthesis and secretion of aldosterone lowering blood pressure. Unlike ACE-inhibitors, ARBs do not interfere with bradykinin pathway, hence cough is not a side effect of these drugs, but they too can cause hyperkalemia because of the effect on the secretion of aldosterone [131, 137]. Among the active substances of this category, Candesartan has been shown to reduce hospital admissions for worsening heart failure and to increase survival, [138] but also Valsartan, Losartan and Irbesartan are commonly used in heart failure.

- Diuretics: these drugs have not significant effects on prognosis of heart failure, so they are used to improve congestive symptoms [1]. Indeed, they increase sodium and water

removal through urine reducing blood volume together with preload and venous congestion and help to control oedema. In chronic heart failure, two types of diuretics are commonly used: loop diuretics and thiazides. The former have a faster and more powerful effect at the level of the ascending loop of Henle where salt reabsorption occurs thanks to the Na/Cl/K symporter; loop diuretics inhibit this transporter so that $Na^+$ and $Cl^-$ are not reabsorbed and water is excreted with urine causing a reduction in blood volume and preload. Furthermore, as calcium and magnesium reabsorption depend on sodium and chloride concentration, loop diuretics interfere with the reabsorption of these ions too, helping diuresis. The most used loop diuretic in heart failure is Furosemide. As of thiazide diuretics, they have a slower but long-lasting effect at the level of the distal convoluted tubule where they inhibit $Na^+/Cl^-$ symporter, thus preventing salt reabsorption and encouraging water excretion [131]. The use of thiazides in co-administration with loop diuretics in heart failure is limited to those patients that have become refractory to loop diuretics or have resistant oedema; in fact, the combined use of both diuretics has a synergic effect [1]. Moreover, thiazides are contraindicated in patients with gout or hyperuricemia because they reduce the clearance of uric acid competing for the same transporter. Both loop and thiazide diuretics could cause hypokalemia as side effect because all sodium and chloride, which are not absorbed in the ascending loop or in the distal tubule, activate $Na^+/K^+$ ATPase in the collecting duct that promotes sodium reabsorption and potassium excretion in urine. The subsequent hypokalemia can result in significant Q-T interval prolongation and in an increased risk of arrhythmias. Mild hypokalemia is countered by the use of aldosterone antagonists such as spironolactone or the use of other potassium-sparing diuretics like amiloride and triamterene, which block $Na^+$ channels in the collecting duct preventing sodium absorption and potassium excretion. However, a more severe hypokalemia could be corrected adding $K^+$ supplement too, but potassium serum levels and renal function need to be checked frequently [131].

- Angiotensin receptor neprilysin inhibitor (ARNI): this is a new therapeutic class of drugs acting on both the RAA system and the metabolism of natriuretic peptides. The first molecule of this class is LCZ696 that combines valsartan and sacubitril in a single substance. The former is an angiotensin II type 1 receptor blocker, while the latter is

an inhibitor of the enzyme neprilysin that promotes natriuretic peptides degradation. Using this drug, ANP and BNP levels increase because of the inhibition of their degradation and this enhances diuresis, natriuresis, myocardial relaxation and an anti-remodeling effect through the augmented cGMP. In the PARADIGM-HF study, the superiority of Sacubitril/Valsartan over enalapril in reducing hospitalization for worsening of heart failure, cardiovascular mortality and overall mortality was demonstrated [139, 140]. However, symptomatic hypotension can occur as a side effect of this drug, so it must be administered with caution and combined treatment with an ACE-inhibitor (or ARB) is contraindicated [1].

- If-channel inhibitor: Ivabradine is the only molecule of this kind of drugs and it is an inhibitor of the funny current that origins in the sinoatrial node and controls the spontaneous depolarization of pacemaker cells. Ivabradine slows heart rate blocking this mixed $Na^+$ and $K^+$ inward current, so that the heart reduces its work together with oxygen demands. Because of its mode of action, Ivabradine can be used only in patents in sinus rhythm with a LVEF $\leq 35\%$ and with a resting heart rate $\geq 75$ bpm, according to the *European Medicines Agency* (EMA) [141].

## 1.11. Angiotensin receptor neprilysin inhibitors

Neurohormonal pathways are of critical importance in the pathogenesis and progression of HF. Previous HF therapies mainly focused on blocking the detrimental effects of long-term neurohormonal activation (ACE inhibitors, ARBs, beta-blockers and MRAs) and largely ignored the physiological compensatory effect of the natriuretic peptide system and other endogenous vasodilator mechanisms. On the other hand, the inhibition of NEP results in an increase in the activity of natriuretic peptides and other vasoactive peptides that potentially exert favorable long-term compensatory effects. However, NEP is also involved in the degradation of angiotensin II, therefore its inhibition leads to an increase of this oligopeptide, which is a major mediator of HF development and progression [142]. Accordingly, the full compensatory benefit of NEP inhibition can only be leveraged if both the RAAS and NEP system are inhibited simultaneously [143].

An early example of a dual ACE/NEP inhibitor, omapatrilat, was no more effective than an ACE inhibitor alone in reducing the risk of death and HF hospitalization in the OVERTURE

study; a possible cause could have been its once-daily dosing, which did not provide 24-hour NEP and ACE-inhibition [144]. In addition, omapatrilat was associated with an increased incidence of serious angioedema with airway compromise requiring mechanical support [145]. Even if the compound was never marketed, these studies confirmed the correct approach in the simultaneous inhibition of the RAAS and NEP systems leading to the development of a new therapeutic class: the angiotensin receptor neprilysin inhibitors (ARNI).

The first in class is LCZ696, a fixed dose combination of an ARB (valsartan) and a NEP inhibitor (sacubitril), which has recently shown superiority to an ACE inhibitor (enalapril) in reducing the risk of death and of hospitalization for HF in the PARADIGM-HF trial [146]. Sacubitril/valsartan is therefore recommended in the outpatient setting as an alternative to ACE inhibitors in HFrEF patients who remain symptomatic despite optimal therapy with an ACE inhibitor, a beta-blocker and a MRA [1].

### 1.11.1 Sacubitril/valsartan: pharmacokinetics

LCZ696 is a fixed-dose hemipentahydrate sodium salt complex of sacubitril and valsartan in a 1:1 molar ratio for oral administration, presented as film-coated tablets.

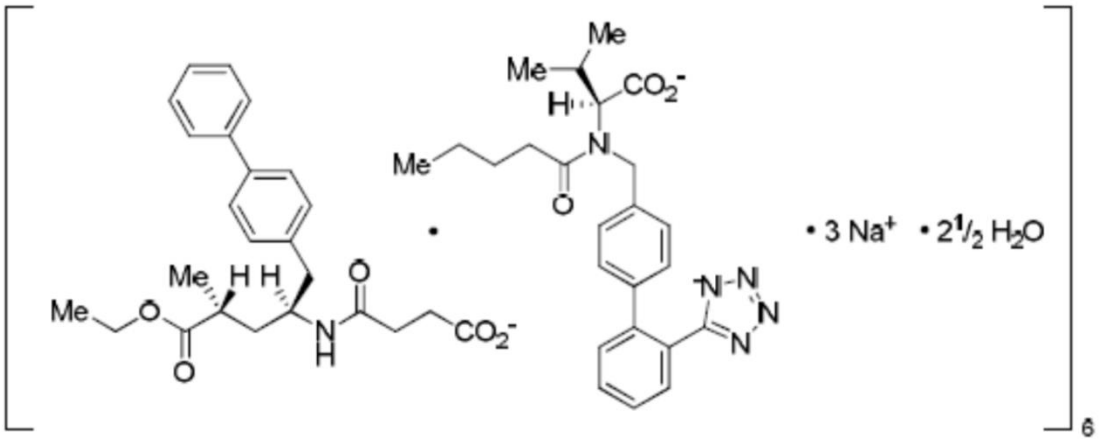



**Figure 21:** Structure of the sacubitril/valsartan complex

Three strengths are proposed containing 24 mg sacubitril and 26 mg valsartan (low strength), 49 mg sacubitril and 51 mg valsartan (middle strength) and 97 mg sacubitril and 103 mg valsartan (high strength).

Following oral administration, LCZ696 dissociates into valsartan and the pro-drug sacubitril (AHU377), which is further hydrolyzed by carboxyl esterase 1 to the NEP inhibitor sacubitrilat (LBQ657), responsible for the therapeutic effect.

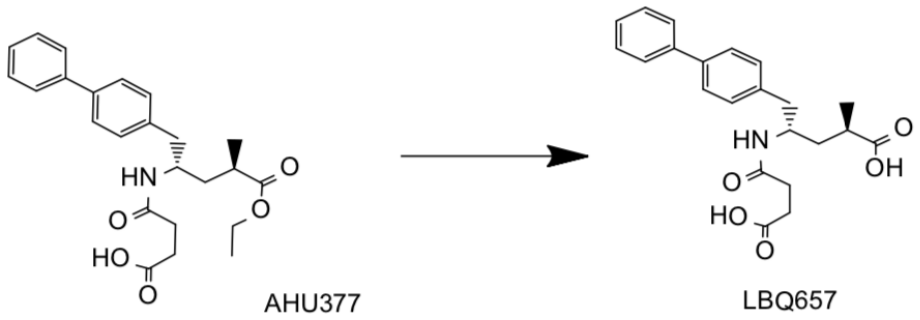



**Figure 22:** Conversion of sacubitril (AHU377) to sacubitrilat (LBQ657)

Both sacubitril and valsartan are absorbed rapidly after ingestion, with maximum plasma concentrations reached within 0.5 to 1.1 and 1.7 to 2.2 hours, respectively, after dosing. Sacubitril is rapidly converted to its active metabolite, with peak sacubitrilat concentrations reached 1.9 to 3.5 hours after oral administration. This explains the rapid onset of activity of LCZ696 and its suitability for once- or twice-daily dosing [147].

The pharmacokinetics of sacubitril, its active metabolite and valsartan are linear over the dose range of 20–600 mg [148, 149].

It is estimated that the oral absolute bioavailability for sacubitril is 60%. The bioavailability of valsartan administered as sacubitril/valsartan is 60% greater than it is when valsartan is administered in single-agent formulations [147].

Following twice-daily dosing, steady-state levels of both sacubitrilat and valsartan are reached in 3 days. At steady state, sacubitril and valsartan do not accumulate significantly, whereas sacubitrilat accumulates by 1.6-fold [150].

Sacubitril, sacubitrilat, and valsartan are highly (94–97%) bound to plasma proteins and undergo substantial distribution to tissues. Average apparent volumes of distribution for sacubitril and valsartan are 103 and 75 liters, respectively. Mean half-life values for sacubitril range from 1.1 to 3.6 hours, whereas valsartan and sacubitrilat are eliminated with estimated half-lives of 8.9 to 16.6 and 9.9 to 11.1 hours, respectively [147].

The primary route of elimination is renal for sacubitrilat (52–68% is eliminated unmetabolized in urine) and hepatic for valsartan (86% is excreted via the feces) [150].

Exposure to sacubitrilat and valsartan results increased by approximately 110 and 132% respectively, in patients with HFrEF, compared with healthy subjects. This raise is consistent with the increase in the estimated half-life for sacubitril, its active metabolite, and valsartan in these patients, which reflects a reduction in the clearance of LCZ696 analytes potentially due to altered hepatic and/or renal function [151].

### 1.11.2 Sacubitril/valsartan: mechanism of action

LCZ696 exhibits the novel mechanism of action of an ARNI by simultaneously inhibiting NEP via sacubitrilat, the active metabolite of the pro-drug sacubitril, and by blocking the angiotensin II type-1 (AT1) receptor via valsartan. As a result, the cardiovascular benefits of LCZ696 in HF patients are attributed to the enhancement of protective peptides that are degraded by NEP, such as natriuretic peptides, and to the simultaneous inhibition of the deleterious effects of angiotensin II.

