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PLASMATIC microRNAs IN ADVANCED HEART FAILURE PATIENTS SUPPORTED BY LEFT VENTRICULAR ASSIST DEVICES (LVAD)

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I. INTRODUCTION

Nowadays, heart failure remains a global problem responsible of a high mortality. Despite spectacular advances made in the past three decades in cardiovascular medicine and surgery, the prognosis of heart failure is worse than that of most cancers (1).

The gold standard therapy for refractory end-stage heart failure is currently heart transplantation. However, the increasing shortage of donors has led to the development of mechanical assist devices to support and improve the organ function of patients while on waiting for transplantation. The good results of ventricular assist devices "as a bridge to transplantation" has expanded the application of VAD to be implanted "as a bridge to decision", "as a bridge to candidacy", "as a bridge to recovery" and, increasingly, "as a destination therapy" (DT) (2). In 2001 the REMATCH (Randomized Evaluation of Mechanical Assistance for the Treatment OF Congestive Heart Failure) study first demonstrated that long-term support with a left ventricular assist device resulted in substantial improvement in survival in patients with severe heart failure who were not candidates for cardiac transplantation compared with any known optimal medical therapy addressed to optimize organ perfusion and minimize symptoms of congestive heart failure (3).

Several mechanisms have been advocated to contribute to pathophysiology of heart failure, including genetic mechanisms. In this

context, short non-coding RNAs called microRNA (miRNAs) block gene expression and protein translation. These molecules are crucial to calcium cycling and ventricular hypertrophy. The actions of miRNAs can be blocked by a new class of drugs, antagomirs, some of which have been shown to improve cardiac function in animal models. Moreover, the microRNAs have been proposed as biomarkers of heart failure or cardiac function recovery. For example, increased concentrations of miRNA34, 192 and 194 are predictive of development of heart failure in patients after acute myocardial infarction (4).

Matkovich and coworkers (5) reported that the miRNA-499 levels of patients with heart failure were greatly increased and almost completely normalized after they had been placed on left ventricular assist device. In selected patients, LVAD can lead to myocardial recovery and explantation of the device. Maybe circulating miRNA could be useful prognostic biomarkers of cardiac reverse remodeling in LVAD patients but further investigations are warranted to understand the real role of miRNAs in this setting and their potential utility.

II. CHAPTER I : LITERATURE REVIEW

a. HEART FAILURE

Heart failure (HF) is a worldwide problem, with an estimated prevalence of 38 million patients (6). Every year, 550000 new cases of heart failure with low ejection fraction are diagnosed in USA (7) and despite the progress in the treatment of heart failure (8), a high mortality rate still occurs (42% at 5 years after diagnosis). The clinical syndrome of HF is the final pathway for myriad diseases that affect the heart (9).

Heart failure is nowadays the most common diagnosis for hospital admission in patients aged 65 years and older in high-income countries. Every year, about 1 million hospital admissions occur for heart failure in the USA and a similar number occur in Europe. A 50% increase in the number of new patients with heart failure every year is estimated in 15 years associated with aging of population (10).

The prevalence of HF increases with age and affects an estimated 10% of people over 70 years of age. In patients older than 65 years in the USA, the 30-day hospital mortality rate for patients admitted to the hospital with heart failure is fairly constant at about 11% and the 30-day hospital readmission rate is around 30% (11).

Primary therapy for HF is based on pharmacologic treatment, and the armamentarium of drugs available to clinicians has progressively grown

including potent diuretics, beta blockers, angiotensin converting enzyme (ACE) inhibitors, aldosterone receptor blockers and new classes of drugs such as neutral endopeptidase inhibitors that cause an increase of atrial natriuretic peptide (ANP), among others . Moreover, some patients need long-term inotropic therapy. In addition to pharmacological and primary therapy for coronary or valvular disease, other therapeutic options are available, including cardiac resynchronization therapy by way of biventricular pacing (12).

Currently, the average mortality from the time of diagnosis of heart failure is >60% at 5 years, with higher rates of mortality when patients are in the most advanced stages (13).

The most recent view of HF pathophysiology includes a complex blend of structural, functional, and biologic alterations that are involve in the progressive nature of heart decompensation and that can explain the efficacy or failure of therapies used in clinical trials (14). Moreover, the mechanism of HF is nowadays explained combining several pathophysiological models and current therapy often targets more than one system (15). The main models are the hemodynamic and the neurohormonal model. The hemodynamic model underlies the effect of an altered load on the failing ventricle and was designed in the era of vasodilators and inotropic drugs. On the other hand, the neurohormonal model emphatizes the role of the activation of renin-angiotensin-aldosterone axis and the sympathetic nervous system in the progression of cardiac dysfunction. Currently, the attention has shifted to detect abnormal signaling, gene expression, or altered contractile protein structure in the base of HF.

Therefore, understanding the genetic and molecular pathways that lead from established cardiovascular disease to heart failure may drive to the development of new biomarkers and therapeutic targets (16).

Table 1. Biologically active tissue and circulating substances in the syndrome of HF

Renin-angiotensin-aldosterone system
Sympathetic nervous system (norepinephrine)
Vasodilators (bradykinin, nitric oxide, and prostaglandins)
Natriuretic peptides
Cytokines (endothelin, tumor necrosis factor and interleukins)
Vasopressin
Matrix metalloproteinases

Modified from Jessup M. N Engl J Med 2003;348:2007-18

The heart responds to stressors such as hypoxia (in myocardial infarction), increased wall stress (in valvular heart disease), and neurohormonal/metabolic stress (in diabetes mellitus and hypertension) by cardiomyocyte hypertrophy and fibrosis. Although initially compensatory for increased wall stress or myocyte loss, the molecular pathways that underlie pathological hypertrophy are ultimately maladaptive, creating further hypertrophy, contractile dysfunction, apoptosis, and fibrosis. The progression to HF is associated with a characteristic cascade of altered intracellular signaling and gene expression, representing a final common pathway to ultimate decompensation (16).

Left ventricle remodelling is the process by which mechanical, neurohormonal, and possibly genetic factors alter ventricle size, shape and function (9) and occurs in several clinical conditions such as myocardial infarction, cardiomyopathy, hypertension, and valvular heart disease. HF structural remodelling is characterized by rearrangement of the architecture of the cardiac ventricular wall involving hypertrophy of the myocytes, fibroblast proliferation and increased deposition of extracellular matrix proteins (17).

The main components that may independently contribute to the adverse left ventricular remodelling are: changes in the biology of the cardiac myocyte, changes in the volume of myocyte and non-myocyte components of the myocardium, and changes in the geometry and architecture of the left ventricule chamber (18).

For example, in order to support a multi factorial etiology of ventricular remodeling, some authors have demonstrated the positive effects of a combination of mechanical (LV unloading) and a cellular target therapy called clenbuterol (a beta(2)-adrenoceptor agonist able to increase myofilament sensitivity to calcium) in a failing animal model (19).

Table 2. Structural components of LV remodeling

Alterations in myocyte biology

Abnormal excitation-contraction coupling

α-myosin heavy chain (fetal) gene expression

β-adrenergic receptor desensitization

Hypertrophy with loss of myofilaments

Loss of cytoskeletal proteins

Myocyte loss

Necrosis

Apoptosis

Autophagy

Alterations in extracellular matrix

Matrix degradation

Replacement fibrosis

Impaired angiogenesis

Alterations in left ventricular chamber geometry

Left ventricular dilatation

Increased LV sphericity

Increased LV wall stress

Mitral valve incompetence

Wall thinning with afterload mismatch

Modified from Topkara VL, Mann DL. Role of microRNAs in cardiac remodelling and heart failure. Cardiovasc Drugs Ther 2011;25:171-182 (54) and Mann DL, Willerson JT: Left ventricular assist devices and the failing heart: A bridge to recovery, a permanent assist device, or a bridge too Far? Circulation 1998;98:2367-2369 (52)

Table 3. Favorable effects of prolonged LVAD in HF remodelling

Decreased myocyte necrosis and apoptosis
Decreased myocytolysis
Improved myocyte contractility
Favorable changes in LV chamber geometry
Favorable changes LV wall thickness
Favorable changes LV volume
Favorable leftward shift in the LV pressure-volume curve

Modified from Topkara VL.... And Mann DL, Willerson JT: Left ventricular assist devices and the failing heart: A bridge to recovery, a permanent assist device, or a bridge too Far? Circulation 1998;98:2367-2369

Table 4. Other factors contributing to the Syndrome of HF

- **Genetic, sex**
- **Age**
- **Environmental factors and habits (alcohol, smoke, etc)**
- **Coexisting medical conditions (such as Diabetes mellitus, Hypertension, Renal disease, Coronary artery disease, anemia, obesity, sleep apnea)**

Modified from Jessup M. N Engl J Med 2003;348:2007-18

Table 5. Functional abnormalities in the Syndrome of Heart Failure

Mitral regurgitation
Intermittent ischemia or hibernating myocardium
Induced atrial and ventricular arrhythmias
Altered ventricular interaction

Modified from Jessup M. N Engl J Med 2003;348:2007-18

b. STATE OF THE ART OF VENTRICULAR ASSIST DEVICES

The gold-standard therapy for end-stage heart failure is heart transplantation (HTx). However, the main limitation to transplantation is the organ shortage. In 2015, 1059 patients in Italy were in list for heart transplantation but only 246 hearts were transplanted. The mean time in waiting list was 2,8 years and the mortality while in waiting list reached 7,2 % (20). Moreover, a decline in one-year survival after HTx from 85% to 76% in the EuroTransplant region has been observed and it is probably due to increasing donor age and recipient comorbidity (21). In this context, ventricular assist devices represent a valid therapeutic option in order to replace the lack of organs either as a temporary bridge to transplantation or as a destination therapy (DT) (22). According to the ISHLT registry more than 30% of patients awaiting HTx need mechanical circulatory support with left ventricular assist devices (LVAD) as bridge to transplant (BTT) (23).

In addition, LVAD has become a new treatment option for end stage heart failure as destination therapy for patients either too old or not suitable for transplantation due to the presence of other medical conditions (24). As a result, an exponential increase of LVAD implantation has occurred in last five years.

Mechanical circulatory support restores adequate cardiac output and organ perfusion and consequently improves patient's clinical condition, quality of life and life expectancy (3, 25). Mechanical assist devices are now commonly used to support the failing heart: as a bridge to transplant to support cardiac

function before heart transplantation; as a bridge to recovery; as a bridge to decision until a determination can be made regarding the patient's eligibility for cardiac transplantation; as a bridge until a definitive device can be implanted; finally, as a destination therapy. (26,27).

In case of DT, mechanical circulatory support clearly provides better haemodynamic support compared with pharmacological treatment, restoring adequate organ perfusion and preventing organ dysfunction (3, 28).

b1. Types of VADs

In 1966, DeBakey and colleagues implanted the first pneumatically driven LVAD (29). The next steps were the implantation of a total artificial heart (TAH) as a bridge to transplantation (30) and the implantation of a more biocompatible TAH as permanent destination therapy (31). Due to the high rate of complications, the concept of TAH was never established as a real alternative (32) and to date the proportion of TAH in mechanical assist devices is below 1% (33).

First generation VADs

The first development of VAD started with the shift of the concept of TAH as heart replacement, towards the development of single chamber pumps as cardiac support (32). In fact, VAD generates additional blood flow in parallel with the specific ventricle. These first generation VADs consisted in either

pneumatically or electrically driven pumps generating pulsatile flow with artificial valves as inlet and outlet. These pumps could be used either as isolated left, right or biventricular assist devices. In case of left ventricular support the devices could be positioned intracorporeal. Conversely, in case of biventricular support the device was only positioned extracorporeal.

The main limits of first generation VADs consisted in large size, noise emission, infections of cannulas and malfunction of the valves (3,32). The main examples of first generation VADs include Berlin Heart EXCOR (Berlin Heart, Berlin, Germany) and Thoratec PVAD (Thoratec, Pleasanton, CA, USA).

Figure 1. First generation BiVAD EXCOR Berlin-Heart (Berlin Heart, Berlin, Germany)

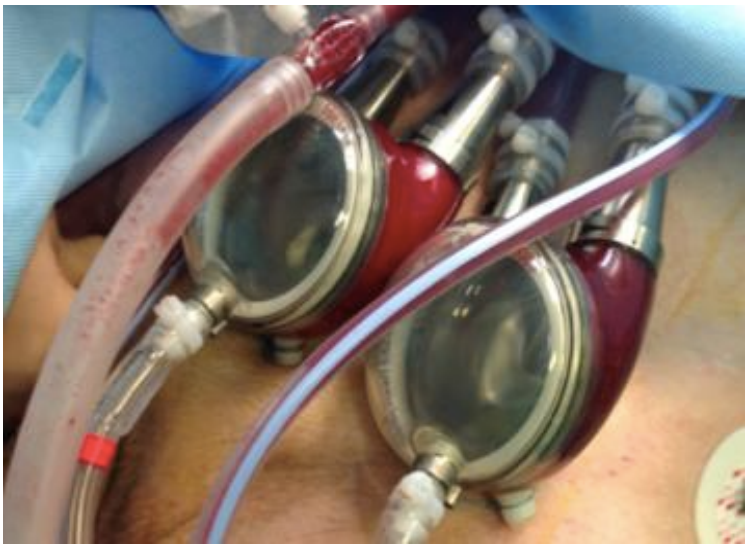

Figure 2. First generation BiVAD EXCOR Berlin-Heart (Berlin Heart, Berlin, Germany). Intraoperative period (with permission)

Second generation VADs

In 1990's second generation of VADs worked out. They consisted in continuous flow centrifugal pump devices. Their main advantages were the reduced size, noise and rate of infections that improved both the quality of life and the outcome of patients. Due to their size, these pumps were designed only for intrathoracic implantation and left ventricular support. The most frequently implanted second generation LVAD is the HeartMate II (HMII; St. Jude Medical, Inc. [Thoratec Corporation], Pleasanton, CA). It consists in a propeller surrounded by a metal case called impeller that presents a combined mechanical and magnetical positioning. Heartmate II presents both BTT and DT Food and Drug Administration approval (34).