As previously explained, natriuretic peptides exert their effects by activating guanylyl cyclase-coupled receptors (NPR-A and -B), resulting in increased concentrations of the second messenger cGMP. Therefore, the latter could be used as a biomarker indicative of NEP inhibition, alongside plasma and/or urinary ANP and BNP levels in patients with HF. Pharmacodynamics studies also assessed markers of RAAS blockade: valsartan, by blocking the activity of angiotensin II at its receptor AT1, reduces the normal feedback inhibition of renin release from the kidneys and therefore increases renin concentration, plasma renin activity (PRA), and angiotensin II levels.

Pharmacodynamics assessments in healthy subjects demonstrated a significant increase in plasma cGMP levels following multiple-dose administration of LCZ696, consistently with

the inhibition of NEP activity. The increase in cGMP peaked at 4 and 12 hours after dosing, shortly after the maximum plasma concentrations of sacubitrilat were reached. The cGMP response returned to baseline at 24 hours post dose, suggesting the need for a twice daily administration of LCZ696 to sustain the cGMP response throughout the dosing interval. Following the high strength dose administration, urinary ANP excretion was significantly increased [147]. A prospective comparison of ARNI with an ARB in HFpEF patients also showed a greater reduction in plasma concentrations of NT-proBNP, a strong marker of left ventricular wall stress, in patients assigned to LCZ696. Because NT-proBNP is not a NEP substrate, it could be a suitable biomarker of HF in patients treated with LCZ696. This result was associated with improvements in some echocardiographic parameters, such as left atrial dimension and volume, consistent with the hypothesis that LCZ696 reduced left ventricular pressures and wall stress [152]. Finally, plasma endothelin levels were measured in both healthy and HF patients, showing a reduction that demonstrates the NEP inhibition.

Moreover, LCZ696 stimulates significant, dose-dependent increases in renin concentration, PRA, and angiotensin II levels, indicative of blockade of the AT1 receptor. Notably, the high strength dose increased renin concentration by 3.1-fold, PRA by 4.9-fold, and angiotensin II by 3.7-fold relative to placebo. These increases are comparable with those previously observed with the administration of valsartan 320 mg in healthy participants. Significant increases in all RAAS biomarkers were sustained 24 hours after LCZ696 administration, in accord with the observed long plasma half-life of valsartan (15-22 hours) [147]. Even though sacubitril/valsartan increases the circulating angiotensin II levels, the overall effect is a dose-dependent decrease in blood pressure due to the simultaneous NEP inhibition that prolongs the beneficial actions of natriuretic peptides [153]. LCZ696, in fact, results in a transient larger natriuretic and diuretic effect in HF patients compared to valsartan, consistent with the expected effects of natriuretic peptides. They also exert their inhibitory effects on aldosterone release, explaining the decrease in aldosterone plasma concentration measured after administration of sacubitril/valsartan [154].

These findings suggest that LCZ696 could have favourable effects in patients with HF.

1.11.3 Clinical use and development of sacubitril/valsartan

According to the most recent ESC guidelines, sacubitril/valsartan is recommended as a replacement for ACE inhibitors to further reduce the risk of HF hospitalization and death in outpatients with HFrEF who remain symptomatic despite optimal treatment with an ACE inhibitor, a beta-blocker and an MRA [1].

There were no dedicated dose-finding studies. LCZ696 contains valsartan, already approved for HF, in a comparable dose. For sacubitril, the dose was chosen following the results of available pharmacodynamics studies that used biomarker data to determine the amount of inhibition of NEP provided by different LCZ696 doses. In healthy volunteers, LCZ696 50, 100, 200 and 400 mg once daily were compared and a dose of 200 mg (corresponding to sacubitril 97 mg and valsartan 103 mg) was selected, as higher doses would not provide additional NEP inhibition.

A twice daily administration of LCZ696 200 mg delivered valsartan exposures that were similar to those delivered by the marketed formulation of valsartan 160 mg bis in die (bid), which was the approved target dose for the treatment of HF. Because NEP inhibition causes an increase of angiotensin-II, adequate blockade of the AT1 receptor was especially important. As already assessed, none of the doses tested could provide 24h NEP inhibition, as none increased cGMP after 24 hours. Thus, the choice of twice daily dosing was accepted.

The main phase III study to support the proposed indication for sacubitril/valsartan was the PARADIGM-HF trial, which aimed to evaluate the superiority of LCZ696 as compared to enalapril on morbidity and mortality reduction in HFrEF. The study involved 8442 patients with NYHA class II, III, or IV symptoms, elevated BNP levels and a LVEF of 40% or less (then amended to 35% or less), randomly assigned to receive either LCZ696 97/103 mg bid or enalapril 10 mg bid, after a run-in period, during which all patients firstly received enalapril and then LCZ696, to ensure an acceptable side-effect profile of the study drugs at target doses. Over a median follow-up period of 27 months, sacubitril/valsartan proved superior to enalapril in reducing the risk of death from cardiovascular causes or hospitalization for HF, providing strong evidence that the combined inhibition of NEP and the angiotensin II receptor is superior to the inhibition of RAAS alone in patients with chronic HF.

As regards the safety, because of its greater vasodilator effects, treatment with LCZ696 was associated with a higher rate of symptomatic hypotension, but rarely required discontinuation of treatment. On the other hand, renal impairment or elevated serum creatinine and potassium levels were reported less frequently in the LCZ696 than in the control group. Also, sacubitril/valsartan was not associated with an increased risk of serious angioedema as, to minimize this risk caused by overlapping ACE and NEP inhibition, enalapril was withheld one day before the initiation of treatment with LCZ696 [146].

While the PARADIGM-HF population included patients pre-exposed to optimal doses of enalapril, many HFrEF patients introduced to sacubitril/valsartan are not at target doses of ACE inhibitor/ARB. The TITRATION trial addressed whether the tolerability of initiating sacubitril/valsartan was affected by the duration of the initiation and uptitration regimen over a 12-week period. Patients were stratified according to the dose of ACE inhibitor/ARB at screening. All patients received a starting dose of LCZ696 24/26 mg bid during a 5-day run-in period, equivalent to the recommended starting dose of valsartan for HF patients. If the initial dose was tolerated, patients were randomized to a 3-week or 6-week up-titration regimen to achieve the target dose of 97/103 mg bid. The study results show that more patients transitioned from a low ACE inhibitor/ARB dose were able to achieve and maintain the sacubitril/valsartan target dose if they were uptitrated more gradually. This difference was due to fewer hypotension, hyperkalemia, and renal dysfunction-related adverse events with uptitration over 6 weeks compared to 3 weeks. Conversely, approximately 84% of patients pre-treated with a higher dose of ACE inhibitor/ARB achieved and maintained the target dose for the 12-week study period regardless of the duration of sacubitril/valsartan uptitration. In conclusion, the more gradual uptitration regimen is more successful, especially in patients taking a low dose (or naïve to) ACE inhibitor/ARB [155].

The recommended starting dose of sacubitril/valsartan is 49/51 mg twice daily. The dose should be doubled at 2-4 weeks to the target maintenance dose of 97/103 mg twice daily, as tolerated by the patient.

However, in patients taking a low dose (or naïve to) ACE inhibitor/ARB, a starting dose of 24/26 mg twice daily and slow dose titration (doubling every 3-4 weeks) are recommended. Also, a lower starting dose should be considered for patients with systolic blood pressure 100

to 110 mmHg and other co-morbidities, such as severe renal or moderate hepatic impairment. LCZ696 should not be co-administered with an ACE inhibitor or an ARB. Due to the potential risk of angioedema when used concomitantly with an ACE inhibitor, it must not be started for at least 36 hours after discontinuing ACE inhibitor therapy [150].

# CHAPTER II

# ACUTE HEART FAILURE: MATERIALS AND METHODS

## 2.1. Background and rationale

Pulmonary function is largely involved in both chronic and acute heart failure, though in a different way; in fact, in the former there is a chronic remodeling of alveolar-capillary units, while in the latter there is an acute stretching of alveoli. Furthermore, the symptom of dyspnea, typical of heart failure, arises from the lung and all the alveolar-capillary membrane abnormalities present in heart failure influence its clinical course [58, 59, 156]. At present, there are no specific biomarkers to assess pulmonary function in heart failure, but surfactant proteins could be good candidates as biological indicators of alveolar damage and above all, surfactant protein type B has been proposed as a biomarker of an alveolar capillary barrier damage [66-68]. Indeed, as mentioned before, its immature form plasma levels are increased in chronic heart failure and, being produced only at the level of the alveolar cells, SP-B acquires a prognostic role in chronic heart failure [66, 157]. The drug used in this study, levosimendan, is an inodilator that has inotropic, vasodilator and cardioprotective effects and, more importantly, does not increase myocardial oxygen demand; in addition, it improves symptoms and hemodynamics in heart failure immediately after its administration reducing BNP levels [120]. Levosimendan has been used not only in patients with acute decompensation, but also in patients with ACHF where it has significantly improved CPET parameters and exercise performance compared to placebo, like reported before [158].

## 2.2. Study objectives

The present study is monocentric and observational. The aim of the study is primarily to determine the different surfactant proteins isoforms changes after a single levosimendan infusion, together with respiratory function changes; secondly, changes in BNP and CPET parameters after treatment administration are evaluated, too.

## 2.3. Study population

Only patients who had advanced chronic heart failure according to the *European Society of Cardiology* definitions [1] and hospitalized due to acute heart failure were evaluated for this study. They belonged to a cohort of patients with chronic HF regularly followed at Centro Cardiologico Monzino in Milan and with a previous experience with cardiopulmonary exercise test (CPET). In particular, to be eligible in the present study patients had to meet some inclusion criteria.

Inclusion Criteria:

They had to have severe acute heart failure symptoms (NYHA classes III to IV), multiple episodes of fluid retention and/or peripheral hypoperfusion and objective evidence of severe cardiac dysfunction with a LVEF $\leq 35\%$ at echocardiography. Furthermore, they had severe impairment of functional capacity, history of more than one hospitalizations for heart failure in the past 6 months and all these features had to be present despite optimal medical therapy. Other inclusion criteria were age $\geq 18$ years old, peak$VO_2 \leq 12$ mL/min/Kg, the ability to perform a maximal CPET with a peak respiratory quotient $\geq 1.05$ and standard heart failure therapy. Patients had to be free from both inotropic agents and other intravenous therapies for at least 48 hours prior to study inclusion, except for intravenous diuretics therapy that was allowed. Even though patients were hospitalized because of acute hemodynamic decompensation, at the study enrollment they were returned to a stable clinical condition.

Exclusion Criteria:

Ongoing mechanical ventilation, recent or acute coronary and respiratory syndromes, recent sustained ventricular tachycardia/ventricular fibrillation/cardiopulmonary resuscitation maneuvers, severe aortic, pulmonary or mitral valve disease (or known malfunctioning heart valve), hypertrophic cardiomyopathy, uncorrected thyroid disease, presence of any comorbidities which might *per se* influence exercise performance. In addition, patients with left ventricle assist device as well as those with a pacemaker guided heart rate at rest or during exercise and patients in which levosimendan was not indicated were also excluded from this study.

Patients' eligibility was assessed after 48 to 72 hours of clinical stabilization including medical history, physical examination, and standard echocardiography. All patients provided written informed consent before entering the study and after that, blood samples were taken for both general chemistry and surfactant proteins determination. Afterwards, global spirometry and CPET were performed, while Levosimendan infusion started 3 hours after all these tests had been completed. Levosimendan infusion was not preceded by any bolus administration, but it started at 0.05 μg/Kg/min, progressively increasing up to 0.2 μg/Kg/min, based on patient clinical status, and blood pressure, until the entire infusion of 12.5 mg of the drug in 500 mL in 5% glucose solution had been administered.

## 2.4. Study procedures

As mentioned before, patients in stable clinical conditions who met the inclusion criteria and provided informed consent, were enrolled in the study. Firstly, a blood sample was taken for general chemistry including BNP, hemoglobin, creatinine, sodium levels, and blood urea nitrogen together with a sample for the determination of the surfactant proteins SP-B, SP-A and SP-D. In particular, to determine SP-B, fresh blood was drawn into Vacutainer tubes with citrate 0.129 mol/L as an anticoagulant, then blood was immediately centrifuged at 1500 x g for 10 minutes at 4° C and the obtained plasma was divided into aliquots and frozen at -80° C until assayed. As mentioned before, an ELISA was performed to determine mature SPB levels, while Western Blotting was used to find the immature form levels [77, 80]. After that, expert medical personnel performed standard spirometry where the patients had to breathe in a mouthpiece linked to a spirometer. This exam permitted the evaluation of static pulmonary volumes like vital capacity (VC) and dynamic pulmonary volumes like the forced expiratory volume in 1 second (FEV1) that were both calculated according to the American Thoracic Society criteria using a mass flow sensor (Sensor Medics 2200, Sensor Medics Co., Yorba Linda, CA, USA) [159, 160]. After spirometry, DLCO and its subcomponents Dm and Vc were measured by the single breath method (Sensor Medics 229D, Sensor Medics Yorba Linda, CA) by breathing gas mixtures containing three different O2 concentrations (21%, 40% and 60%) with 0.3% CO and 0.3% methane as described by Roughton and Foster [56]. After these respiratory tests, CPET was performed on a cycloergometer (Sensor Medics Ergo 800S, Sensor Medics, Yorba Linda, CA) using a personalized ramp protocol, which was

calculated on clinical status and previous CPET, in order to achieve peak exercise in 10 minutes [161]. If the test duration was inferior to 7 minutes, the CPET was repeated the following day as well as the blood chemistry measurements. Regarding CPET parameters, expiratory $O_2$, $CO_2$, and ventilation (VE) were measured breath by breath, while peak VO2 was considered as the highest $VO_2$ achieved during the exercise and percentage of predicted peak $VO_2$ was derived from Hansen and Wasserman regression equation [162]. The $VE/VCO_2$ relationship was calculated as the slope of the linear relationship between VE and $VCO_2$ from the beginning of the loaded exercise to the end of the isocapnic buffering period. Twelve-lead electrocardiograms were also continuously recorded (Case 800, Marquette, Milwaukee, WI, USA) and blood pressure was measured during CPET every two minutes, by sphygmomanometer. Once these tests were completed, Levosimendan infusion started and within 24 hours after the drug infusion completion, blood samples, global spirometry, and CPET were repeated. (See Figure 23)

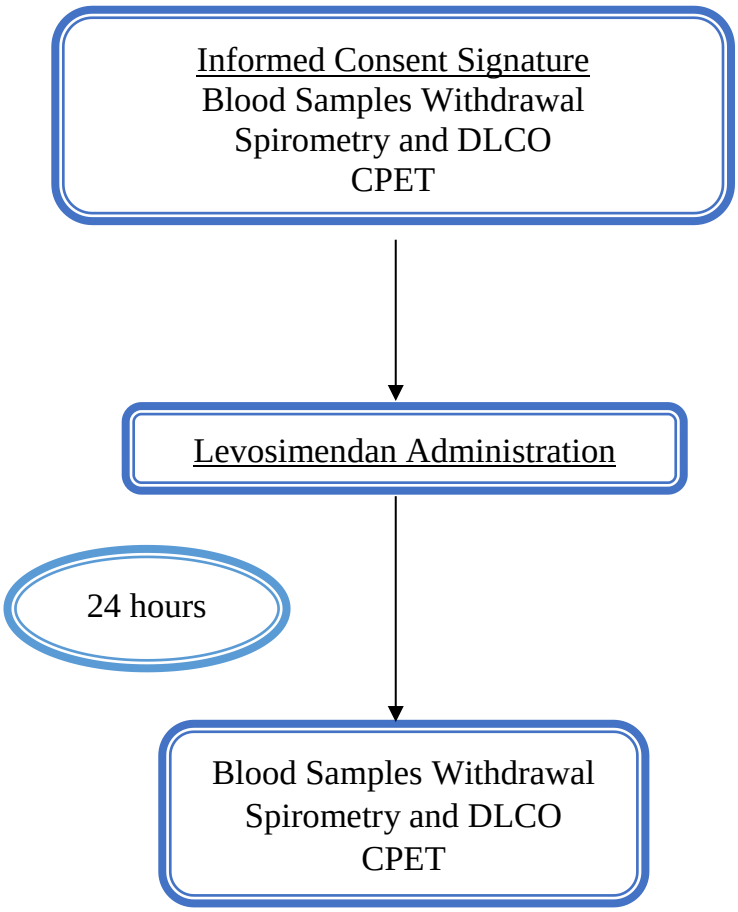



**Figure 23:** Design of the study

# CHAPTER III

# CHRONIC HEART FAILURE: MATERIALS AND METHODS

## 3.1 Background and rationale

Sacubitril/valsartan has emerged as a novel therapy in the treatment of HFrEF, proving superiority to standard therapy in reducing the risk of death from cardiovascular causes or hospitalization for patients with HF [146]. The most recent ECS guidelines for the diagnosis and treatment of HF recommend the replacement of ACE inhibitor or ARB with sacubitril/valsartan in outpatients who remain symptomatic despite optimal treatment [1].

Although its recent widespread use in clinical practice, little is known about the effects of this treatment combination on cardiopulmonary function and short-term functional capacity. Recent studies provided evidence of an improvement in exercise tolerance in the 6-minute walk test in patients with HFrEF treated with sacubitril/valsartan [163, 164]. However, the first study demonstrating a significant increase in peak$VO_2$ is limited by the small sample size and the short follow-up [165]. Therefore, more studies were undertaken to validate these beneficial effects of sacubitril/valsartan on cardiopulmonary parameters and, in February 2019, promising results were published from the largest prospective study to date [166].

Moreover, in a series of small preliminary studies, sacubitril/valsartan has recently demonstrated efficacy in improving LVEF and other echocardiographic left ventricular remodelling parameters [167, 168], besides positive effects on functional status [169], if administered on top of the standard medical therapy.

In conclusion, more evidence is needed to investigate the potential beneficial effects of sacubitril/valsartan on exercise capacity, pulmonary function and left ventricular remodelling in HFrEF and to assess its impact on DLCO in patients on treatment, where only limited data is available.

## 3.2. Study objectives

In the present study we aimed to evaluate changes in exercise capacity, pulmonary function, left ventricular remodelling and NT-proBNP levels and to assess the relative dose-effect relationship in a cohort of patients affected by HFrEF and eligible for treatment with sacubitril/valsartan.

Therefore, the primary endpoint was an increase in the exercise tolerance through the evaluation of the peak$VO_2$ during CPET. Secondary endpoints were an improvement in pulmonary function assessed through spirometry and DLCO, an increase in cardiac performance, lower end-diastolic and end-systolic volumes with a higher LVEF, and a reduction in circulating concentrations of NT-proBNP.

## 3.3. Study population

We enrolled a cohort of outpatients in the Heart Failure Unit of Centro Cardiologico Monzino in Milan, who were eligible for treatment with sacubitril/valsartan in accordance with Italian reimbursement criteria.

Inclusion criteria:

- over 18 years of age;
- symptomatic HF defined as NYHA functional class II or III, despite optimized treatment for HF;
- stable clinical conditions;
- LVEF ≤ 35%, as measured using echocardiography;
- previous treatment with an individual optimal dose of ACE inhibitor or ARB;
- eligible for treatment with sacubitril/valsartan (systolic blood pressure ≥ 100 mmHg, no history of angioedema);
- ability to perform CPET.

Exclusion criteria:

- inability to provide informed consent;
- moderate-to-severe chronic obstructive pulmonary disease (COPD);
- estimated glomerular filtration rate (eGFR) < 30 mL/min/1.73m$^2$;
- inability or contraindication to perform CPET;
- chronic oxygen therapy;
- recent major cardiovascular treatment;
- cardiac resynchronization therapy defibrillator (CRT-D) implantation or revascularization procedure (coronary artery bypass/percutaneous coronary intervention) planned during the study period.

## 3.4. Study design

This prospective, observational study was approved by the Centro Cardiologico Monzino, IRCCS Ethical Committee (CCM-898) and complies with the principles of the Declaration of Helsinki and the Good Clinical Practice standards. All participants provided written informed consent.

The target number of participants expected for this study is 100 patients, enrolled and prospectively followed in four Italian cardiology centers. Centro Cardiologico Monzino, as the study coordinator site, enrolled the biggest number of patients. In this interim analysis, we only considered data collected up to June 2019 at this site.

At enrollment, medical history and baseline characteristics of the patients were recorded before replacing ACE inhibitor or ARB with sacubitril/valsartan. Initially, patients were prescribed the lower dose of sacubitril/valsartan (24/26 mg twice daily) for four weeks, in order to assess tolerability. In case of previous ACE inhibitor therapy, patients were instructed to discontinue this treatment for at least 36 hours before introducing sacubitril/valsartan, to reduce the risk of angioedema. Patients were reviewed every four weeks for three times, in order to re-evaluate the treatment and, if possible, gradually titrate the dose up to the maximal tolerated dose or the optimal dose of 97/103 mg twice daily, as per the ESC guidelines [1].

Once the maximal tolerated dose was reached, a final follow-up appointment was scheduled five months later.

## 3.5. Study procedures

| | Baseline | 1 month (24/26mg) | 2 months (49/51mg) | 3 months (97/103mg) | 6 months (max dose) |
|---|---|---|---|---|---|
| **Clinical assessment** | X | X | X | X | X |
| **ECG** | X | X | X | X | X |
| **Blood venous sample collection** | X | X | X | X | X |
| **PFT** | X | X | X | X | X |
| **Maximal CPET** | X | X | X | X | X |
| **QoL (KCCQ)** | X | X | X | X | X |
| **Echocardiography** | X | X | | | X |
| **NYHA classification** | X | X | X | X | X |
| **SPB** | X | | | | X |
| **DLCO** | X | | | | X |

**Table 2.** Study procedures flowchart

At each appointment, after the physical examination, laboratory analyses were performed for general chemistry including hemoglobin, creatinine, eGFR, sodium and potassium levels, and NT-proBNP. A plasma sample was also collected at baseline and end of study visits to dose the circulating concentrations of surfactant protein type B (SPB), a strong prognostic marker of alveolar-capillary membrane damage. The plasma samples, divided into aliquots, are currently stored at -80° C in our local laboratories, and will be assayed only once the study has ended, therefore these data are not currently available.

Subsequently, expert medical personnel performed a standard spirometry, inviting patients to breathe in a mouthpiece linked to a spirometer for the evaluation of static and dynamic pulmonary volumes, calculated according to the American Thoracic Society criteria using a

mass flow sensor [159, 160]. During the first and last visits, DLCO was also measured by the single breath method with a gas mixture of $O_2$, CO and helium.