Right ventricular failure is one of the most important concerns when a LVAD is implanted and is associated with increased postoperative morbidity and

mortality. It is therefore crucial to evaluate the right ventricle risk profile preoperatively and predict which support strategies will be needed in the operative setting (inotropes, ECMO or even a temporary right ventricle assist device) (35).

The main perioperative side effects or complications following LVAD implantation are (I) bleeding, due to coagulation factor alteration and activation, platelet modifications and abnormal liver function related to refractory heart failure; (II) cerebral and peripheral thromboembolism (III) infections, related to foreign materials and the skin exit sites of cables; (IV) hemolysis, due to red blood stress created by the device impeller; and (V) arrhythmias, related to electrical instability of the myocardium, apical scarring and suction physiology (35).

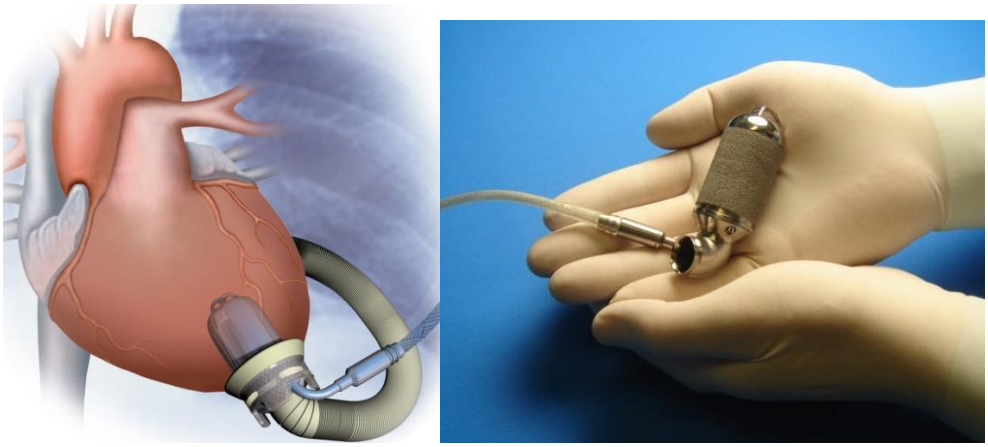

Figure 3. Second generation LVAD. Jarvik 2000 (Jarvik Heart, Inc.; New York, NY, USA) (with permission)

Jarvik 2000 LVAD

The Jarvik 2000 (Jarvik Heart, Inc.; New York, NY, USA) is a valveless electrically powered continuous (non-pulsatile) axial-flow left ventricular assist device. It consists in a miniaturized intraventricular blood pump positioned in the apex of left ventricle without a real inflow conduit. The pump generates a blood flow towards either the ascending or descending aorta through an outflow conduit. It has been approved as a bridge to transplant but also as a destination therapy (36).

The Jarvik-2000 is 2.5 cm wide, 5.5 cm long and weighs 85 grams. The pump has one moving part, an impeller that is a neodymium-iron-boron magnet and which is housed inside a welded titanium shell. It is supported by ceramic bearings and spins blood to generate an average flow rate of 5 L/min (up to 7 L/min) with a rotation speed of 8,000-12,000 rpm (37). The device is connected to an external controller via a tunneled driveline from the abdominal wall or left retro-auricular region, that delivers power to the impeller. This controller permits manual adjustments of the pump speed and shows the current battery charge level.

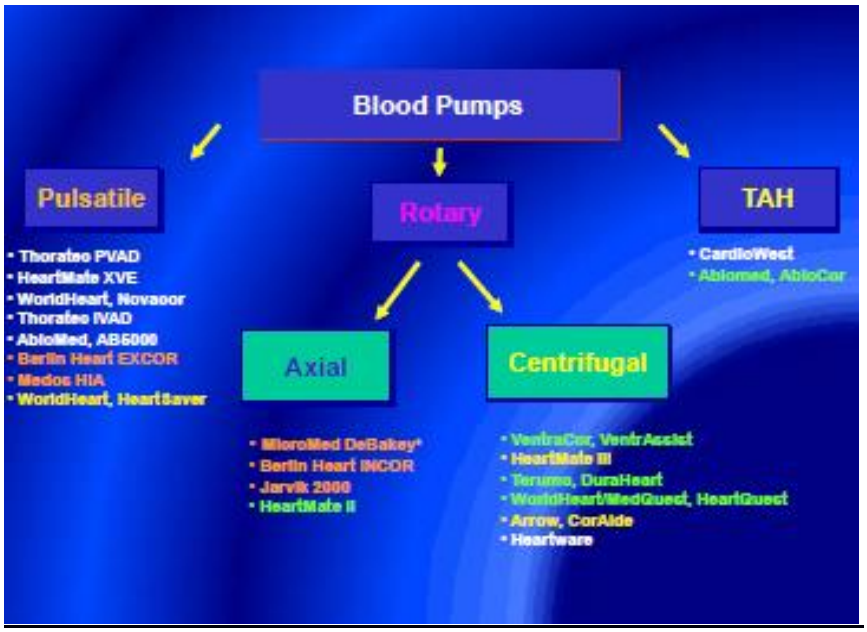

Figure 4. Blood pump technology.

Third generation VADs

The third generation LVADs add another significant reduction in size. The main example is the HeartWare (HeartWare Inc., Framingham, MA, USA). It is a miniaturized centrifugal pump that sits entirely within the pericardial cavity. It is designed as a radial pump. Impeller blades are held in place by a hybrid magnetic and hydrodynamic bearing system. Its main advantages are its estimated durability of 10 years and the capability of produce a flow up to 10 L/min, taking over complete circulatory support also in overweight patients. (32). Other advantage is that the titanium-coated surfaces reduce thrombogenicity.

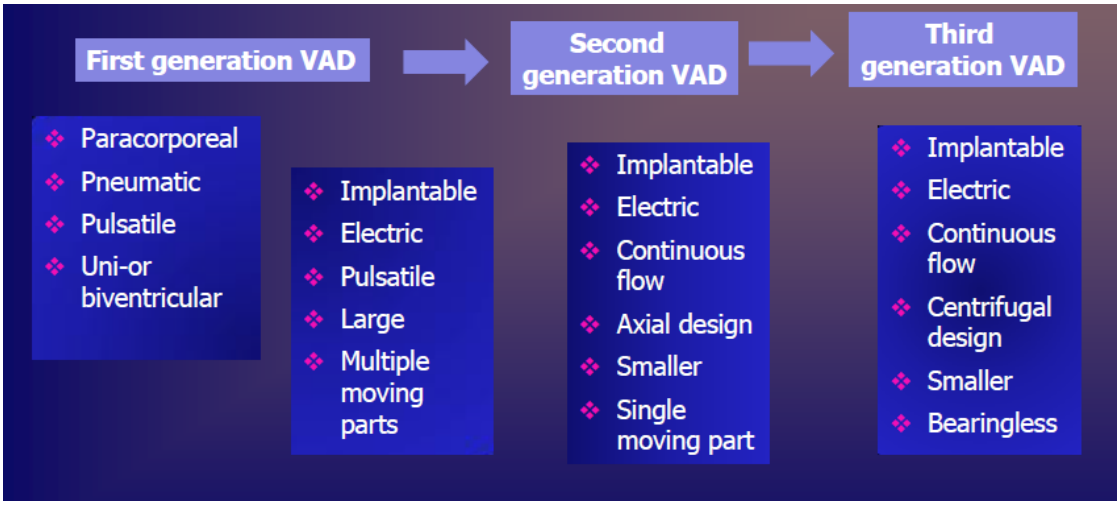

Figure 5. Evolution of mechanical support devices (Modified from Slaughter MS, Singh R. The role of ventricular assist devices in advanced heart failure. Rev Esp Cardiol 2012;65(11):982–985)

Indications to LVAD

LVAD implantation is indicated in patients with end-stage heart failure. The main criteria for LVAD implantation are NYHA class IV heart failure refractory to medical therapy, LVEF less than 25%, systolic blood pressure < 80 mmHg, pulmonary capillary wedge pressure > 20 mmHg, cardiac index <2.0 l/min/m^2 despite continuous inotropic therapy and intra-aortic counterpulsation (37,38,39), some cases of malignant cardiac arrhythmias and patient on transplant waiting list (32).

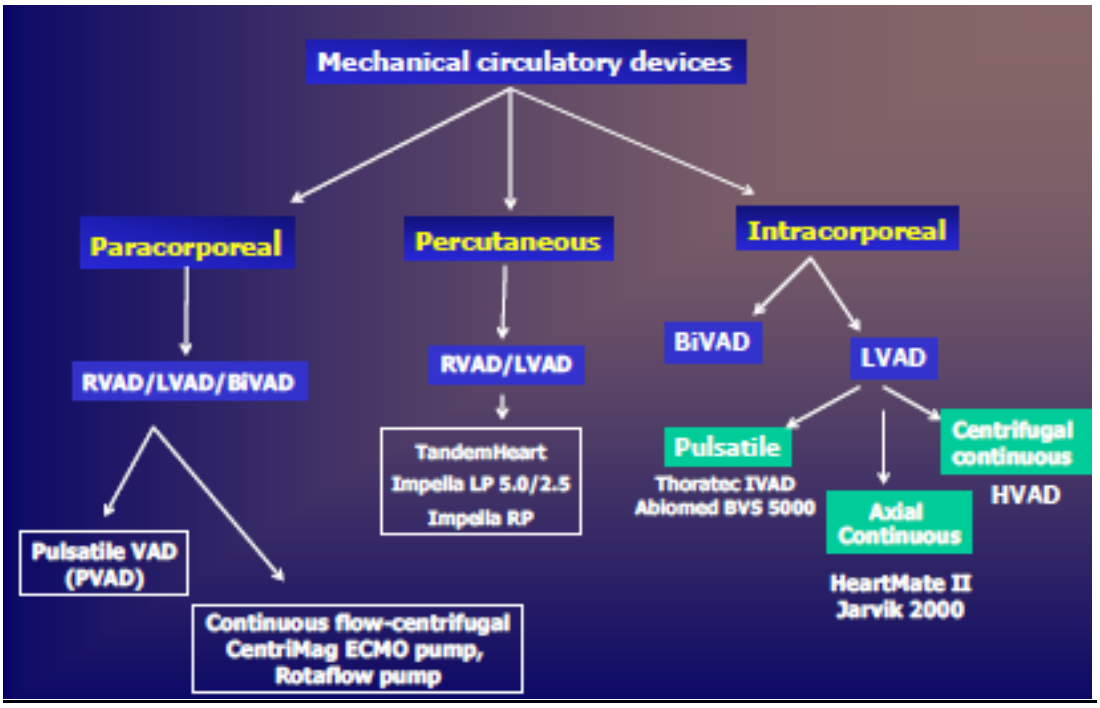

Figure 6. Classification of mechanical circulatory support (modified from Sen A, Larson JS, Kashani KB et al. Mechanical circulatory assist devices: a primer for critical care and emergency physicians. Crit Care 2016; 20:153)

The success and long term results of LVADs implantations highly depend on the timing of implantation. If the LVAD is implanted too early, benefits of medical therapy with potential recovery of heart function are not fully exhausted. If the LVAD is implanted too late, the outcome of the patient may worsen due to secondary organ dysfunction caused by prolonged heart failure (32). The Interagency registry of Mechanical Assisted Circulatory Support (INTERMACS) categorizes patients awaiting LVAD implantation on a scale from level 1 to level 7 (Table 6) (41). Long-term survival has been demonstrated to be best for patients with INTERMACS level 3 (stable with inotropic support) and worst for patients with level 1 (cardiogenic shock) (38).

Category	Description	Shorthand	Life expectancy
INTERMACS 1	Critical cardiogenic shock	Crash and burn	Hours
INTERMACS 2	Progressive decline	Sliding fast	Days to weeks
INTERMACS 3	Stable on inotropic agents	Stable but dependent	Weeks
INTERMACS 4	Recurrent advanced HF	Frequent flyer	Weeks to months
INTERMACS 5	Exertion intolerant	Housebound	Weeks to months
INTERMACS 6	Exertion limited	Walking wounded	Months
INTERMACS 7	Advanced NYHA III	-	-

Table 7. INTERMACS scale. Modified from Stevenson LW, Pagani FD, Young JB et al. INTERMACS profiles of advanced heart failure: the current picture. J Heart Lung Transplant 2009;28:535-41 and Prinzing A, Herold U, Berkefeld A et al. Left ventricular assist devices-current state and perspectives. J Thorac Dis 2016;8(8): E660-8

Outcome

In the last 10 years, design devices, implantation techniques and prognoses have changed dramatically. Devices providing best long-term results are continuous flow, rotary or axial blood pumps implanted using minimally invasive techniques on a beating heart.

The randomized controlled trial REMATCH published in 2001 analyzed the rates of survival and rates of serious adverse effects (such as infection, stroke

and malfunction of the device) in patients long-term supported by a pulsatile LVAD

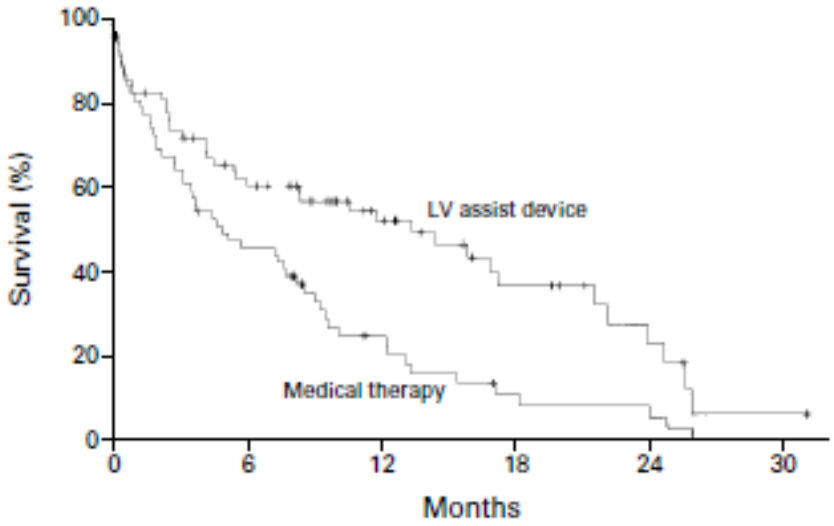

Figure 7. Kaplan-Meier curves of survival in the group that received LV assist devices and the group that received optimal medical therapy. REMATCH study. *From Rose EA, Gelijns Ac, Moskowitz AJ et al. Long-term use of a left ventricular assist devices for end-stage heart failure. N Engl J Med 2001;345:1435-43*

compared to best medical therapy. A significant improvement in survival could be shown for patients receiving treatment with LVAD (52%) compared with best medical treatment (25%). After two years the rate of survival of LVAD group was 23% vs 8% in medical group (3) (Figure 6).

In another randomized clinical trial Slaughter and colleagues compared a first-generation pulsatile-flow device with a second-generation continuous-flow

device. Second-generation devices with continuous flow were observed to have a significant superiority regarding survival, frequency of adverse events, quality of life and functional capacity (58% vs 24% in first-generation devices) (38) (Figure 7).

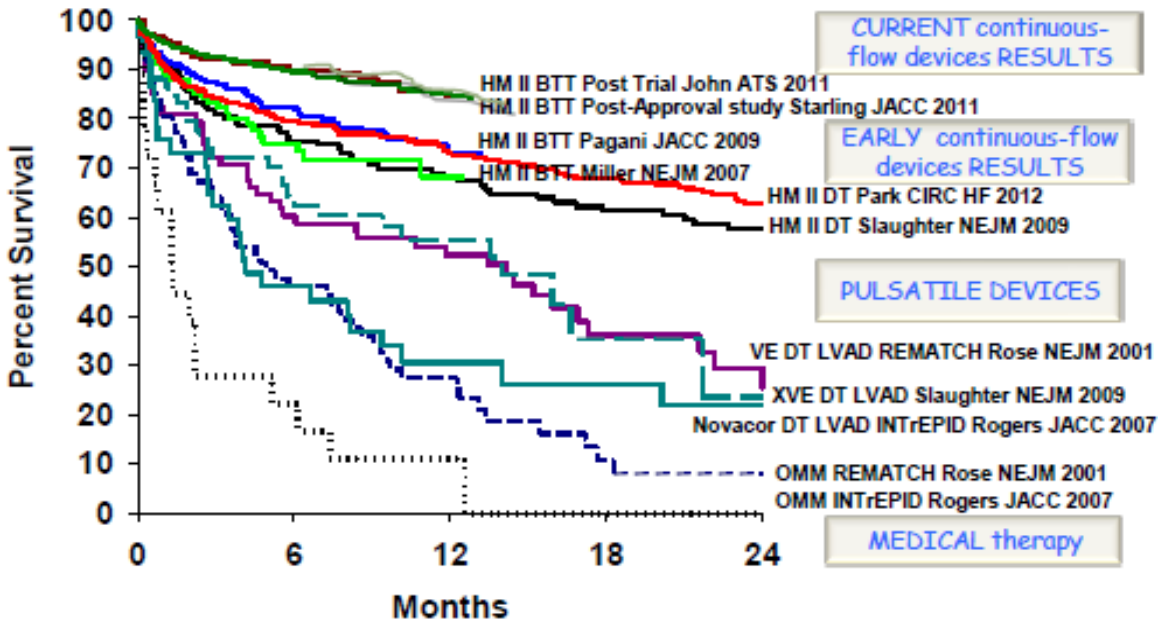

Figure 8. Improving survival and LVAD trials. From several authors.

The rates of survival and freedom from adverse events are higher with third-generation. The ReVOLVE-trial demonstrated 87% of survival after six months, 85% after one year and 79% after three years in patients supported by HeartWare (42).

Similar results have been reported for another third-generation LVAD, the new HeartMate III. Six month survival rates of 92% exceeded the INTERMACS performance goal. The rates of reexploration for bleeding, driveline infections,

gastrointestinal bleeding and stroke were 14%, 10%, 8% and 8% respectively (43).

The most current data regarding mechanical assisted circulatory support is provided by the INTERMACS report (28). The most recent is the seventh annual that was published on December 2015 and include 15,745 VAD's implantations performed between June 2006 and December 2014 in 158 participating hospitals. For patients undergoing continuous-flow VAD, one-year survival is 76% at INTERMACS-level 1, 80% for INTERMACS-level 2-3 and 82% for patients with INTERMACS-level 4-7. The one and two year survival rates of LVAD therapy are comparable to HTx . The mean leading causes of death for INTERMACS-level 1 are neurological events (18%), right ventricle failure (4,5%) multi-organ failure (15,6%). The most frequent adverse events are bleeding (7,8%), cardiac arrhythmia (4,1%), infection (mainly driveline infections) (7,3%), respiratory failure (2,7%) and stroke (1,6%) (28).

The VADs program at University Hospital of Udine started on 2088. From September 2008 to December 2016, 42 VAD implantations have been performed. The global survival at 6-, 12- and 24-months is 85%, 71% and 43% respectively.

The key to successful LVAD implantation remains appropriate patient selection and timing. One of the main clinical challenges is the right ventricle failure after LVAD implantation than remains a serious complication, leading to an estimated 19 to 43% increase in perioperative mortality (44). The risk of developing RV failure is clearly associated with the underlying disease (more frequent in dilated idiopathic than in ischemic cardiomyopathy). Currently,

there are no general consensus on the best parameters predicting RV failure (24).

Figure 9. Third generation LVAD. HVAD (HeartWare Inc., Framingham, MA, USA) (Source : www.heartware.com)

LVAD and Heart failure

The abnormal structure, function and molecular configuration of dilated failing hearts can be partially normalized in patients supported by LVAD, a process called reverse remodeling. This leads to recovery of some degree of function, but the rate of full recovery is low and in many cases temporary, leading to the concept of heart remission, rather than recovery. For this reason, very few

patients exhibit sufficiently improved function to justify device explantation (45).

The treatment of heart failure by resting the failing heart was firstly suggested by Burg and colleagues in the 1969s (46). Left ventricular assist devices provide chronic pressure and volume unloading of the dilated failing left ventricle. This is associated with reverse structural remodeling (normalization of the passive pressure-volume relationship), reverse molecular remodeling (increased expression of several genes involved in calcium metabolism that are down-regulated in heart failure), improved baseline contractility, and improved contractile response to increased heart rate and to beta-agonist stimulation. These findings indicate the possibility of recovery of myocardial properties in hearts previously considered to have irrecoverable end-stage heart failure (47).

LVAD can induce reverse left ventricular remodelling leading to partial recovery of ventricular function, improved condition of patients, reduced cardiomyocytes size and changes in contractile fibers (48).

When compared with the cardiac tissue obtained at device implantation, following several months of LVAD the supported ventricles exhibited regression of cardiomyocyte hypertrophy and increase in the density of beta-adrenergic receptors, improvement of calcium cycling, with more rapid calcium entry into the cardiomyocytes, enhanced RYR2 function, and SERCA2[a] activity. It is important to distinguish between ventricular reverse remodelling, characterized by normalization of cardiac chamber size and regression of myocyte hypertrophy, and myocar-

dial recovery, in which previously failing heart regains sufficient function to be able to support the circulation (49).

LVADs as a bridge to transplantation has consistently shown dramatic improvements in cardiac output (49), and New York Heart Association functional class (50). These clinical changes are associated to significant decreases in plasma levels of neurohormones such as epinephrine, norepinephrine, plasma rennin activity, angiotensin, atrial natriuretic peptide and arginine vasopressin (51) and decrease of cytokines such as interleukins 6 and IL 8 (52), suggesting that LVAD support may alter the heart failure "milieu" (53). Conversely, other authors have demonstrated an increased fibrosis and non complete cessation of myocyte necrosis after LVAD implantation, suggesting that there was ongoing cell death and replacement fibrosis during the period of mechanical support (54). The same authors reported that using exercise testing helps to identify which patients could be weaned successfully from LVAD support. LVAD patients able to exercise to a VO2 > 20 mL/kg/min and/or peak cardiac output of 10 L/min had sufficient cardiac reserve to tolerate LVAD explantation (54).

c. **Micro RNAs and heart failure**

MicroRNAs (miRNAs) were firstly described in 1993. They are short, non-coding RNAs which are present in almost all higher eukaryocytes. These small RNA particles (~ 22 nucleotides in length) have the ability to regulate gene expression at the post-transcriptional level (56).

Almost 2000 miRNAs have been isolated in human beings. miRNAs are gene products processed first in the nucleus and then in the cytoplasm; they silence messenger RNA (mRNA) by pairing with its messenger sites, leading to translational repression or degradation of the mRNA and thereby preventing protein translation and gene expression. Each miRNA might attach to several mRNAs, while each of the mRNAs can bind several microRNAs.

miRNAs are thought to play a role in a variety of pathophysiological mechanism, including various cardiovascular disorders, especially the development of heart failure (57).

Preclinical research into miRNAs has shown the important role of these molecules in the control of calcium cycling and in the development of ventricular hypertrophy and heart failure. Recent evidences suggest that miRNA are differentially expressed in the failing myocardium and play an important role in progression of heart failure by targeting genes that govern diverse functions in cardiac remodelling process including myocyte hypertrophy, excitation-contraction coupling, increased myocyte loss, and myocardial fibrosis. (55). For example, the genetic knockout of miRNA22 reduces the activity of SERCA2a pump, causing heart failure (58). miRNA208 stimulates ventricular hypertrophy in mice

secondary to transverse aortic constriction, whereas its knockout prevents hypertrophy.

MiRNAs have been reported to be able to enter the bloodstream where due to their enhanced stability, they could become useful potential diagnostic and prognostic biomarkers for multiple diseases including heart failure such as B-Natriuretic Peptide (BNP) is currently used (59). However, the meaning and role of these circulating miRNA levels in some specific clinical settings such as Acute Heart Failure remain to be established (60).

Recently, it has been shown that intracellular regulation of gene expression is regulated at least in part by small RNA molecules: micro RNA. These miRNAs are highly expressed in heart tissue. The number of microRNAs involved in heart disease is increasing and their exact function and role has not been elucidated yet (48).

In heart failure structural and physiological modifications of the myocardium occur.

Various cellular processes are controlled at the level of mRNA transcription by miRNAs. In the heart, the role of these miRNA has been described in some processes (Table 8).

Some evidences suggest that miRNAs play an important role in the pathogenesis of heart failure throught their ability to negatively regulate expression levels of genes that govern the process of adaptative and maladaptative cardiac remodelling (55).

The expression profile of miRNAs in failing heart has been investigated in microarray studies performed in animal models of heart failure, such as thoracic

aortic constriction (TAC) (61), coronary artery ligation, ischemia reperfusion injury (62), calcineurin overexpressin (61), and Akt overexpression (63), as well as in myocardial tissue obtained from patients with end-stage heart failure (64,65,66). The critical points of these studies that can/could influence the interpretation of the results are the following:

1. the expression analysis of the miRNAs has been carried out using microarray platforms that are continually evolving while the ongoing discovery of novel miRNAs, so as the technology improves it may allow for the discovery and validation of new miRNAS with higher sensitivity and specificity.
2. human heart failure studies of miRNAs expression are limited by the lack of standardized protocols, small sample size, and the variability between different groups.
3. In studies performed in myocardial samples, differential expression of a specific miRNA may not always be ascribed to the expression of cardiomyocytes since other myocardial cell types such and fibroblasts, endothelial cells and inflammatory cells may contribute to differential expression of miRNAs (55)
4. in addition, differential expression of a circulating specific miRNA may not always be attributed to the expression on the myocardium due to potential expression in other tissues.

Schematically, miRNAs may be classified according to their function and in relation to their role in the pathogenesis of cardiac remodeling (55):

1. miRNAs involved in myocyte hypertrophy

2. miRNAs involved in myocyte contraction
3. miRNAs involved in non-myocyte myocardial alterations
4. miRNAs involved in regulating cellular viability
5. miRNAs involved in regulating extracellular matrix
6. miRNAs involved in electropysiological alterations
7. miRNAs as biomarkers in heart failure

- **<u>miR-1</u>**

  miR-1 is a cardiac and skeletal muscle specific miRNA that is expressed from bicistronic units with members of the miR-133 family. It was discovered in 2002 (67) in a screen for muscle-specific miRNAs in mouse. In the mouse heart, miR-1 accounted for about 40% of all miRNA transcripts in the cell (57). Cardiac expression of miR-1 is regulated by serum response factor transcription factor (58). miRna -1 seems to have a crucial role in cardiac development. In a mice model, Zhao et al have demonstrated that targered delection of miR-1-2 led to increased late embryonic/postnatal secondary to ventricular septal defects (69). In addition, miR-1 seems to be a negative regulator of cardiac hypertrophy. Sayed and coworkers reported that cardiac expression of miR-1 was downregulated at 1 and 7 days after thoracic aortic constriction in a mouse model (70). Care et al has demonstrated reduced expression of miR-1 in various models of hypertrophy such as TAC, Akt overexpression and exercise induced hypertrophy (71). Some studies have reported down-regulation of miR-1 levels in the failing heart, whereas others

have reported opposite findings, so currently exact role of miR-1 in the development of human heart failure remains unclear (72).

miR-1 is thought to be involved in excitation-contraction coupling and myocyte apoptosis (72) and arrhythmia.