A CPET was then performed on an electronically braked cycle ergometer (Vmax 229D Sensormedics and Quark PFT, Cosmed) using a personalized ramp protocol, which was calculated on clinical status and reported exercise tolerance, in order to reach peak exercise in 10±2 minutes [161]. The ramp protocol was set for each patient at the baseline visit and applied unchanged to the following CPETs. In the absence of clinical events, tests were stopped as patients reported maximal effort. The patients wore a mask to measure ventilation and respiratory gases breath by breath. Heart rate and 12-lead ECG were monitored continuously, haemoglobin saturation recorded by an oxymeter, and blood pressure measured with a cuff sphygmomanometer at rest and every two minutes throughout the test. Peak$VO_2$ was calculated as the 20 seconds average of the highest $VO_2$ recorded, while the $VE/VCO_2$ slope was calculated as the slope of the linear relationship between VE and $VCO_2$ from 1 minute after the beginning of the loaded exercise and the end of the isocapnic buffering period [170]. Percent predicted peak$VO_2$ was derived from Hansen and Wasserman regression equation as *(height - age) x 20* for men and *(height - age) x 14* for women, with height expressed in cm and age in years [171]. The anaerobic threshold was measured by V-slope analysis of $VO_2$ and $VCO_2$, and confirmed by ventilatory equivalents and end-tidal pressures of $CO_2$ and $O_2$ [172]. $VO_2$/work relationship was measured through the entire exercise protocol. Other data are reported as 20 seconds average.

The patients were also asked to complete the Kansas City Cardiomyopathy Questionnaire (KCCQ) to evaluate their quality of life.

At baseline and during the last follow-up appointment expert medical personnel performed an echocardiogram to noninvasively provide measures of ventricular function and detect the hemodynamic and morphologic changes in HF over time. The assessment was also repeated at the low dose control visit in a subgroup of patients.

# CHAPTER IV

## ACUTE HEART FAILURE: RESULTS

In the present study, 65 patients who fulfilled the study inclusion criteria, were enrolled, and completed the study without unwanted side effects. The characteristics of the study group are described in Table 3 from which it is clear that most of the patients were males and the mean age was 70 ± 9 years. Furthermore, it is shown that ischemic heart disease was the origin of heart failure only in 40% of cases, while the remaining 60% had a primitive cardiomyopathy. In *Table 3*, some important parameters taken from standard echocardiography are showed too, such as left ventricle ejection fraction, left ventricle end-diastolic volume and systolic volume and pulmonary systolic pressure.

| Characteristic | Value |
|---|---|
| Male [n(%)] | 57 (88) |
| Age (years) | 70 ± 8,99 |
| Ischaemic cardiomyopathy [n(%)] | 25 (38,5%) |
| Left ventricle ejection fraction (%) | 25 ± 7,23 |
| Telediastolic volume (mL) | 213 ± 74,18 |
| Telesystolic volume (mL) | 162 ± 66,03 |
| Pulmonary artery systolic pressure (mmHg) | 44 ± 13,30 |

**Table 3:** Characteristics of the study group

Before and after levosimendan infusion, blood samples were taken to assess general chemistry and, in particular, to evaluate the circulating BNP levels and kidney function. As it is showed in *Table 4*, the drug infusion was associated with unchanged renal function and a significant BNP reduction (See figure 24).

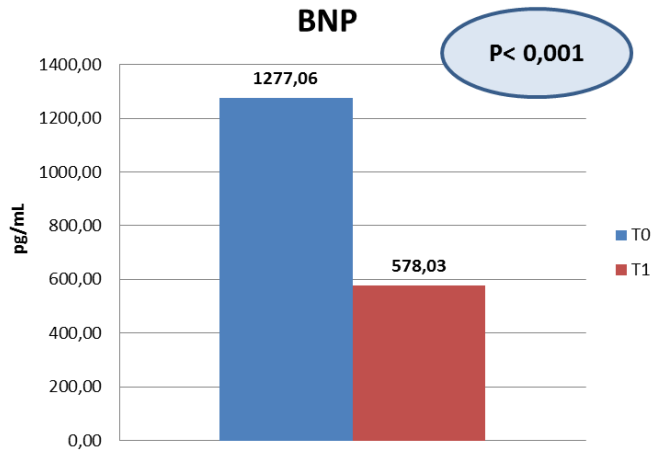



**Figure 24:** BNP levels before and after levosimendan infusion

Standard spirometry before levosimendan administration showed reduced FEV1 and VC with a decreased DLCO due to a low membrane diffusion that was partially compensated by an increase in capillary volume (Vcap). Regarding spirometry parameters (Table 4), the inotropic agent caused an increase in FEV1 (from 2.09±0.56 to 2.26±0.63, p<0.001), VC (from 2.87±0.74 to 3.09±0.83, p<0.001) and alveolar volume, while DLCO (see figure 25) and its subcomponents remained unchanged.

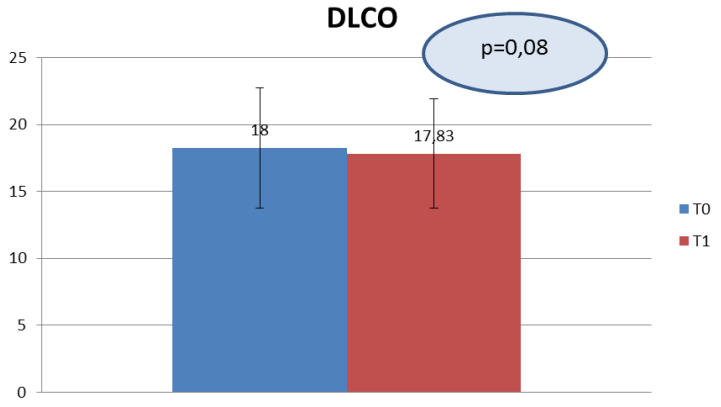



**Figure 25:** DLCO changes before and after levosimendan infusion

Afterwards, CPET was performed in all patients, but in three cases exercise performance was extremely limited so that the test was judged not evaluable. In general, baseline peak $VO_2$ was severely reduced with a decrease in peak workload, oxygen pulse and the $VO_2$/work relationship; on the other hand, $VE/VCO_2$ slope was higher than normal values (42±9.86). During CPET, exercise-induced periodic breathing was observed in 32 cases (54% of the study group) and it was defined as a cyclic fluctuation of ventilation (VE) during exercise

[173]. As it is demonstrated in Table 4, levosimendan infusion caused a significant increase in peak $VO_2$ (from 10.1±2.36 to 11.50±2.30, p<0.001) (see figure 26), together with reduction in $VE/VCO_2$ slope (see figure 27) and general improvement of all the measured CPET parameters. Moreover, periodic breathing disappeared in 9 out of 32 cases, while in patients with this respiratory pattern at baseline, its length was reduced from 71% ± 25 to 38% ± 33 and none of the patients enrolled developed periodic breathing after the infusion. This reduction in periodic breathing may be due to the positive inodilator effect of levosimendan that causes an increase in cardiac output and general hemodynamic improvement or to an action on the respiratory center [174].

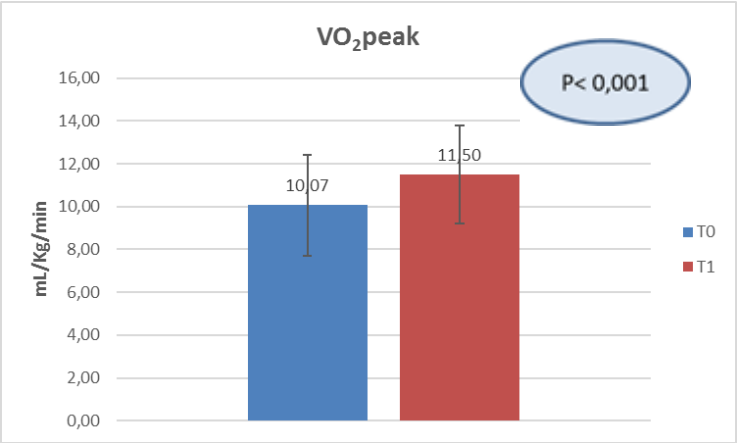



**Figure 26**: Peak$VO_2$ before and after levosimendan infusion

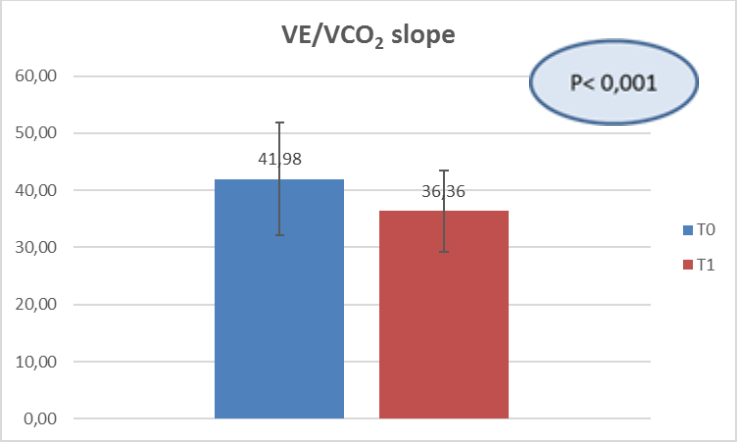



**Figure 27:** $VE/VCO_2$ slope before and after levosimendan infusion

| | ***Levosimendan*** | | |
|---|---|---|---|
| ***Parameter*** | ***Pre-infusion*** | ***Post-infusion*** | ***P- value*** |
| **<u>Blood chemistry</u>** | | | |
| **BNP *(pg/mL)*** | 954 (540-1736) | 370 (165-874) | <0.001 |
| **BUN *(mg/dL)*** | 69 (56.0-94.5) | 70 (49.0-98.0) | 0.59 |
| **Creatinine *(mg/dL)*** | 1.46 (1.16-1.80) | 1.40 (1.09-1.93) | 0.12 |
| **<u>Spirometry</u>** | | | |
| **VC (*L*)** | 2.87±0.74 | 3.09±0.83 | <0.001 |
| **FEV1 *(L)*** | 2.09±0.56 | 2.26±0.63 | <0.001 |
| **DLCO *(mL/mmHg/min)*** | 18±4 | 17.93±4.07 | 0.17 |
| **Vcap *(L)*** | 113.7 (85.5-177.0) | 120.5 (103.7-171.0) | 0.79 |
| **VA *(L)*** | 4.67±1.18 | 4.84±1.17 | 0.01 |
| **DM *(mL/min/mmHg)*** | 24±9.03 | 22.49±6.09 | 0.07 |
| **<u>Cardiopulmonary exercise test</u>** | | | |
| **Peak $VO_2$ *(L/min)*** | 0.74±0.23 | 0.84±0.22 | <0.001 |
| **Peak $VO_2$ *(mL/Kg/min)*** | 10.1±2.36 | 11.50±2.30 | <0.001 |
| **Peak VO2 (% pred)** | 44±12.40 | 49.63±11.09 | <0.001 |
| **Peak workload *(watt)*** | 42.5 (31.7-57) | 49.5 (38.7-61.0) | <0.001 |
| **Peak $O_2$ pulse *(mL/beat)*** | 8.2±2.64 | 8.80±2.65 | 0.002 |
| **$VO_2$/workload slope *(mL/min/W)*** | 9.0±1.87 | 9.64±1.51 | 0.001 |
| **VE/$VCO_2$ slope** | 42±9.86 | 36.36±7.13 | <0.001 |

*VC= Vital Capacity; FEV1= Forced Expiratory Volume in 1 second; DLCO= Diffusing capacity of the Lung for Carbon Monoxide; Vcap= Capillary Volume; VA= Alveolar Volume; DM= Membrane Diffusing capacity; peakVO2= Oxygen consumption at peak of exercise; Peak O2 Pulse= O2 pulse at peak; VO2/workload slope= Oxygen consumption related to workload; VE/VCO2 slope= minute ventilation vs CO2 production*

**Table 4**: Laboratory, spirometry and CPET parameters before and after levosimendan infusion

All surfactant proteins showed significant but different changes after levosimendan infusion. In particular, SP-A, SP-D and the immature form of SP-B (see figure 28) decreased (from 39.4±18.7 to 34.4±17.9, p< 0.001), while the mature form of SP-B (see figure 29) increased (from 424.3±218.1 to 460.7±243.0, p<0.001), as it is showed in Table 5.