- **miR-133**

  The miR-133 family includes three members, miR-133a-1, miR-133a-2, and miR-133b. MiR-133a-1 and -2 are expressed in cardiac and skeletal muscle, however miR-133b is expressed exclusively in skeletal muscle (63). miR-133 expression levels are downregulated in a number of cardiac hypertrophy models. Moreover, the partial knockdown by specific antagomiRs resulted in attenuation of hypertrophic growth and fetal gene expression (63). In contrast, Liu et al reported that genetic ablation of either copy of mR-133a in mice not lead to exaggerated hypertrophic response in a thoracic aortic crossing model (73). In fact, majority of human heart failure studies did not demonstrate differential regulation of miR.133a levels (74), so further studies are necessary to determine the real role that miR-133 could play in heart failure.

- **miR-21**

  miR-21 is one of the few miRNAs which demonstrate a consistent pattern of upregulation in the failing myocardium (55). miR-21 is also highly expressed in various types of cancer tissues and cell lines, suggesting a common feature of

miR-21 in stress response and pathological cell growth (75) but its exact role in development of cardiac hypertrophy remains controversial. Knockdown of miR-21 using an antisense oligonucleotide was shown to be sufficient to attenuate phenylyephrine or angiotensin-II induced cell growth and protein synthesis in cultured neonatal cardiac myocytes (76).

A carefully designed study by Thum and colleagues revealed that miR-21 is predominantly expressed in cardiac fibroblasts (77). Moreover, expression levels of miR-21 in isolated cardiomyocytes did not change in the failing verses non-failing hearts, suggesting a limited role of miR-21 in cardiomyocytes. Therefore, the change in miR-21 levels in the failing myocardium could be primarily related to an increase in fibroblast cell number or activity rather than changes in cardiomyocyte function (55). To date, miR-21 has no validated gene targets that are directly related to cardiac myocyte hypertrophy.

- **miR-378**

  miR-378 is a muscle-enriched miRNA that is expressed in cadiomyocytes but not in fibroblasts. In some studies, there was a decreased in miR-378 in the heart after TAC, and mantainance of miR-378 levels by genetic overexpression of miR-378 decreased hypertrophy and improved LV function. So miR-378 seems to be a regulatory of hypertrophy and a better remodeling (16).

- **miR-1202**

  Myocardium tissue is rich of miR-1202. Some authors had reported high levels of plasmatic miR-1202 in LVAD patients. According to INTERMACS miR-1202

is the miRNA that correlates better with NT-proBNP at 3 months after LVAD implantation (78).

- **miR-96**

  miRNA-96 has been associated with a reduction in high platelet reactivity (79).

- **miR-223**

  Platelets are rich in non coding RNA like microRNA and long non coding miRNAs. miR-223 has been reported to be released by activated platelets. Landry *et al* (80) have previously demonstrated that miR-223 regulates the expression of P2Y12 receptor . Moreover, platelet miR-223 is low expressed in case of high levels of plasmatic factor XIII, so is associated with a reduced bleeding time (79).

- **miR-26**

  Platelet expression of miRNA-26a has been reported to be associated with high platelet reactivity and with associated with clopidogrel resistance following coronary stenting (81).

Table 7. miRNAs associated to heart failure process (54-76)
Physiologic exercise increases miR-29
Promoters of hypertrophy: 23a, 208, 499
Inhibitors of hypertrophy: 1,9,98,133,378
Ischemia-reperfusion, oxidative stress: increased miR-499, miR-378, miR-21
Ischemia-reperfusion, oxidative stress: reduced miR-24, miR-29
Overexpression of 133 blocks adverse effects of beta-adrenergic stimulation,
Overexpression of 133 enhaces the effects of beta bloclers in heart failure.
Antagomir to miRNA 208a preventes cardiac remodelling and improved survival in a hypertensive rat model
Myocardial infarction and TAC in mice cause pathologic remodelling and upregulation of miRNA34. Antagomir of miRNa 34 improved cardiac function
miRNA-25 is upregulated in mice with heart failure secondary to TAC in which it interferes with cardiomyocyte calcium cycling by blocking the SERCA2[a] pump. Antagomir25 improves LVEF. miRNA-25 is increased also in patients with HF
miRNA 499 greatly increased in HF patients and completely noprmalised after they had been placed on LVAD
miRNA 21,24, 299, 30c, 101a, 101b, 214 are regulators of fibrosis
miRNA21 is expressed in cardiac fibroblasts and is important to development of cardiac fibrosis after TAC; when blocking by its antagomir fibrosis is suppressed
miRNA 29 seems to exert an antifibrotic effect
Cardiomyocyte hyperplasia: miR-1, miR-2
Ventricular arrhythmias: miR-1

e. P-Selectin

One of the main complications of VAD are thrombotic events. Platelets play a major role in the understanding of thrombotic events in prolonged mechanical support. It has been demonstrated that in patients with implanted external ventricular assist device, the platelet activation profile displays a persistent activation with a preserved reactivity associated with a persist high inflammatory state and endothelial activation. (82). P-selectin is a cell surface glycoprotein involved in inflammatory and thrombogenic process.

P-Selectin, also known as GMP-140, LECAM-3, PADGEM, and CD62P, plays a critical role in the migration of lymphocytes into tissues. (83). It is found in a preformed state in the Webel-Palade bodies of endothelial cells and in the alpha granules of platelets . This stored P-Selectin is mobilized to the cell surface in response to inflammatory and thrombogenic stimuli. The mobilized P-Selectin remains on the cell surface for only a few minutes after which is recycled to intracellular compartments (84).

Evidence indicates that P-selectin is involved in the adhesion of myeloid cells, B cells and a subset of T cells to activated endothelium. P-Selectin is also involved in the adhesion of platelets to monocytes and neutrophils, playing a central role in neutrophil accumulation within thrombi (84). P-Selectin, acting in cooperation with L-Selectin, is implicated in the mediation of the initial weak interactions between leukocytes and neutrophils and the surface of endothelium that produce a characteristic “rolling” motion of the leukocytes and neutrophils on the endothelium surface. (84). P-Selectin is found in the plasma of normal individuals at ng/mL concentrations (85). Several

studies have reported that levels of sP-Selectin in biological fluids may be elevated in subjects with a variety of pathological conditions such as connective tissue diseases, thrombotic thrombocytopenic purpura, haemolytic uraemic syndrome (86), scleroderma (87) and myocardial infarction (88).

III. CHAPTER II: STUDY

A) Aim of the study:

To evaluate the modification of some miRNAs related to myocardial fibrosis, ventricular remodelling and platelet function in patients with heart failure compare with healthy volunteers and in heart failure patients after LVAD positioning at short and long term periods (6-10 day, 2-3 months, 12-18 months).

To analyze the modification of P-Selectin in patients supported by VAD and its correlation with thrombotic events.

B) Methods:

B1. PATIENT SELECTION AND BLOOD SAMPLE COLLECTION

All LVAD patients included in this observational prospective study were recruited at the Department of Cardiac Surgery of the University Hospital of Udine between August 2011 and January 2016. All patients underwent the same LVAD (Jarvik 2000) positioned as a bridge to transplant or as a destination therapy.

Ethics Committee approval was obtained.

Healthy controls were matched to the demographic data of patients including age, gender and ethnicity, to remove potential confounding factors.

12 ml of venous peripheral blood were collected in EDTA tubes from LVAD patients at T0, T1, T2 and T3 (Table 8) while from healthy controls at a single time-point (T0).

Overall, a total of 50 samples were included in this study: 39 from LVAD patients and 11 from healthy donors.

At T0, T1, T2 and T3 miRNAs 26a-5p, 21, 96, 1202, 223, 378 and endogenous miR-92a were analyzed. At the same times BNP, P-selectin, and platelet count were performed.

At T0, T1, T2 and T3 patients also performed transthoracic echocardiography (LVEF, RV TAPSE, LV volumes) and the 6 minutes walking test.

Time	
T0	Pre-implantation of VAD- end stage HF
T1	Between 6-10 days after VAD implantation
T2	Between 2-3 months after implantation
T3	Between 12-16 months after

Table 8. Times of blood sample

B2. miRNA DETERMINATION

I. PLASMA ENRICHMENT

The plasma was separated from venous blood within 4 hours after the collection by a two-step centrifugation protocol. Samples were first centrifuged at 1500*g for 15 minutes at room temperature (RT) and then the supernatant was centrifuged again at 14,000*g for 20 minutes at 4°C to obtain cell- and platelet-free plasma. Enriched plasma samples were stored at -80°C until analysis to preserve RNA integrity and stability.

II. RNA and miRNA ISOLATION FROM PLASMA

Total RNA was extracted from plasma samples using the miRvana™ PARIS™ kit, according to the manufacturer's instruction. Briefly, 200uL of plasma were pooled equally with the 2X Denaturing Solution and immediately mixed thoroughly. The mixture was incubated on ice for 5 minuromote the sample disruption. Before the protein precipitation step, 25 fmoles of *C. elegans* cel-miR-39 synthetic spike-in (miVana miRNA Mimic, Life Technologies) were added to the lysate.

Then, two volumes of acid-phenol: clorophorm were put in each sample and then the mixture was centrifugated for 5 minutes at maximum speed (≥ 10,000*g) at RT. After this centrifugation step, the upper aqueous phase containing RNA was carefully removed and transferred into a fresh tube. For the RNA and the miRNA purification a glass-fiber binding protocol was applied.

Ethanol was added to the samples and then they were passed through a filter cartridge which immobilized the RNA and the small-size RNAs. The filter was then washed few times with ethanol-based solutions and finally RNA and miRNAs were diluted using 50uL of RNAs free water. The elution solution was preheated at 95°C to enhance the filter absorption and the RNA and miRNA recovery. Nanodrop (Thermo Scientific) spectrophotometer quantification was not reliable because the amount of RNA and miRNAs is very low in the plasma samples.

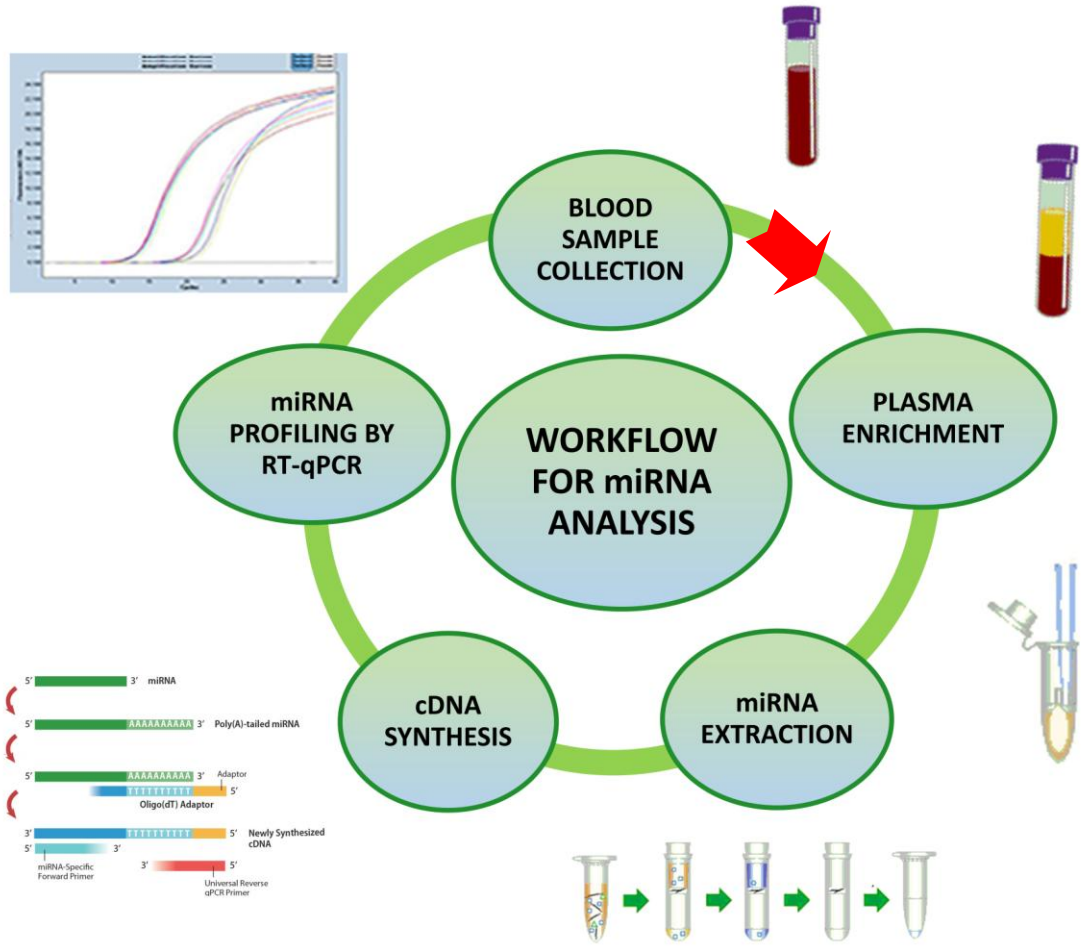

Figure 10. Workflow: miRNAs expression determination procedure

III. cDNA SYNTHESIS AND PRE-AMPLIFICATION

A three-step Reverse transcription (RT) protocol, using the *TaqMan® Advanced miRNA cDNA Synthesis Kit* (Applied biosystems), was performed employing 2.5uL of the RNA extracted previously.

First, a poly(A)-tailing reaction was performed to add a 3'-adenosine tail to the miRNA, then, an adaptor ligation reaction was required because the adaptor at the 5' terminal may act as the forward-primer binding site for the miR-Amp reaction. This two modifications are necessary for the cDNA template preparation.

After these preliminary steps, the reverse transcription (RT) reaction was carried out using universal random primers. Then, 5uL of cDNA were pre-amplified according to the manufacturer's protocol. The use of a universal forward and reverse primer mix increases the number of cDNA molecules to analyze by real-time qPCR.

IV. miRNA EXPRESSION PROFILING BY RT-qPCR

2uL of the preamplification product were used to perform the RT-qPCR assays. The detection of miRNA was performed using the TaqMan® Advanced PCR Kit implemented in the ABI 7300 Real-time PCR System (Applied Biosystems). The qPCR tests were performed in a 96 multiwell optical plate. The reaction started with a first step at 95°C for 2 minutes, followed by 40 cycles of 95°C for 20 seconds and 60°C for 30 seconds. The expression levels of target miRNAs

miR21-5p, miR96-5p, miR1202, miR378, miR-26a-5p and miR223-5p) and control miRNAs (normalized endogenous controls miR-92a-5p) were measured simultaneously. All reactions, including no template controls, were performed in duplicate. The relative expression levels of target miRNAs were calculated with the $2^{-\Delta\Delta CT}$ method.

B3. P-SELECTIN DETERMINATION

P-Selectin was measured in plasma samples using the Human P-Selectin/CD62P Immunoassay that consists in a 1.25 hour solid phase ELISA that measures P-Selectin in cell culture supernates, serum, and plasma. It contains recombinat human P-Selectin and antibodies raised against the recombinat factor.

I. PRINCIPLE OF THE ASSAY:

A monoclonal antibody specific for human P-Selectin has been pre-coated onto a microplate. Standards, samples and control are pipetted into the wells together with a polyclonal antibody specific for human P-Selectin which has been conjugated to horseradish peroxidase. Following a wash to remove any unbound conjugated antibody, a substrate is added and color is developed which is proportional to analyte concentration.- the color development is stopped and the intensity of the color is measured.

Blood samples of patients and healthy volunteers were collected in citrated tubes and centrifugated for 15 minutes at 1000x g within 30 minutes of collection. All samples and the P-Selectin Control were diluted at least 20-fold into sample diluent.

II. REAGENT PREPARATION

Standards were reconstituted before use with 1.0 mL of distilled or deionized water. Vials sit at room temperature for at least 10 minutes at room temperature and mixed by gentle inversion and swirling until all the contents were completely dissolved.

P-Selectin Controls were reconstituted immediately before use with 500 uL of distilled or deionized water. The control sit at room temperature for at least 10 minutes and mixed by gentle inversion and swirling. The P-Selectin Control were diluted 20-fold in sample diluent prior to assay.

The P-Selectin Conjugate was prepared transfering 250 uL of the conjugate concentrate into the bottle of conjugate diluent.

III. ASSAY PROCEDURE

All reagents were brought to room temperature before use. All samples were assayed in duplicate. An amount of 100uL standards, previously diluted control and diluted samples were added to each well.

The plate was cover with a plate sealer and incubated at room temperature for 1 hour. Three washes of each well were made. An amount of 100 uL of substrate was added to each well. The plate was cover with a new plate sealer and incubate at room temperature for 15 minutes. The Stop solution (100 uL)

was added to each well. Finally, the optical density (OD) of each well was

determined within 30 minutes, using a microplate reading set to 450 nm. The results were calculated averaging the duplicate readings for each standard, control, and sample. A standard curve was created using a computer software able to create a four parameter logistic curve-fit. We determined the concentration of each unknown sample by calculating the concentration of P-Selectin corresponding to the mean absorbance from the standard curve. The P-Selectin Control was run in each assay.

B4. PLATELET COUNT DETERMINATION

Platelet count was established with UniCel®DxH 600 coulter cellular analysis system (Beckman Coulter, Miami, FL, USA) based on impedance variation.

C) Statistical Analysis

Statistical analysis consisted in descriptive statistics. Categorical and continuous data are presented by number (%) and by mean (SD). miRNA correlation with other miRNA or parameters were analyzed using the Spearman's rank correlation coefficient or Spearman's rho. The Kruskal–Wallis test was used for comparing two or more independent samples. Continuous variables were compared using the t-test or Mann-Whitney U test as appropriate. Statistical significance was $p<0,05$.

D) Results:

11 Caucasian healthy volunteers (8 men and 3 women) and 10 patients (8 men and 2 women) with refractory and-stage heart failure were included in this study. All patients underwent second generation LVAD Jarvik 2000 implantation as a bridge to cardiac transplantation or as a destination therapy.

Patients supported with LVAD had a mean age of 58,5 years. Patients were 80% (n=8) male. Healthy volunteers were matched by age and gender (mean age 57,9 years). The main cause of end-stage heart failure was idiopathic cardiomyopathy (80% of cases). The rest 20% of population was affected by post-ischemic cardiomyopathy. 60 % of patients were implanted as a bridge to cardiac transplantation and 40% of patients received LVAD as a destination therapy. The most frequent New York Heart Association Functional (NYHA) class at implantation was NYHA IV (60% of cases). Fifty percent of patients were on Level 2 of INTERMACS classification and 40% of patients on level 3 at implantation time.

The mean left ventricular ejection fraction (LVEF) at implantation was 26,6%, with a left ventricular end diastolic volume (LVEDV) of 219,4 ml, a LVESV of 175,2 mL and a mean PAPs of 50 mmHg.

Regarding right ventricular function the main TAPSE at implantation was 15,42 mm. Two patients suffered from RV failure in the immediate postoperative period and 1 patients after 1 year of LVAD implantation.

The median assistance level of LVAD after 3 months of LVAD positioning was 3.

The mean duration of LVAD was 497,6 days (± 280,12) (range 107 to 873 days).

The rate of patients affected by composite thrombotic events were 40 % including transient ischemic attacks, stroke and one pump thrombosis

Number of patients	**10**
Male (%)	**80**
Mean age at implantation (years)	**58,5**
Idiopathic DCM/Ischemic HD (%)	**80/20**
BTT/DT (%)	**60/40**
NYHA III/IV at implantation (%)	**40/60**
INTERMACS 2/3/5 levels at implantation (n)	**5/4/1**
Patients with thrombotic events (n)	**4**
Patients with hemorrhagic events (n)	**3**
Pump thrombosis (n)	**1**
Mean LVEF at implantation (%)	**26.6**
LVEDV at implantation (mL)	**219,4**
LVESV at implantation (mL)	**175,2**
Mean PAPs at implantation (mmHg)	**50**
Mean CI at implantation (mL/min/m^2)	**2,02**
Bilirrubin tot at implantation (mg/dL)	**1,96**
Mean creatinine at implantation (mg/dL)	**1,01**
Median Jarvik assist level at T2	**3**
Patients on ASA associated to warfarin %)	**20**
Mean distance at 6MWT at T3 (m)	**407**
Mean Borg score at 6MWT at T3	**0**

Table 9. Demographic, hemodynamic and laboratoristic data

The rate of patients affected by hemorrhagic events were 20% including both gastrointestinal and cerebral bleedings. All patients assumed warfarin in order to maintain a INR target between 1,8 and 2,2. Only 20% of patients associated ASA to warfarin.

Periodic 6-minute walking-tests (6MWT) were performed in the postoperative period. The mean distance walked at three months after LVAD support was 325 m (290-414) and the mean distances walked at three months after LVAD support was 403 m (321-501). There was not a significant difference in patient functional performance between after three months of LVAD support and after 12 months measured by 6MWT ($p=0.2$).

The survival rate of our case series was 90% at 6 months, 90% at 1 year and 67 % at 24 months.

BNP

The mean BNP in healthy volunteers was 24,54 pg/mL. In end-stage heart failure patients the mean BNP was 1071,71 pg/mL before VAD implantation, that immediately normalized after few days of LVAD assistance (table) reflecting an immediate LV unloading process. Significant differences of BNP between healthy volunteers and patients ($p<0.001$) and in patients before implantation, 6 days, 3 months and more that 12 months of VAD support were observed ($p<0.0001$) (fig. 17).

	Control	T0	T1	T2	T3
BNP (mean, pg/mL)	24,54	1201,8	344,1	293,1	317,33
miR26 (mean fold-change)	0,2227	15,37	36,96	620,26	1,27
miR 21 (mean f-c)	0,6992	441,81	138,198	1432,133	152,01
miR96 (mean f-c)	0,1972	3,31	1,33	3,96	1,66
miR 223 (mean f-c)	0,3780	3,87	6,678	243,16	7,057
miR 378 (mean f-c)	0,05309	134,14	380,99	113,88	22,26

Table 10. Modification of BNP and miRNAs during time

miRNAs and BNP

In 11 healthy volunteers' samples and 39 HF patients' samples, levels of the following miRNAs were measured: miR-21, miR-26, miR-92a, miR-96, miR-223, miR-378, miR-39 and miR-1202.

Micro-ribonucleotides levels were expressed in folds compared to the expression of endogenous miR-92a in the healthy control population according to the following formula (82):

$$\text{Amount of miRNA target} = 2^{-\Delta\Delta C_T}$$

$$\Delta\Delta C_T = (C_{T,\,\text{target}} - C_{N,\,\text{normalizator}})_{\text{Time X}} - (C_T - C_N)_{\text{mean of controls}}$$

In our population, significant differences were observed in levels of miRNA 21a between healthy volunteers and failing patients. No significant differences were observed in levels of miRNA 21a before and after LVAD implantation at any postoperative time.

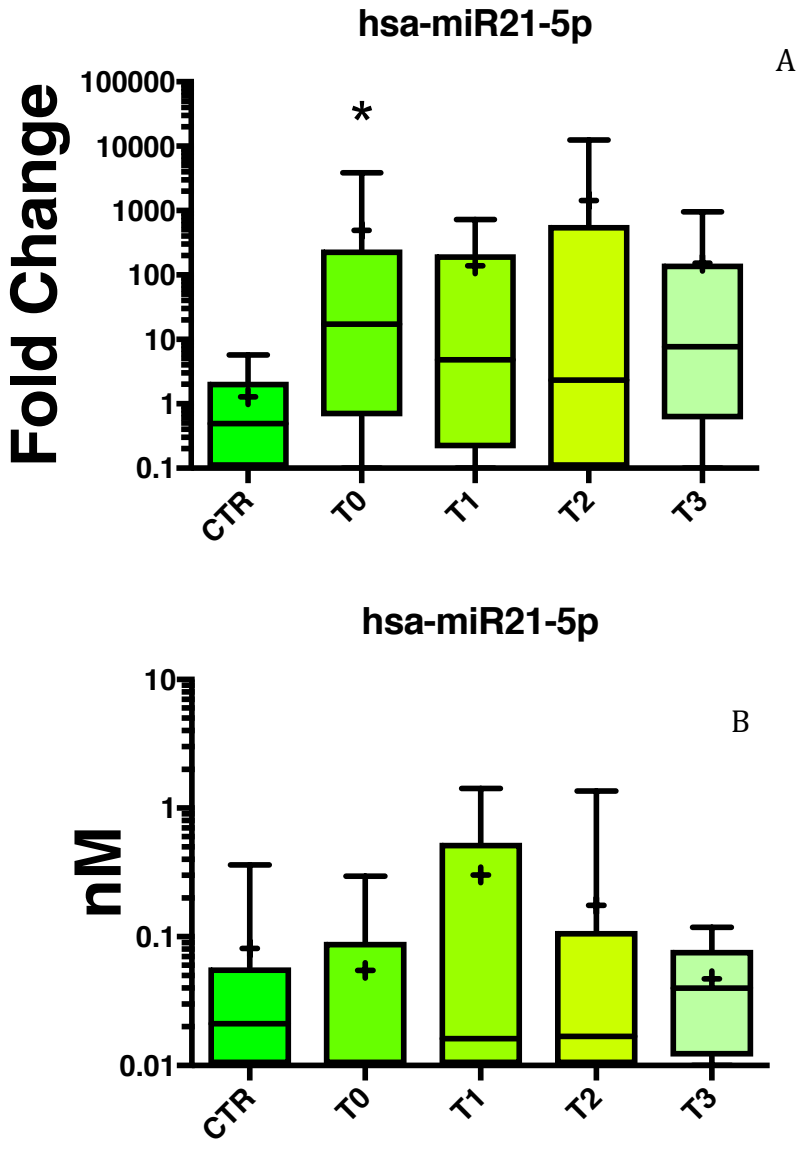

Figure 11. Fold-change (A) and absolute (B) miR21 expression

No significant differences were found between non-failing and failing subjects and at any time in postoperative period for miR-223, miR-96a, miR-26 and miR-378.