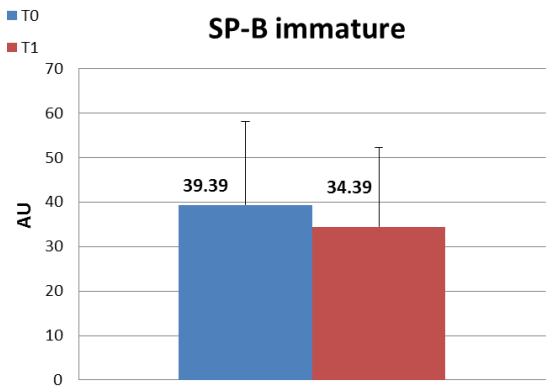



**Figure 28:** Immature SP-B levels before and after Levosimedan infusion

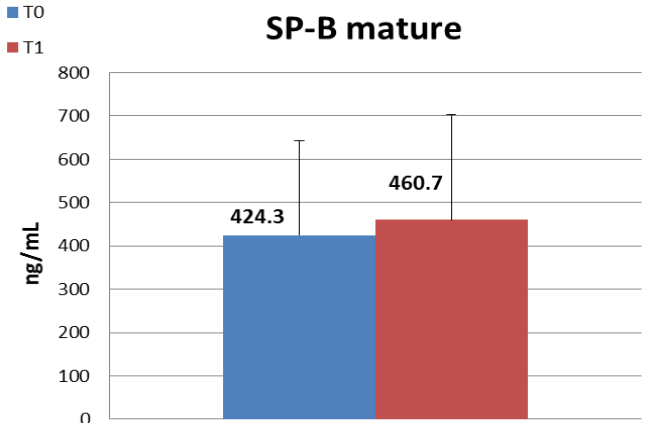



**Figure 29:** Mature SP-B levels before and after Levosimendan infusion

| | ***Levosimendan*** | | |
|---|---|---|---|
| ***Parameter*** | ***Pre-infusion*** | ***Post-infusion*** | ***P- value*** |
| **SP-A *(ng/mL)*** | 73.7±25.3 | 66.3±22.7 | <0.001 |
| **SP-D *(ng/mL)*** | 246.8±121.3 | 223.0±109.6 | <0.001 |
| **SP-B immature *(ng/mL)*** | 39.4±18.7 | 34.4±17.9 | <0.001 |
| **SP-B mature *(ng/mL)*** | 424.3±218.1 | 460.7±243.0 | <0.001 |

*SP-A= Surfactant protein type A; SP-D= Surfactant protein type D; SP-B immature= immature form of surfactant protein type B; SP-B mature= mature form of surfactant protein type B*

**Table 5:** Surfactant proteins changes

# CHAPTER V

## CHRONIC HEART FAILURE: RESULTS

A total of fifty-three patients were enrolled at Centro Cardiologico Monzino in Milan from July 2018 to June 2019. The baseline characteristics of the entire population are reported in table 6. In the entire population the mean age was 66.6±9.2 years, 77% were males and 49% had ischemic heart disease. At enrollment all patients were on optimized medical treatment for HF, as reported in table 7: 38 patients (72%) were treated with an ACE inhibitor and 15 (28%) with an ARB. Medical history and cardiovascular risk factors are reported in figure 28.

During the course of the study, only three patients (0.06%) discontinued treatment due to hypotension, worsening of renal function and referred intolerance. Moreover, three patients interrupted the study participation because of death, CRT-D implantation and terminal cancer.

| | **Mean ± SD**<br>***n (%)*** | |
|---|---|---|
| **Age** | 66,6 | ± 9,2 |
| **Gender (females)** | *12* | *(23%)* |
| **Systolic blood pressure (mmHg)** | 112,0 | ± 16,4 |
| **Diastolic blood pressure (mmHg)** | 71,0 | ± 9,3 |
| **Heart rate (bpm)** | 67,0 | ± 11,6 |
| **Body max index (Kg/m2)** | 26,9 | ± 4,0 |
| **NYHA class II** | *43* | *(81%)* |
| **NYHA class III** | *10* | *(19%)* |
| **Ischemic Etiology** | *26* | *(49%)* |
| **MECKI score (%)** | *4,7* | *(2,2-8,6)** |

*expressed ad median (IQR)

**Table 6:** Population characteristics (n=53)

| | n | (%) |
|---|---|---|
| **ACE inhibitor** | 38 | (72%) |
| **ARB** | 15 | (28%) |
| **Beta-blocker** | 52 | (98%) |
| **Mineralcorticoid antagonist** | 31 | (58%) |
| **Diuretic** | 44 | (83%) |
| **Ivabradine** | 5 | (9%) |
| **Digoxin** | 3 | (6%) |
| **Implantable cardioverter defibrillator** | 19 | (36%) |
| **Cardiac resynchronization therapy** | 13 | (25%) |

**Table 7:** Baseline HF medical therapy (n=53)

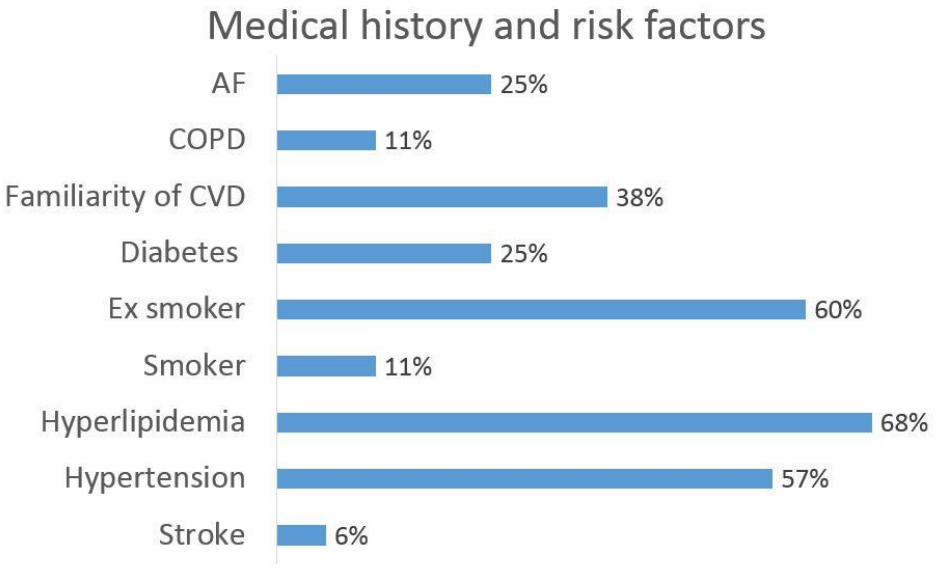



**Figure 30:** Medical history and risk factors of the entire study population (n=53)

For the purpose of this interim analysis, among the patients still on treatment, we only considered those who had been treated with sacubitril/valsartan for at three months and that we tried to uptitrate to the maximum dose. Therefore, only twenty-five patients were examined in this preliminary study.

At a mean follow-up period of 169±74 days, 92% of these patients were on sacubitril/valsartan 97/103 mg, while one subject was on the low strength dose and another on 49/51 mg because of hypotension at higher doses.

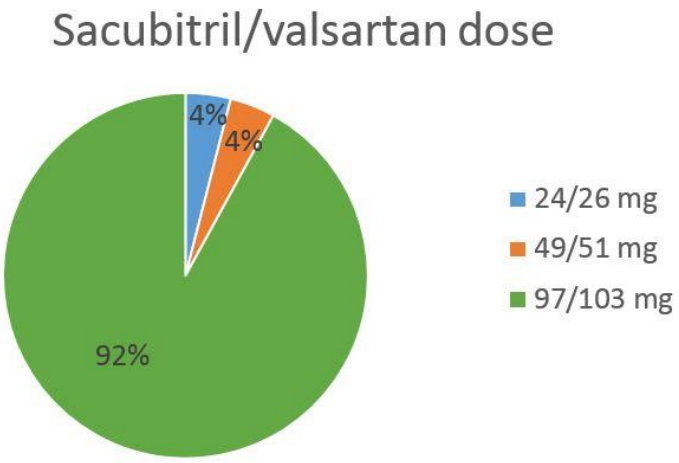



**Figure 31:** Sacubitril/valsartan dose at follow-up (n=25)

By comparing the baseline analysis with the follow-up assessment, we observed a non-statistically significant decrease in systolic and diastolic blood pressure (respectively, 112±15 vs. 109±18 mmHg, p=ns; 73±8 vs. 70±10 mmHg, p=ns) and a stability of renal function (eGFR 67.5±17.3 vs. 65.0±19.2 ml/min/1.73m$^2$; p=ns) and potassium levels (4.40±0.35 vs. 4.48±0.41 mmol/l, p=ns). Plasma NT-proBNP concentrations, on the other hand, decreased significantly (p=0.004) from a median 1067 (IQR 648-2547) to 1066 (391-1255) pg/ml, as displayed in the box-plot (see Figure 32).

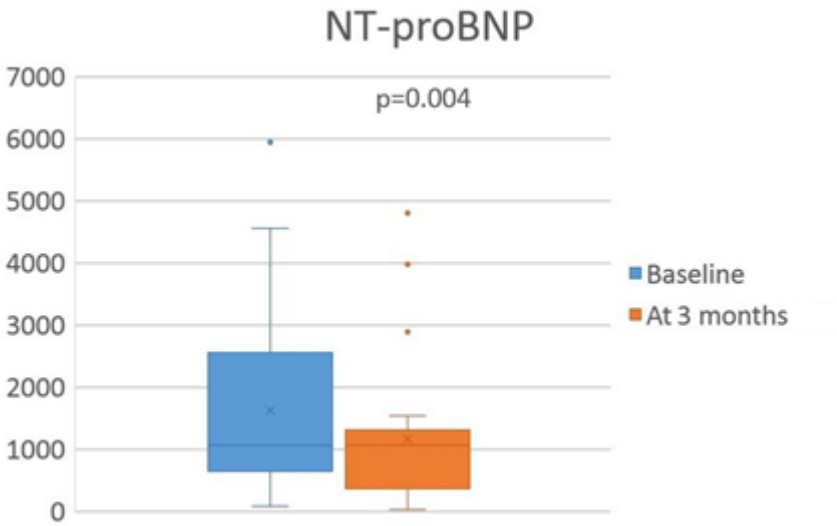



**Figure 32:** NT-proBNP concentrations at baseline and follow-up (n=25)

As per the cardiac performance, the echocardiographic assessments performed in thirteen patients showed a significant increase in LVEF (31.0±5.4 vs. 37.2±9.6 %; p=0.009) (see figure 33), along with a reduction of both left ventricular end-diastolic and end-systolic volumes (respectively, 116.8±31.4 vs. 90.5±21.3 Δ=-26.2 ml, p=0.011; 80.9±24.5 vs. 58.2±21.4, Δ=-22.8 ml, p=0.004) as represented in Figure 34.