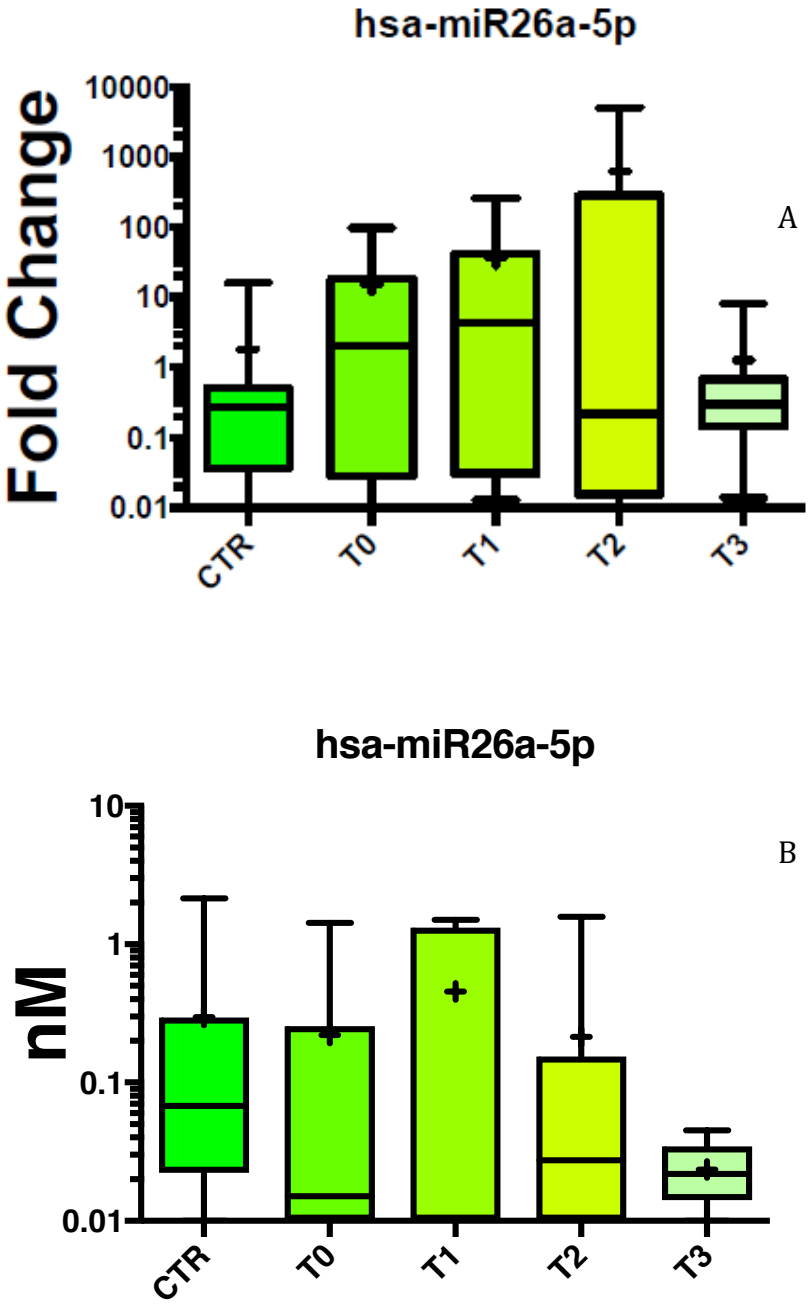

Figure 12. Fold-change (A) and absolute (B) miR26 expression

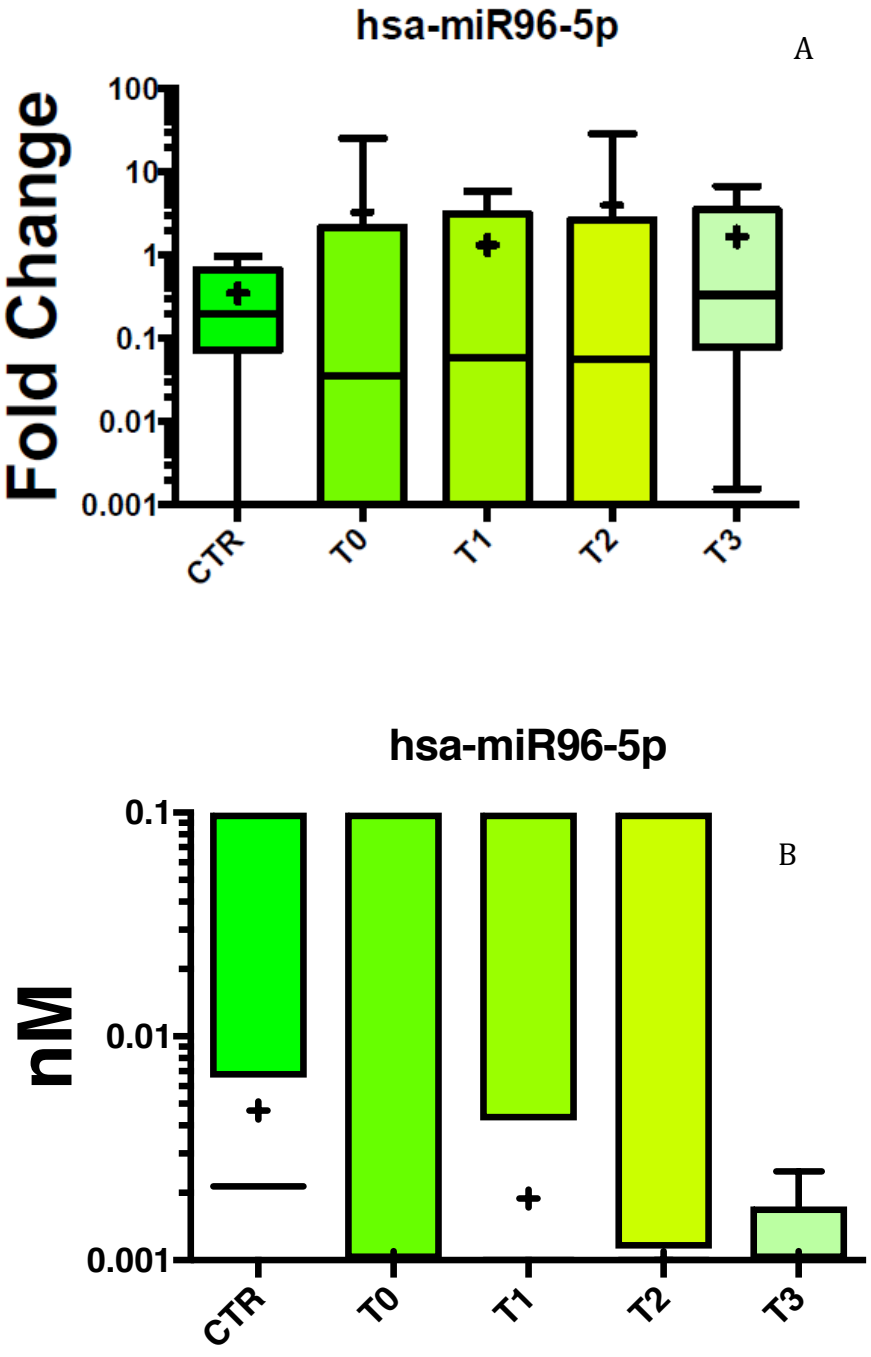

Figure 13. Fold-change (A) and absolute (B) miR96 expression

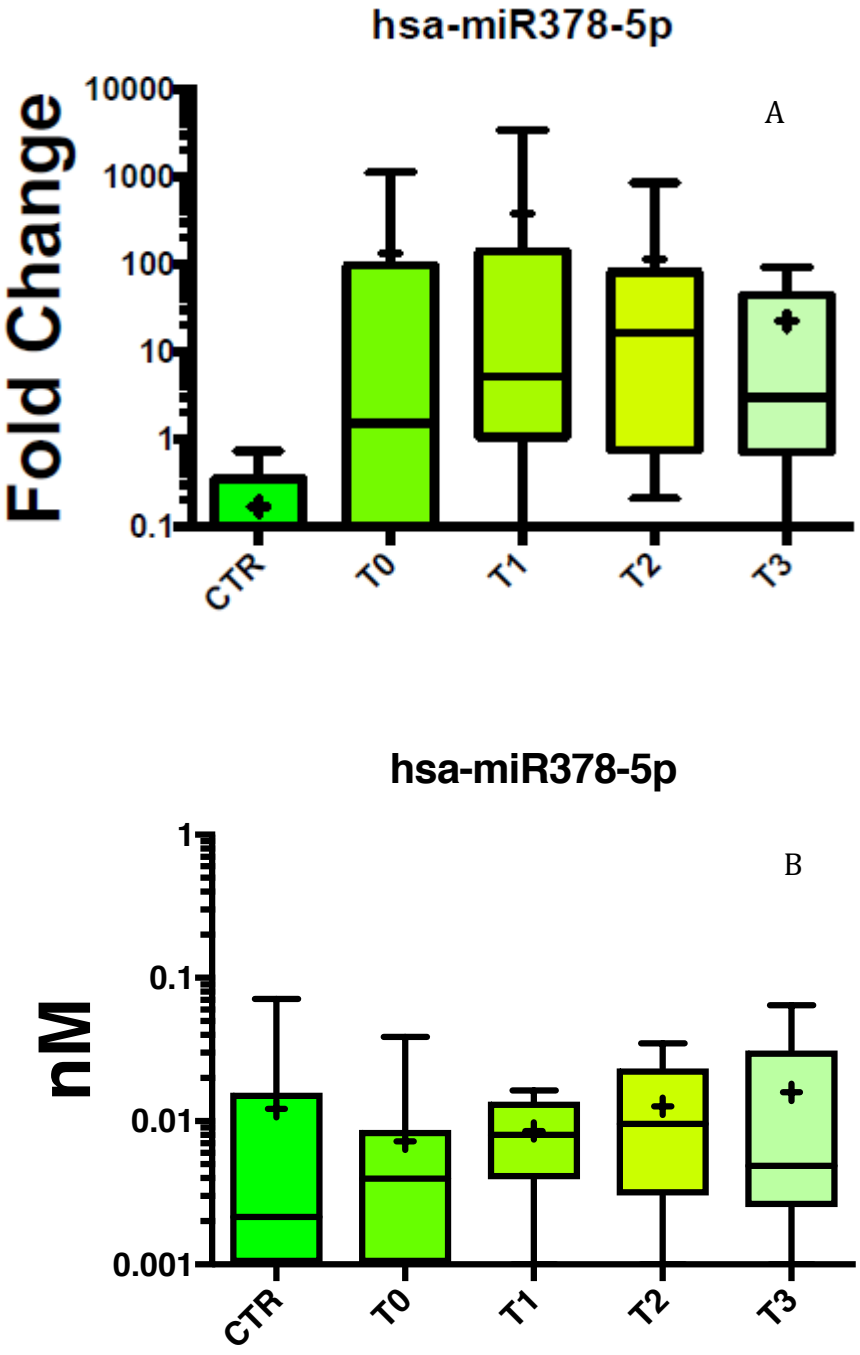

Figure 14. Fold-change (A) and absolute (B) miR2378 expression

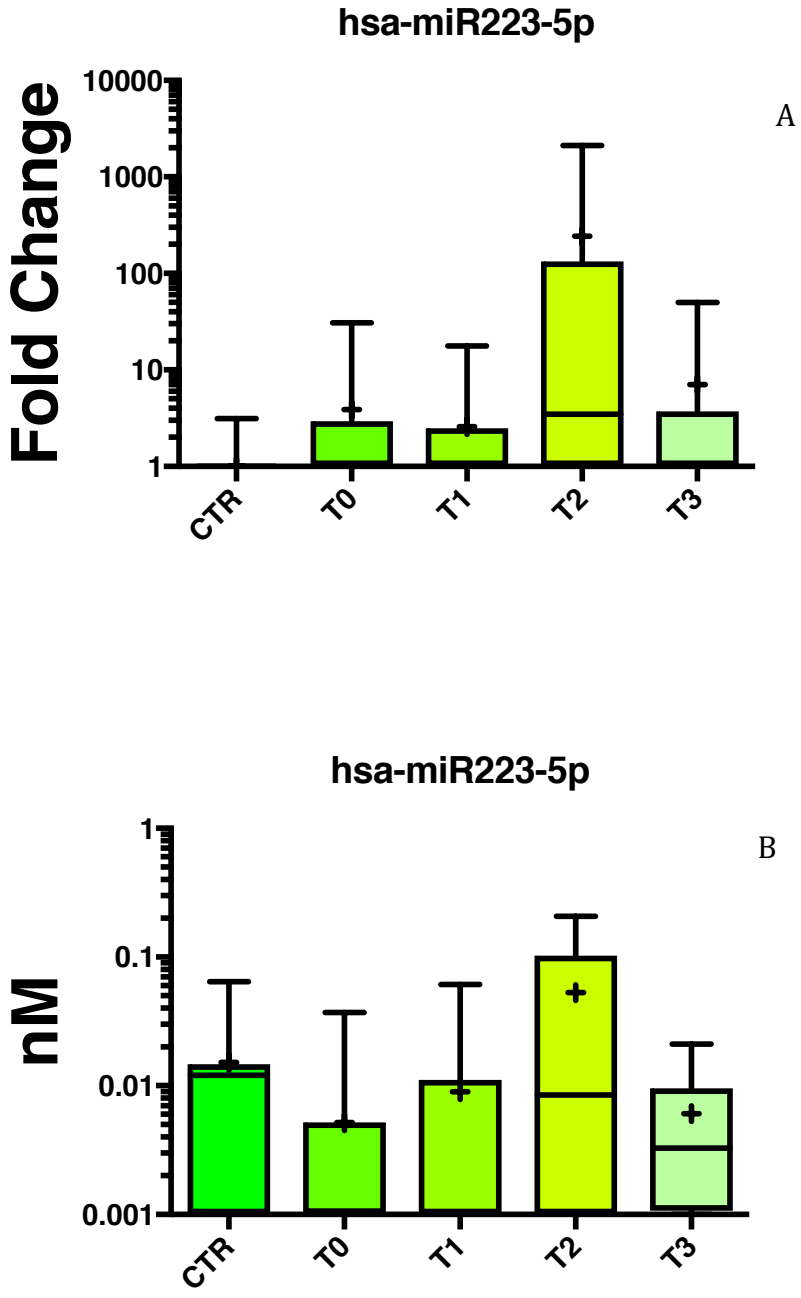

Figure 15. Fold-change (A) and absolute (B) miR223 expression

Unexpectedly, miRNA-1202 were not detectable in our population unless for two samples that present the minimum range concentration (Figure 11).

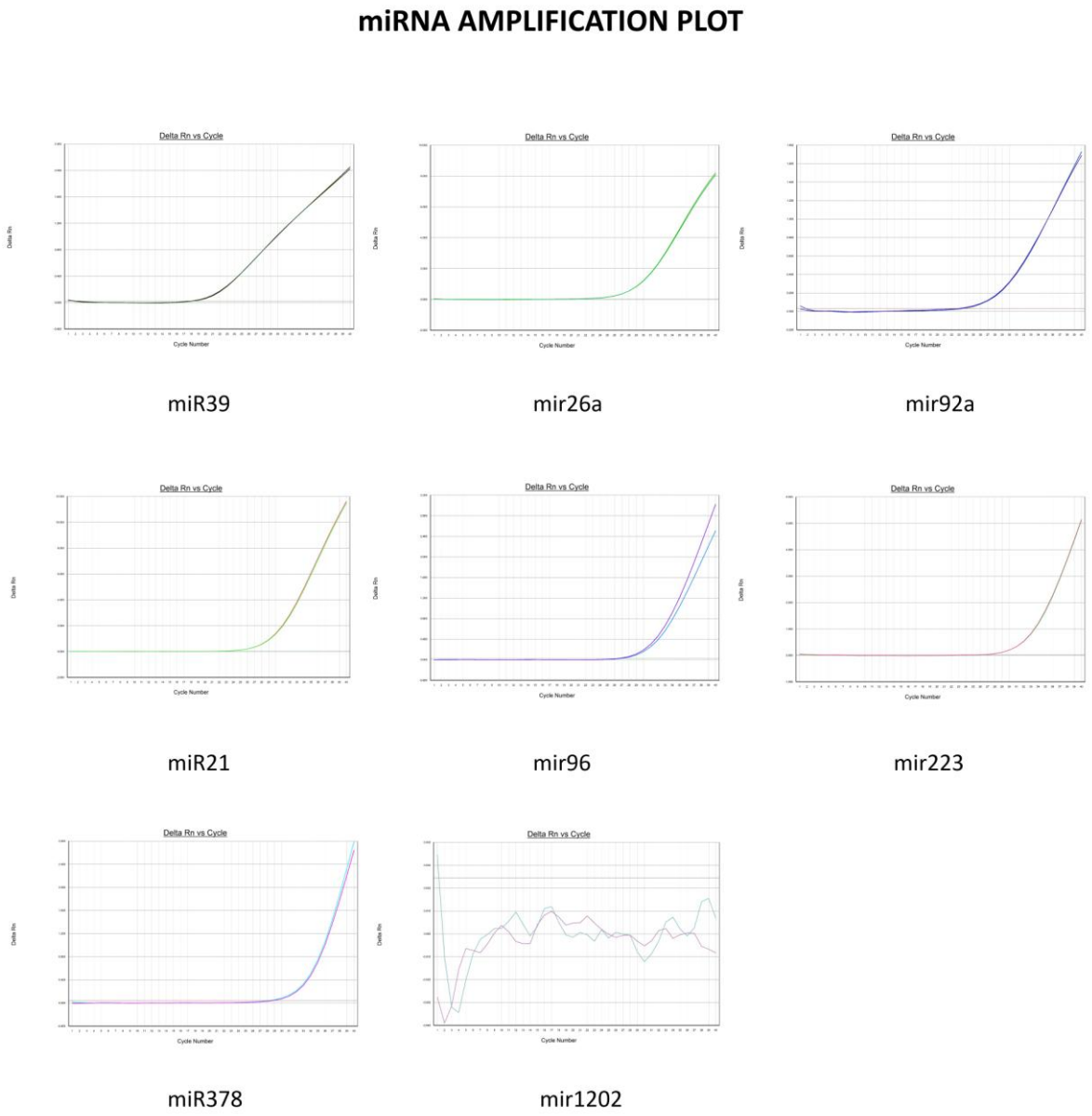

Figure 16. Example of a patient miRNA amplification plot

At T0 (failing patients before LVAD implantation) any miRNA didn't present a good correlation with BNP. The same result was observed at phase T2 and T3. At T1 (6 to 10 days after LVAD implantation) a significant correlation between miR-223 and BNP was observed ($p < 0.05$).

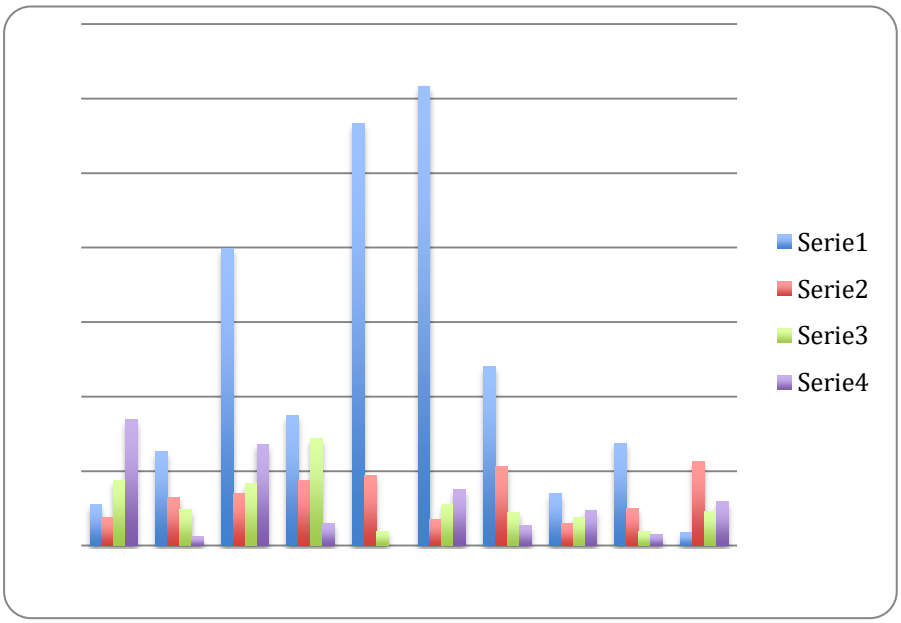

Figure 17. BNP for each patient at T0, T1, T2 and T3

In failing patients a significant correlation between mir378 and mir96 was observed. There was a significant correlation also between the following miRNA: miR-26 and miR96 ($p < 0.05$). , miR-223 and miR-21 ($p < 0.05$) and miR-21 and miR-96 ($p < 0.01$) at T1; miR-21 with miR26, miR-96 and miR-223 ($p < 0.05$) at T2. No correlation

between any miRs was observed at T3 (12 months after LVAD implantation). Despite the same trend of variation between failing and non-failing patients and during the time after LVAD implantation observed for miR-378 and miR-21, no significant correlation between both miRs was found.

Correlation between miRNAs, LVEDV, LVESV, LVEF, INTERMACS, and NYHA

In failing patients (T0), NYHA correlates with BNP and miRNA-26 ($p< 0.05$); BNP correlates with LVEDV ($p< 0.05$) and LVESV ($p< 0.01$) and INTERMACS level with miR-26 and LVESV ($p< 0.01$). In conclusion, both functional scores NYHA and INTERMACS correlates with miR-26 in refractory end-stage HF patients but any miRNA don't correlate with BNP the current gold-standard biomarker of heart-failure. One year after LVAD implantation, inverse correlations between left ventricular ejection fraction and miR-26 and LVEF and total Bilirrubin have been observed (p0.05).

P-Selectin variation during LVAD support

In our study serum P-Selectin levels were analyzed in heart failure patients before and after LVAD implantation at 10 days, 3 months and 12 months of mechanical support. No significant variation was observed before and after LVAD implantation and during the follow up (figure 18).

In our population thrombotic events happened in four patients including one stroke, two transient ischemic attack and one pump thrombosis. All events happened after one year of LVAD implantation.

No statistical differences were observed in P-Selectin levels between patients affected by thrombotic complications and patients without thrombosis (Mann-Whitney test, p=0,95).

According to ROC analysis, P-Selectin was not able to predict thrombotic events in our population .

Only miRNA 378 (expressed in absolute value) was able to predict thrombotic events (p 0.01) in our population.

An inverse significant correlation was found between the levels of P-Selectin and the miRNAs associated to platelet activation mir96-5p and miR223-5p (p 0.043 and p 0,045 respectively)

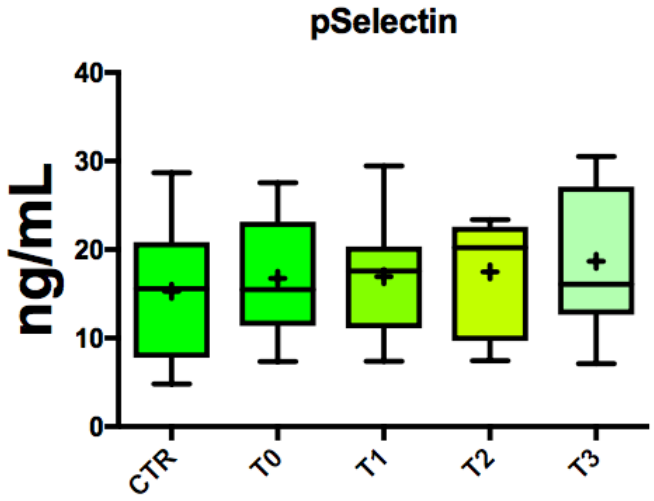

Figure18. P-Selectin variation in LVAD patients

Platelets

The mean platelet count at any time is shown in figure 19. Significant differences were found between T1 and T2 (p 0,043) with an increase in platelet count that was no associated with thrombotic events. A significant difference between platelet count at T2 and T3 (p 0,014) was found with low mean platelet count at T3.

No significant differences were observed between mean platelet count of patients within thrombosis at T3 and patients that didn't develop thrombotic complications at the same time.

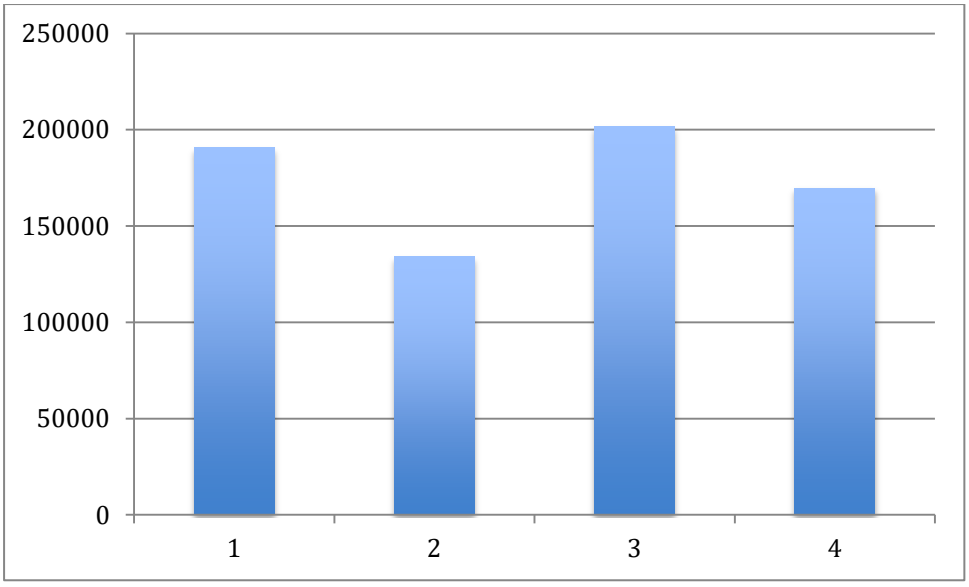

Figure 19. Platelet count variation before LVAD implantation (1:T0), after 6 days (2:T1), 3 months (3:T2) and 12 months (4:T3) of LVAD implantation.

E) Discussion

Micro-RNAs are small, non-coding RNAs that bind mRNAs at their 3'-untranslated regions, stimulating mRNA degradation or inhibiting protein translation (90). Currently, miRNAs are emerging as important post-transcriptional regulators of gene expression, with each miRNA predicted to regulate hundreds of target genes (56). Recent data indicate that miRNAs are key regulators of cardiac development, contraction, and conduction and can be altered in heart disease. These miRNA changes likely contribute to heart disease pathogenesis by mediating pathological changes in gene expression. A different pattern of miRNA expression has been observed between heart disease etiologies (65). One of the main aim of transcriptional profiling is that RNA from tissues or plasma may increase the accuracy of clinical diagnosis and prognosis in several settings including cardiovascular diseases, leading to optimal management and potential improve of survival. Nowadays, the investigation of non-coding RNA represents a new research field in the context of heart failure. The non-coding RNAs include microRNAs, long non-coding RNAs and circular RNAs. MicroRNAs regulate cellular process and long non-coding RNAs are also involved in gene expression and protein levels. Recently, non-coding RNAs have been shown to be of great value as therapeutic targets in adverse cardiac remodeling and also as diagnostic and prognostic biomarkers for heart failure (91). The ultimate current treatment for end-stage heart failure is cardiac transplantation, but in the lack of unlimited organs and considering the discrepancy between the number of donor hearts available and the number of patients awaiting heart transplant, ventricular assist devices represent a valid therapeutic option both as a bridge to transplant or as a destination therapy. LVADs provide volume and pressure

unloading of the left ventricle, reversing the compensatory responses of the overloaded myocardium, resulting in partial "reverse remodeling" (92,93,94). Clinical experience with LVAD support has shown that a subset of patients could be weaned from the device after restoration of basic cardiac function, so called bridge to recovery (95,96.97). Nowadays, the main life-threatening complications of VAD remain still associated to bleeding and thrombosis events, infections and right ventricular failure. In this study, the aims were to analyze the expression of some less known miRNAs related to fibrosis and platelet function in patients with end stage heart failure compared to a healthy population, and to analyze the dynamic modification of these miRNAs' expression after short and long-term periods of mechanical support and to evaluate the correlation between miRNAs levels and some clinical and laboratory parameters.