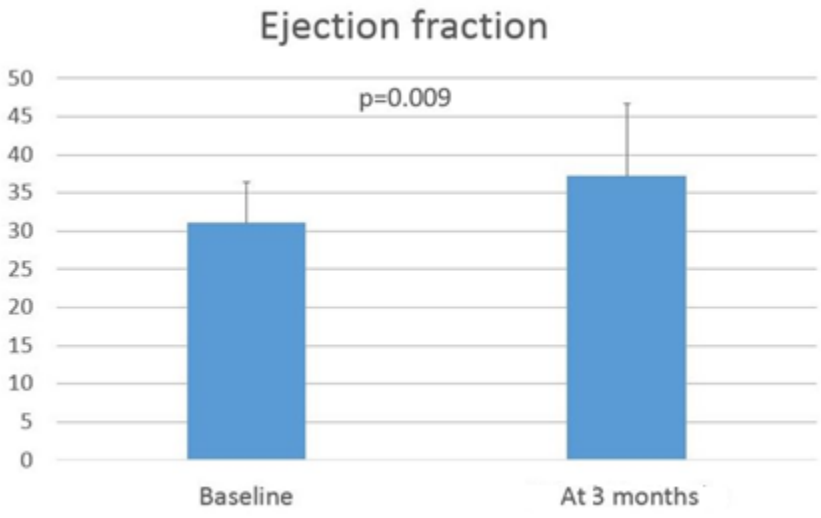



**Figure 33:** LVEF comparison (n=13)

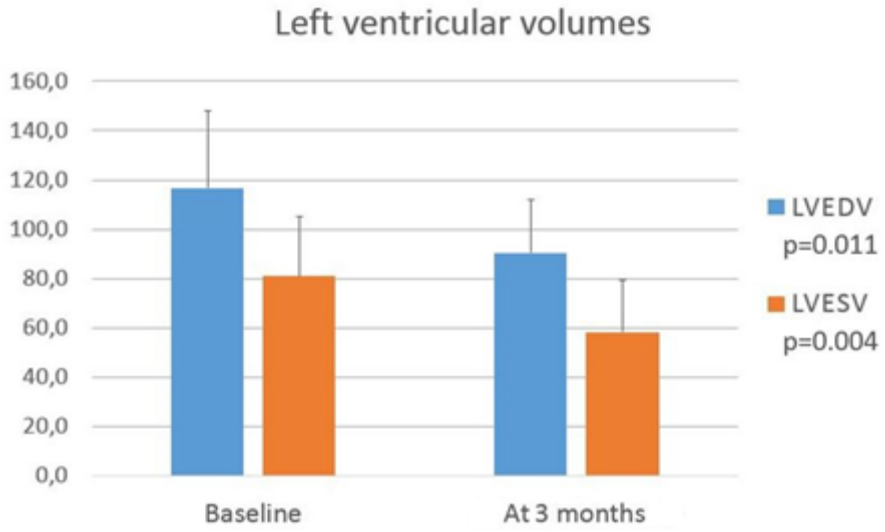



**Figure 34**: Left ventricular end-diastolic and end-systolic volumes (respectively, LVEDV and LVESV) at baseline and after at three months of treatment (n=13)

Regarding exercise capacity, the cardiorespiratory analysis performed on twenty-three patients before starting sacubitril/valsartan treatment showed a mean peak$VO_2$ of 16.0±3.9 ml/kg/min, corresponding to a mean 63% of the predicted value, with a VE/$VCO_2$ slope of 30,8±4,5. These results document the HF severity in our study group.

The comparison with follow-up CPETs showed an improvement in exercise tolerance represented by a significant increase in the $VO_2$ uptake at peak exercise: Δ=+115.2 ml (p=0.001), Δ=+1.25 ml/kg/min (p=0.006), Δ= +6.9% of predicted (p=0.002) (see figure 35). The workload reached at maximal exercise also augmented (97.0±39.3 vs. 103.7±39.7watt, p=0.001). In addition, AT-$VO_2$ increased from 881.2±278.8 to 1056.0±350.8, Δ=+203.2 ml/min, p=0.012.

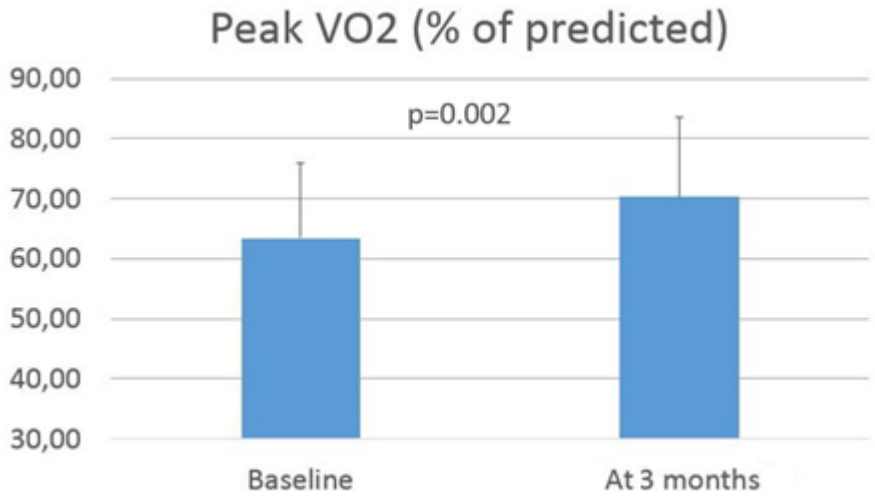



**Figure 35.** Percent of predicted peak$VO_2$ improvement at follow-up (n=23)

On the other hand, ventilation (VE) and ventilatory efficiency during exercise (VE/$VCO_2$) did not show a significant enhancement during the follow up assessment.

Values of percentage of predicted peak$VO_2$ and VE/$VCO_2$ slope were combined with laboratory (hemoglobin, sodium and kidney function by means of MDRD) and echocardiographic (LVEF) results to calculate MECKI score for the thirteen patients for

whom all six values were available. The median MECKI score was 3.0% (IQR 1.7-6.3) at baseline and significantly decreased to 1.8% (0.8-3.6) at follow-up (p=0.009).

A significant improvement in the NYHA functional class was also observed (p=0.004). In fact, at baseline, 84% of patients were in NYHA class II and 16% in class III; at the follow-up physical examination none of the subjects were in NYHA class III and 24% improved to a NYHA class I (see figure 36).


**Figure 36.** NYHA class at baseline and after at three months of sacubitril/valsartan treatment (n=25).

Finally, in pulmonary function tests, significant increases in vital capacity (VC, 3.63±1.03 vs. 3.82±0.86 l, p=0.002) and peak expiratory flow (PEF, 7.46±2.44 vs. 7.91±2.19 l/s, p=0.043) were observed, while DLCO remained unchanged (n=12, 20.56±5.48 vs. 21.01±5.89 ml/min/mmHg; p=ns) (see figure 37).

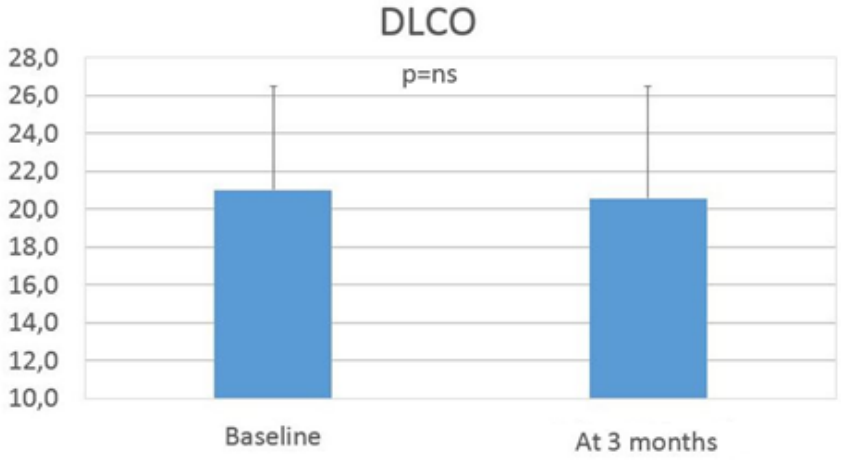



**Figure 37:** DLCO at baseline and follow-up comparison (n=12)

All the results are summarized in table 8.

| Variable | Baseline mean ± SD *n (%)* | Follow-up mean ± SD *n (%)* | p-value |
|---|---|---|---|
| ***Physical status and laboratory analysis §*** | | | |
| **SBP (mmHg)** | 112 ± 15 | 109 ± 18 | 0,509 |
| **DBP (mmHg)** | 73 ± 8 | 70 ± 10 | 0,183 |
| **eGFR (ml/min/1.73m$^2$)** | 67,5 ± 17,3 | 65,0 ± 19,2 | 0,391 |
| **$K^+$ (mmol/l)** | 4,40 ± 0,35 | 4,48 ± 0,41 | 0,421 |
| **NT-pro-BNP (pg/ml)** | 1067 (648-2547)* | 1066 (391-1255)* | 0,004 |
| ***Echocardiographic parameters*** □ | | | |
| **LVEF (%)** | 31,0 ± 5,4 | 37,2 ± 9,6 | 0,009 |
| **LVEDV (ml)** | 116,8 ± 31,4 | 90,5 ± 21,3 | 0,011 |
| **LVESV (ml)** | 80,9 ± 24,5 | 58,2 ± 21,4 | 0,004 |
| ***CPET parameters #*** | | | |
| **Peak$VO_2$ (ml)** | 1282,7 ± 403,0 | 1397,9 ± 408,3 | 0,001 |
| **Peak $VO_2$ (ml/kg/min)** | 16,0 ± 3,9 | 17,3 ± 3,7 | 0,006 |
| **Peak$VO_2$ (% pred)** | 63,4 ± 12,5 | 70,3 ± 13,3 | 0,002 |
| **Peak workload (watt)** | 97,0 ± 39,3 | 103,7 ± 39,7 | 0,001 |
| **AT-$VO_2$ (ml/min)** | 881,2 ± 278,8 | 1056,0 ± 350,8 | 0,012 |
| **VE/$VCO_2$ slope** | 30,8 ± 4,5 | 32,4 ± 5,7 | 0,181 |
| ***Functional status*** | | | |
| **NYHA class I** | *0* | *6 (24%)* | 0,004 |
| **NYHA class II** | *21 (84%)* | *19 (76%)* | 0,004 |
| **NYHA class III** | *4 (16%)* | *0* | 0,004 |
| **NYHA class IV** | *0* | *0* | 0,004 |
| ***Pulmonary function parameters*** □ | | | |
| **VC (l)** | 3,63 ± 1,03 | 3,82 ± 0,86 | 0,002 |
| **PEF (l/s)** | 7,46 ± 2,44 | 7,91 ± 2,19 | 0,043 |
| **DLCO (ml/min/mmHg)** | 20,56 ± 5,48 | 21,01 ± 5,89 | 0,688 |
| ***MECKI score (%) $*** | 3,0 (1,7-6,3)* | 1,8 (0,8-3,6)* | 0,009 |

** expressed as median (IQR); § Clinical characteristics of the study population (n=25); □ Echocardiographic assessment performed in 13 patients; # Cardiopulmonary exercise testing performed by 23 patients; □ Standard pulmonary function testing for 25 patients and DLCO for 12 subjects; $ Values*

*required for the MECKI score calculation were available for 13 patients*

**Table 8:** Comparison of baseline and follow-up parameters

*SBP*, systolic blood pressure; *DBP*, diastolic blood pressure; *eGFR*, estimated glomerular filtration rate; $K^+$, serum potassium; *NT-proBNP*, N-terminal pro-hormone BNP; *LVEF*, left ventricular ejection fraction; *LVEDV*, left ventricular end-diastolic volume; *LVESV*, left ventricular end-systolic volume; $VO_2$, oxygen consumption; *AT-*$VO_2$, oxygen consumption at anaerobic threshold; *VE/*$VCO_2$, minute ventilation-carbon dioxide production; *NYHA*, New York Heart Association; *VC*, vital capacity; *PEF*, peak expiratory flow; *DLCO*, lung diffusion of carbon dioxide; MECKI, Metabolic Exercise test data combined with Cardiac and Kidney Indexes

Of the twenty-five patients considered in this study, twelve (48%) have reached the end of the study. The comparison of baseline characteristics and follow-up assessment in these subjects evidenced a statistically significant, but not clinically relevant, decrease in systolic blood pressure (112.0±16.4 vs. 102.9±17.1 mmHg, p=0.040) and a slight, however not significant, improvement in VE/$VCO_2$ slope (34.0±7.3 vs. 30.9±4.9, p=0.058). The most relevant findings previously displayed have been confirmed in this subgroup. Nevertheless, the smaller sample size negatively affected the significance, in particular for highly variable parameters such as NT-proBNP concentrations.

# CHAPTER VI

## ACUTE HEART FAILURE: DISCUSSION

The effects of a single levosimendan infusion on laboratory, spirometry, DLCO and CPET parameters together with surfactant proteins levels were evaluate in patients with advanced chronic heart failure. The study group was a population with ACHF who had a very recent hemodynamic decompensation, although they were returned to a stable clinical condition in order to be enrolled. The idea for this study came from the results obtained in a previous double-blind placebo controlled study, where a single levosimendan infusion was associated with exercise performance improvement and BNP reduction [158]. Therefore, the present study wants to assess the effects of these hemodynamic improvements on DLCO and surfactant proteins. As in the previous study [158], CPET was performed before and after the infusion and it showed a significant increase in peak $VO_2$ and a significant decrease in $VE/VCO_2$ slope, while blood samples analysis showed a > 50% reduction of BNP. Peak $VO_2$, $VE/VCO_2$ slope and BNP are all parameters associated with heart failure severity and prognosis, so their improvement is very important for the patients and, in addition, peak$VO_2$ is seen as a marker to assess the efficacy of a therapy and it is a criterion to include, exclude or withdraw a patient from a heart-transplant list. Indeed, peak $VO_2$ had to be $\leq$ 12 mL/Kg/min for the patient to be enrolled in this study because this value is the cut-off used to assess patients who are candidates for heart transplantation if they are treated with β-blockers [175, 176]. Besides CPET parameters, standard spirometry measurements improved, too and this amelioration could be related to lung fluid reduction, which is frequently the cause of lung mechanical improvement [177-179]. In addition, acute $VE/VCO_2$ changes are directly linked to lung fluid changes, so that a rise in lung fluid contributes to exaggerate the ventilatory response to exercise increasing $VE/VCO_2$ slope [180-182]. Therefore, BNP, spirometry and CPET parameters changes suggest an hemodynamic improvement and reduction of previously increased lung fluid; however, DLCO did not change albeit alveolar volume increased still thanks to lung fluid reduction. The lack of DLCO improvement was not an unexpected event because in a similar population the use of ultrafiltration, a technique that generates a rapid lung fluid reduction, does not affect DLCO which remains unchanged despite pulmonary mechanics and hemodynamic improvement both at rest and during

exercise [177, 183-185]. Indeed, as mentioned before, in chronic heart failure the alveolar-capillary membrane dysfunction is associated with interstitial fibrosis, local thrombosis and an increase in cellularity on top of lung fluid increase causing a reduced DLCO. Moreover, long term treatment with drugs which affect pulmonary hemodynamic is associated with improvement or worsening of DLCO; for example the use of ACE-inhibitors and mineralcorticoid receptor antagonists has a positive effect on DLCO because of the increase in bradykinin levels and an antifibrotic action respectively. On the contrary, the use of unselective β-blockers has a negative effect on DLCO because of the alveolar β2 receptor blockage [60, 61, 186].

Regarding surfactant proteins levels, there were discordant changes after levosimendan infusion. Indeed, SP-A and SP-D were reduced after drug administration and, having both a widespread multi-organ production and being involved in inflammatory responses, their decrease may be linked with the anti-inflammatory effect of levosimendan [126, 187] As for the reduction of the immature form of SP-B, it indicates a reduction of the hemodynamic stress on the alveolar-capillary membrane because an acute hemodynamic and/or respiratory stress on the membrane is known to increase SP-B plasma levels [74, 79, 188]. Among the SP-B isoforms, the immature one is the most unlikely to be found in the blood stream, so it is a clear indicator of alveolar cell stress, dysfunction if not death and, before the infusion, its higher presence in the blood stream could mean a dysfunction of alveolar cells that are not able to carry out SP-B processing. On the other hand, the increased levels of the mature form of SP-B in the blood after the infusion may suggest a restoration of alveolar cell function with an overproduction of SP-B, which reaches the blood stream once its intracellular katabolic process is terminated [74]. However, the present interpretation of these results is highly speculative, but has relevant functional meaning and what is clear from this study is that surfactant proteins, mostly SP-B, are fast responder markers of the alveolar-capillary membrane function.

It must be said that the present study has several limitations. First of all, the interpretation of the results regarding surfactant proteins is highly speculative with almost no experimental evidence; secondly, only short term, but not long term results of effects of levosimendan or of prolonged hemodynamic improvement by any cause are known. Indeed, multiple blood samples for surfactant proteins determination would have been desirable in order to assess

changes of SPs and DLCO with time in case of prolonged heart failure improvement. Thirdly, the effects of possible confounders like concomitant heart failure treatment or comorbidities are unknown just like what happens on the other side of the alveolar-capillary membrane with the likelihood of surfactant proteins being elevated in the alveolar fluid of HF patients. However, analyzing multiple samples of alveolar fluids was considered inconvenient, if not unethical. Another thing that remains unknown is what happens about DLCO and SPs changes in case of acute heart failure without pre-existing alveolar-capillary damage. Finally, it must be said that levosimendan was not placebo controlled, but experts in a blind fashion performed CPET readings a posteriori and the comparison between placebo and levosimendan about CPET and BNP had already been done in the previous study [158].

# CHAPTER VII

## CHRONIC HEART FAILURE: DISCUSSION

In the present interim analysis, we evaluated the effects of sacubitril/valsartan, administered for at three months, on NT-proBNP plasma concentrations, pulmonary function tests, CPET and echocardiography in outpatients with HFrEF. In particular, the study population consisted of a limited number (n=25) of patients with reduced LVEF, recruited and monitored at Centro Cardiologico Monzino in Milan for a mean of 169±74 days. Despite the small sample size, this interim analysis demonstrated that sacubitril/valsartan has beneficial effects on exercise tolerance and left ventricular remodelling. In particular, after a minimum of three months of treatment, we observed a significant improvement in most of the prognostically relevant CPET parameters, in cardiac performance and circulating HF biomarker levels. Regarding cardiac performance, the significant reduction in left ventricular end-diastolic and end-systolic volumes, as well as the improvement in LVEF, could be the result of the cellular effects of NEP inhibition or a reflection of hemodynamic changes. A reduction in afterload, as a consequence of the lower systolic blood pressure, and the natriuretic and diuretic effects of sacubitril/valsartan which reduce preload, are both beneficial in the failing heart, allowing the heart to operate on the steeper part of the Frank-Starling curve, positively influencing stroke volume. Earlier this year, the largest prospective study on serial CPETs to date, performed after switching patients to sacubitril/valsartan, has provided evidence of significant improvements in peak$VO_2$ and ventilatory efficiency with a positive impact on HF prognosis [166]. In this study, the decrease in $VE/VCO_2$ slope reached a statistical significance only at the 12-month follow-up and in sicker patients with a baseline $VE/VCO_2 > 34$, while healthier patients only improved oxygen consumption [166]. Compared to the results observed by Vitale *et al*., our interim analysis did not confirm the enhancement in ventilatory efficiency, probably because of the smaller sample size and the shorter follow-up period. Another significant difference between these two studies is the fact that Vitale *et al*. encouraged their patients to have a minimum of 2.5 hours a week of moderate intensity anaerobic exercise, while our study participants continued their normal physical activities. Nevertheless, despite the limited population of our interim analysis, the significant improvement in peak$VO_2$ and oxygen consumption at the anaerobic threshold shown in our study is a promising result and

has led to a positive outcome in terms of HF prognosis. The standard spirometry measurements also improved, probably thanks to the lung fluid reduction. However, we did not observe an enhancement in DLCO, possibly because this assessment was only performed at the baseline and final visit, which, at the moment, has been only reached by twelve patients – an even smaller sample size which could be the cause of the limited significance. All these positive evidences could be associated with the functional status improvement represented by the reduction in NYHA class and the decreased circulating NT-proBNP levels observed after initiation of sacubitril/valsartan. Moreover, the significant improvement in MECKI score indicates a lower two-year risk of cardiovascular death, urgent heart transplantation or left ventricular assist device (LVAD) implantation compared to baseline. This result attests the potential of sacubitril/valsartan to enhance the poor HF prognosis and prolonging survival, consistently with the PARADIGM-HF outcomes [146]. Lastly, during the course of the study, the administration of sacubitril/valsartan was not associated with important safety concerns: only few patients (0.06%) interrupted treatment because of adverse events and none of the subjects experienced severe renal impairment, hyperkalemia or angioedema. Furthermore, 92% of our study population reached the maximum recommended dose, probably thanks to the gradual up titration regimen. There are several limitations that need to be addressed in regard to this analysis. Firstly, this represents only an interim analysis performed on some of the parameters collected for the purpose of a larger prospective study that is still underway. The decision to include in this evaluation only patients treated with sacubitril/valsartan for at three months further limited the sample size and did not allow the sub-analysis on the dose-response relationship, which will be the aim for the final study. The multicentric recruitment will also represent a strength of the finished study, allowing a better representation of the real-life population. In addition, the examination of the quality of life questionnaires and the assay of the plasma samples for surfactant protein type B (SPB) were not performed for this preliminary analysis and would provide further information. These initial results only represent a promising starting point, worthy of further analysis, in particular for those variables that did not show significance. Moreover, we should take into account the fact that the improvement observed in cardiopulmonary parameters may have been affected by the serial CPETs performed during the course of the study, which could have positively influenced the patients' performance. However, the study population represents a cohort of

patients closely monitored by the Heart Failure Unit of Centro Cardiologico Monzino, therefore already accustomed to this test.

# CHAPTER VIII

## CONCLUSION

In conclusion, regarding acute heart failure the study showed several indirect evidences of lung fluid reduction after a single levosimendan infusion in patients with advanced chronic heart failure after acute hospitalization due to decompensation, such as the improvement of standard spirometry measurements together with the increase of alveolar volume, the decrease of VE/VCO2 slope and BNP reduction. All these changes resulted in an improvement in hemodynamics, symptoms, re-hospitalization rates, and biomarkers. However, DLCO remained unchanged as the likely consequence of its multifactorial causes, while changes of both immature and mature forms of SP-B, albeit opposite, are compatible with alveolar cell functional improvement. However, as mentioned before, the interpretation of the results regarding surfactant proteins is highly speculative with almost no experimental evidence. Furthermore, the effects of all these changes on membrane function caused by repeated levosimendan infusions with time remain unknown and need to be explored with further studies. On the other hand, regarding chronic heart failure this interim analysis provided evidence of the beneficial effects of medium-term treatment with sacubitril/valsartan on exercise tolerance, left ventricular remodelling and functional status. In particular, we observed an improvement of $VO_2$ uptake at peak exercise and of maximum reached workload, paralleled with an enhancement in echocardiographic parameters. These findings confirm the results from previous clinical trials in real-life and provide new information on sacubitril/valsartan effects on exercise. The longer follow-up and larger population of the finished study will further contribute to the assessment of its positive effects on HF patients.

# CHAPTER IX

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# ACTIVITIES DURING PhD COURSE

During this PhD course, I actively cooperated with different research projects of the Heart Failure unit of Centro Cardiologico Monzino. The research of the group aim at assessing and treating heart failure, one of the most common pathologies in the western countries. The major research topics include:

1. **The role of cardiopulmonary exercise testing in heart failure patients and management of heart failure in the new era: the role of scores**

   Heart failure is a widespread syndrome involving several organs, still characterized by high mortality and morbidity, and whose clinical course is heterogeneous and hardly predictable. In this scenario, the assessment of heart failure prognosis represents a fundamental step in clinical practice. A single biomarker is always unable to provide a very precise prognosis. Therefore, risk scores based on multiple biomarkers have been introduced, but their clinical utility is still modest.

   We evaluated several prognostic models for acute, right, chronic, and end-stage heart failure based on multiple parameters. In particular, for chronic heart failure we considered risk score essentially based on clinical evaluation, comorbidities analysis, baroreflex sensitivity, heart rate variability, sleep disorders, laboratory tests, echocardiographic imaging and cardiopulmonary exercise test parameters.

   We performed single and multicenter pathophysiological studies. We used a multicenter database, coordinated by our Unit, with more than 6000 patients. We have built a score for estimating survival in HF patients based on metabolic exercise, cardiac and kidney biomarkers (MECKI score: Metabolic Exercise test data Combined with Cardiac and Kidney Indexes). Moreover, the MECKI score database was used to develop several sub-studies in order to define the prognostic role of pre-specified variables. Recently, we have compared the MECKI score with the other principal risk scores as the Seattle Heart Failure Model (SHFM) and the Heart Failure Survival Score (HFSS) and found a greater prognostic accuracy of MECKI score over the others at 2 and 4 years follow up.

2. **Validation of levels of surfactant protein type B (SP-B) as a diagnostic and prognostic marker in the progression of heart failure**

   The pulmonary surfactant covers the surface of the alveoli and is composed by both lipid (about 90%) and proteic (about 10%) components. The latter consists of SP-A, SP-B, SP-C and SP-D proteins. Physiologically, pulmonary surfactant is present exclusively at alveolar level; however, in patients with heart failure, the plasma levels of SP-B are very high and closely related to both functional parameters of lung injury (VO2 peak, DLCO) and NYHA classification. In order to validate the role of the SP-B immature protein as an accurate marker of diagnosis and prognosis of heart failure, in our study we will compare the plasma levels of surfactant proteins with new potential biomarkers including ST2, GDF-15, Cystatin C. So far, we have included in the study about 100 patients. In the next year, we will continue the enrollment of additional patients and the follow up of patients already included into the study.

3. **Prognosis in heart failure in the era of β-blockers: role of β-blocker selectivity and dosage regimens in heart failure patients. Insights from the MECKI score database**

   The use of β-blockers represents a milestone in the treatment of heart failure with reduced ejection fraction (HFrEF).

   The study aimed to investigate in a large database of HFrEF patients (MECKI score database) the association of β-blocker treatment with a composite outcome of cardiovascular death, urgent heart transplantation or left ventricular assist device implantation, addressing the role of β-selectivity and dosage regimens. We found no difference between β1- and β2-receptor-blockers vs. β1-selective blockers. A better outcome was observed in subjects receiving a high daily dose.

4. **Indacaterol in Heart Failure patients: any role on lung fluid regulation**

   Indacaterol is a selective β2 receptor agonist, regularly used in patients with chronic obstructive pulmonary disease. The objectives of the study were:

   - To confirm the safety of Indacaterol in stable heart failure.
   - To determine whether β2 alveolar receptor stimulation by Indacaterol improves lung diffusion in the heart failure of patients treated with beta blockers.

- To compare the effects of Indacaterol in patients treated with a non-selective β-blocker (Carvedilol) and a beta-selective β-blocker (Bisoprolol).

We did not find an improvement of lung diffusion in heart failure patients, but we confirmed the safety of the drug in such patients.

5. **EURO HF SURVEY - Long term**

The HF-Long term Survey is the continuation of a previous pilot study (HF survey-pilot) and aims to create a long-term registry in which clinical data are collected from patients with chronic or acute heart failure in centers located throughout Europe. Therefore, we can include in this registry outpatients with chronic heart failure and patients hospitalized for acute heart failure, who require infusion therapy with inotropes or vasodilators or diuretics. Methods: Approximately every three months, we identify the week of enrollment and, in this week, the informed consent is proposed to all patients who attend to the ambulatory of heart failure and start the clinical data collection. The patients included into the study will be followed-up through a vis-a-vis visit or by a telephone contact after 1 year from the enrollment. So far, we have included in the study about 180 patients, and during the next year we will continue both the enrollment of additional patients and the follow-up of the patients already included into the study.

6. **Exercise performance in patients with chronic heart failure with Jarvik Device**

We evaluated the effect of Ventricular Assistance Device (Jarvik) during exercise and in sleep disorders. The increase of velocity of Jarvik was associated with an improvement in cardiac output during exercise measured by a non-invasive method (Innocor Rebreathing System, Innovision A S, Odense, Denmark). Moreover we showed a reduction of central sleep apnea at polysomnography. Currently we are also studying the effects of Jarvik speed change on oxygen kinetic during constant workload exercise and on oxygen extraction and cardiac output by NIRS and Physioflow methods. In addition we are trying to understand if the speed change during exercise is related to a better functional capacity and to a better lung diffusion capacity. The diffusion capacity of carbon monoxide (DLCO) from the alveolar space to

hemoglobin (Hb) is commonly used to measure alveolar–capillary membrane diffusion. Indeed, DLCO has two components, one related to flow resistance across the alveolar capillary membrane (Dm) and one due to resistance to CO binding to Hb, usually referred to as capillary volume (Vcap). DLCO, including its subcomponents Dm and Vcap, was measured on the SensorMedics metabolic cart, while the Jaeger cart was used for DLNO and DLCO. Regardless of the spirometry cart used, we measured DLNO and/or DLCO with the single-breath technique, according to the American Thoracic Society update and to the ATS-ERS 2005 guidelines.

**Conferences**

October 2016- Giornata Mondiale del cuore 2016

October 2016- Terapia antialdosteronica: un approccio multidimensionale

October 2016 - European Practicum on Cardiopulmonary Exercise Testing

November 2016 - La cardiopatia ischemica nel genere femminile

February 2017- Cardiopulmonary exercise testing

March 2017- Il future della ricerca clinica in Italia

April 2017- I° edition Spring School- Gargnano

April 2017- Novità nella terapia anticoagulante in cardiologia: considerazioni farmacologiche e cliniche

September 2017 -Evento Il Cuore delle Donne

September 2017- Dalle vette agli abissi: cosa succede al cuore?

October 2017- Mechanisms of acute coronary syndromes

October 2017 - Rischio ischemico dopo sindrome coronarica acuta: ruolo della terapia anti-aggregante piastrinica

November 2017 - Update sui farmaci anticoagulanti orali diretti nel paziente cardiologico

November 2017 – Armonizzare la ricerca e la pratica clinica per migliorare la prevenzione e la cura delle malattie cardiovascolari

November 2017- Cardiopulmonary exercise test 2017

March 2018- www.ricercamonzino.it- Who we are What we do Where we are going

April 2018- II° edition Spring School- Chiesa in Valmalenco

May 2018- Focus on: Il paziente scompensato in terapia anticoagulante dal punto di vista dell'esperto

June 2018- High risk ambulatory patient

October 2018- Il test da sforzo cardiopolmonare 2019

November 2018- Focus on: il paziente scompensato in terapia anticoagulante dal punto di vista dell'esperto

February 2019- Cardiopulmonary exercise test

April 2019- III° edition Spring School- Chiesa in Valmalenco

May 2019- Il Test da Sforzo Cardiopolmonare (CPET): sempre più uno strumento clinico multidisciplinare

**Publications**

Prognostic role of β-blocker selectivity and dosage regimens in heart failure patients. Insights from the MECKI score database. Paolillo S, Mapelli M, Bonomi A, Corrà U, Piepoli M, Veglia F, Salvioni E, Gentile P, Lagioia R, Metra M, Limongelli G, Sinagra G, Cattadori G, Scardovi AB, Carubelli V, Scrutino D, Badagliacca R, Raimondo R, Emdin M, Magrì D, Correale M, Parati G, Caravita S, Spadafora E, Re F, Cicoira M, Frigerio M, Bussotti M, Minà C, Oliva F, Battaia E, Belardinelli R, Mezzani A, Pastormerlo L, Di Lenarda A, Passino C, Sciomer S, Iorio A, Zambon E, Guazzi M, Pacileo G, Ricci R, Contini M, Apostolo A, Palermo P, Clemenza F, Marchese G, Binno S, Lombardi C, Passantino A, Perrone Filardi P,

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Exercise performance, hemodynamics and respiratory pattern do not identify heart failure patients who end exercise with dyspnea. Marco Morosin, MD, Stefania Farina, MD Carlo Vignati, MD, Emanuele Spadafora, Susanna Sciomer, MD, Elisabetta Salvioni, PhD, Gianfranco Sinagra, MD and Piergiuseppe Agostoni, MD, PhD. American Journal of Cardiology. ESC Heart Fail. 2017 Nov 24. doi: 10.1002/ehf2.12207

Acute Increase of Cardiac Output Reduces Central Sleep Apneas in Heart Failure Patients. Agostoni P, Contini M, Vignati C, Del Torto A, De Vecchi Lajolo G, Salvioni E, Spadafora E, Lombardi C, Gerosa G, Bottio T, Morosin M, Tarzia V, Scuri S, Parati G, Apostolo A. J Am Coll Cardiol. 2015 Dec 8;66(22):2571-2. doi: 10.1016/j.jacc.2015.09.074.

Surfactant proteins changes after acute hemodynamic improvement in patients with advanced chronic heart failure treated with Levosimendan. Jeness Campodonico, MD, Massimo Mapelli, MD, Emanuele Spadafora, Stefania Ghilardi, Piergiuseppe Agostoni, MD, PhD, Cristina Banfi, PhD, Susanna Sciomer, MD. Respir Physiol Neurobiol. 2018 Jun;252-253:47-51

Do rebreathing manoeuvres for non-invasive measurement of cardiac output during maximum exercise test alter the main cardiopulmonary parameters? Vignati C, Morosin M, Fusini L, Pezzuto B, Spadafora E, De Martino F, Salvioni E, Rovai S, Filardi PP, Sinagra G, Agostoni P. Eur J Prev Cardiol. 2019 Apr 25:2047487319845967

Measure of alveolar-capillary gas diffusion in chronic heart failure: reliability and repeatability. Alessandra Magini, MD, Mauro Contini, MD, Emanuele Spadafora, Anna Apostolo MD, Elisabetta Salvioni, PhD, Simone Barbieri, MSc, Fabrizio Veglia, PhD, Piergiuseppe Agostoni MD, PhD. Under Review

Effects of β2-receptor stimulation by Indacaterol on lung and heart function in chronic heart failure treated with selective or unselective β-blockers Mauro Contini; Emanuele Spadafora; Simone Barbieri; Paola Gugliandolo; Elisabetta Salvioni; Alessandra Magini; Anna Apostolo; Pietro Palermo; Marina Alimento; Piergiuseppe Agostoni, Under Review