Most miRNAs are more ubiquitously expressed and are not cell-type specific and this could be a limit when analyzing circulating miRNAs. Many miRNAs are expressed at low levels under basal stable conditions but during pathological stress are strongly down- or upregulated (77). For example, the neurologic-enriched miRNA miR-212/132 family becomes activated during heart failure regulating cardiac hypertrophy in cardiac myocytes and leading to hyperactivation of the hypertrophic calcineurin/NFT signaling pathway (77). In an animal model the inhibition of miRNA 132 has been demonstrated to be a novel therapeutic tool able to rescued cardiac hypertrophy and heart failure (98).

Identifying and monitoring miRNAs associated to adverse remodeling and heart failure may have diagnostic, prognostic and therapeutic impact. Cardiac fibrosis represents an important hallmark in adverse cardiac remodeling. Some authors have

demonstrated that miRNA-21 is enriched in cardiac fibroblast and could be a potential therapeutic target in this setting (99). Furthermore, monitoring the level of miRNAs associated to cardiac hypertrophy could be a useful prognostic and therapeutic tool in adverse remodeling diseases.

The cardiac endothelium seems also to have an important role in heart failure. Fiedler *et al* have demonstrated that blocking miRNA -24 expressed in cardiac endothelial cells could lead to improved cardiac function and survival (100). Even, the evaluation of circulating miRNA patterns is able to distinguish heart failure with reduced ejection fraction and HF with preserved EF (101).

Some authors have already demonstrated the modification of several tissue and circulating miRNAs families in patients supported by LVAD (78, 94,102).

In our study, we selected to analyze a circulating miRNA related to cardiac fibrosis (miRNA-21), and some less frequently studied miRNAs associated with cardiac remodeling (miR-378 and miRNA 1202) and platelet activity (miRNA-223, miRNA-26 and miRNA-96). Based on previous published data, we used miRNA 92a for endogenous normalization and miRNA-39 for exogenous normalization.

Unexpectedly, miR-1202 was not expressed in our population.

Significant differences were observed in levels of miRNA 21a between healthy volunteers and patients before LVAD implantation. On the contrary, no differences were observed before and after LVAD implantation at any time. Same results were observed for miRNA 223, miR-96a, miR-26 and miR-378.

Matkovich et al (103) demonstrated that twenty-eight miRs were upregulated in congestive heart failure, with nearly complete normalization with left ventricular assist device treatment. Moreover, the authors showed that miRNAs are more

sensitive than mRNAs to the acute functional status of end-stage HF and present potential important regulation functions in the myocardial response to stress.

In contrast, several authors have demonstrate that messenger RNA (mRNA) signatures varies little after mechanical support (50,104, 105, 106).

Matkovich et al (95) demonstrated that miR-21 were upregulated in human heart disease. On the contrary, other authors found either invariant or downregulated miRNAs.

Ikeda and coworkers reported downregulation of miR-21 in end-stage HF due to dilated cardiomyopathy and aortic stenosis, although the magnitude of expression changes was generally small (65). In our study there was significant difference in miR-21 between healthy subjects and heart failings and higher levels were observed in heart failure patients. The levels of miR-21 were stable from several days from LVAD implantation to more than a year, concluding that prolonged LVAD support didn't normalized miR-21 expression. So, it could be hypothesized that patients with DCM presented a fibrotic picture at the time of VAD implantation that could not be reversible after long-term mechanical assistance.

Other authors have studied the dynamic expression of microRNA in patients supported by LVAD. Schipper *et al* (48) demonstrated that the trend of regulation of miRNA expression during LVAD depends on the etiology of heart failure. In this study, the authors reported that LVAD support leads to a decrease of the expression of cardiac tissue miR-1, miR-133a, and miR-133b in DCM patients but to an increase in expression in IHD patients. Both DCM and IHD presented lower miRNA levels (downregulation) compared with normal hearts . The small amount of patients affected by ischemic heart disease in our population (20%) didn't allow to perform this type of sub-analysis in our study.

Schipper et al (48) have demonstrated that in DCM patients, non- significant restoration of expression of miRs occurred after LVAD support. According to the authors, the main reason for this is the less dramatic reduction of miRs in DCM compared with IHD patients with heart failure. For this reason, during LVAD support, restoration in DCM is more difficult than in IHD. The authors advocated a cell proliferative reparative mechanism (such as in IHD) rather than a reduction in hypertrophy (such as in DCM) associated to miRs expression. In our study 80% of patients were affected by DCM and, as in Schipper's study, it could be assumed that this could explain the no significant modification of the most of circulating miRs analyzed on our study.

Several factors could be related to the lack of significance in miRNAs variation in our population. Firstly, the small size of our population. Secondly, analyzing plasmatic circulating miRNA instead of tissue miRNA could lead to measure too low plasmatic concentrations that could be undetectable with the current method. Another factor that could explain the lack of significance could be that the 80% of our population was affected by idiopathic cardiomyopathy. Other authors have demonstrated that some miRNAs such as miRNA 21 present less variability depending on the etiology of end stage HF and in case of DCM the degree of variation is less evident. (48, 65)
Other limits of our study could be related to factors associated with the interindividual biological variability of miRA concentration, errors in sample collection and storage. Moreover, other technical limitations could be associated with, the efficacy of RNA extraction and purification procedures and so the variation in the amount of the starting material, and the reverse transcription efficiency that can contribute to quantification errors. For these reasons, it is crucial to perform adequate

normalization controls when quantifying miRNAs. In our study, miR-92a was used for endogenous normalization. In our population miR-92a didn't present constant plasmatic concentrations in healthy volunteers, failing patients and during LVAD. In fact, unlike other endogenous normalizators, a high variability of miR92a concentrations between patients has been observed in our population. For example, some patients showed very low concentrations of endogenous miR-92 so this resulted in very extremely high fold-change values and, in conclusion, miR-92 resulted a non-useful endogenous normalizator. In order to overcome this drawback, miR-39 was used to perform exogenous normalization and the miRNAs' concentrations were expressed as absolute values. Again, no significant differences in miRNAs levels between failing and non failing patients were found in our population. Neither significant differences between all the three phases after LVAD implantation were observed for all analyzed miRNAs.

In our experiment, the expression of fold-change of miR-96 presents a stable pattern (figure 11). Analyzing absolute values instead of fold-change values of miRNA-96, the correlation between controls (healthy patients) and failing patients (T=0) becomes closed to significance (P=0,058).

Analyzing fold change of miR21 there was a significant difference between non-failings (controls) and failings (T0) which agrees with data previously published in literature by other authors (78).

Regarding the curves of miR-378, miR-26 and miR-223, the comparison between non-failing, failing subjects and the three post VAD implantation phases showed that these miRNAs didn't reach the statistical significance but presented an interesting trend. All three miRNAs, presented higher plasmatic levels in failing patients rather than in healthy volunteers, maintained stable levels in T1 (some days after implantation), and

tend to reduction after 3 months of mechanical support (T2) reaching values close to those of healthy subjects after one year of mechanical assistance.

Lok and coworkers (78) analyzed the expression pattern of several myocardial and plasmatic miRNAs including miR-21 and miR-378 in patients with end-stage non–ischemic DCM and supported with pulsatile and continuous LVAD. Circulating miR-21 decreased at 1, 3 and 6 months after LVAD implantation. Other miRNAs such as miR-146a, miR-221 and miR-222 showed a fluctuating pattern post-LVAD. As in our study, Lok and colleagues, observed that myocardial miR-21 changed non-significantly in continuous flow-LVAD (cf-LVAD) but showed a trend of reduction between pre and post- VAD implantation. On the contrary, prior to implantation, levels of plasmatic miR-21 were doubled compared to controls, decreased significantly during support, but did not normalize. In our study we observed the same trend but without significant differences (Figure 12).

MiR-21 is very abundant in cardiovascular system and several studies (76) have revealed that its expression is deregulated in heart and vascular under cardiovascular disease conditions. On the contrary in our study we found an upregulation of plasmatic miR-21 in heart failure patients.

In Lok investigation, several myocardial miRs including miR-378 that have previously been reported to be differentially expressed between pre- and post- pulsatile flow-LVAD (pf-LVAD) did not significantly change in cf-LVAD. In our study, plasmatic miR-378 presented non-significant higher values in failing patients that tend to reduce after LVAD implantation but don't normalize after one year (Figure 16).

In our experiment, the fold-change miRNA-223 expression was the same in healthy and failing patients, and after few days and after 12 months of LVAD support. On the contrary, absolute levels of miR-223on healthy patients were no significantly higher

than in end-stage HF patients (figure 17). miR-223 has a key role mainly in the development and homeostasis of the immune system and hematological system (107). Recently, an emerging role in the platelet function regulation has been investigated. miR-223 is the most abundant miRNAs in megakariocytes and platelets and plays an important role in regulating gene expression (108). Recent clinical cohort study showed that the level of circulating miR-223 is inversely associated to major cardiovascular events (MACEs) in coronary heart disease. In addition, other authors (107) demonstrated that the level of both intraplatelet and circulating miR-223 is an independent predictor for high on-treatment platelet reactivity (HTPR), thus providing a link between miR-223 and MACE (109). These authors suggested that miR-223 may serve as a potential regulatory target for HTPR, as well as a diagnostic tool for identification of HTPR in clinical settings. So we hypothesized that it could be a correlation between miR-223 expression in LVAD patients and thrombotic or hemorrhagic events.

Regarding BNP, as published by other authors before, in our study BNP normalizes immediately after LV unloading by LVAD. In a recent study (110) postoperative BNP levels decreased dramatically in 2 days with the initiation of LVAD support and the pump speed settings (RPM) were correlated with the changes in BNP levels. The levels correlated inversely with the degree of hemodynamic support rendered at various degrees of LVAD support. The effective LVAD support seemed to result in a marked reduction in BNP levels, and the authors concluded that monitoring serial BNP levels may be helpful in managing patients supported on continuous LVAD.
For other authors, BNP has been considered a good marker to guide therapy during the early postoperative period leading to a significantly lower length of hospital stay

(LOS) (111). BNP is also considered a good marker of long-term prognosis in patients implanted with a left ventricular assist device. Sato and coworkers (112) retrospectively analyzed a cohort of 83 patients treated with LVAD and demonstrated that BNP concentration measured 60 days after implantation was significantly associated with all-cause mortality. For these authors, the optimal BNP cutoff value to predict death during LVAD support was 322 pg/ml with a sensitivity of 71,4% and a specificity of 79.8% . Moreover, Hellman et al (113) demonstrated that pre-LVAD implantation BNP levels remained the single most powerful predictor of ventricular arrhythmia post LVAD implantation.

Regarding functional status after LVAD implantation, the mean 6-minute-walk-distance at three months was 325 m (290-414) and this value agrees with previously published data by Miller et al. (39). There was not a significant difference in patient functional performance between after three months of LVAD support and after 12 months measured by 6MWT (P= 0.2) in our study . 6MWD is a sub-maximal exercise test that assesses global functional capacity by measuring distance walked over six minutes. It is volitional and can be limited by musculoskeletal and cardiopulmonary deficits (114). On the other hand, Cardiopulmonary exercise testing (CPET) evaluates maximal exercise capacity by measuring a number of physiological markers during increasing exercise loading, and is limited by exhaustion (115). Both 6MWD and $VO_{2\ max}$ tests have been demonstrated to have prognostic implications in HF (116) and it has recently been found that the postoperative 6MWD was the strongest predictor of late post-LVAD mortality and persistent post-operative exercise intolerance (117). Notwithstanding, only small trials have demonstrated a moderate correlation between 6MWD and $VO_{2\ max}$ in LVAD patients, so in order to establish the real

functional status of the patient and as a prognostic tool, it may be desirable to perform a more accurate test such as cardiopulmonary exercise (CPET) in the follow up of these patients.

Regarding P-Selectin, some studies have demonstrated that serum P-Selectin levels are increased in pathologies associated to inflammation and thrombosis. Katayama et al demonstrated that P-Selectin was present in concentrations ranging from 19 to 521 ng/ml (mean +/- SD = 121 +/- 84 ng/ml). Plasma P-Selectin levels were significantly elevated in patients with thrombotic thrombocytopenic purpura (12 patients, 332 +/- 184 ng/ml, $P < 0.001$) and haemolytic uraemic syndrome (17 patients, 297 +/- 191 ng/ml, $P < 0.0001$), as compared to the normal levels.

Matsubayashi et al demonstrated that following LVAS (Novacor) placement, resting platelets shown increased expression of activated platelets such as P-selectin. Houël et al analyzed platelet activation in patients supported by Thoratec VAD after six weeks of implantation measuring the spontaneous expression of P-Selectin (CD62P) on the platelet membrane surface. CD62P values remained increased during a 5-week postoperative period. The platelet activation profile displays a persistent activation with a preserved reactivity associated with a persistent high inflammatory state and endothelial activation (82).

On the contrary, in our study levels of levels of P-Selectin in heart failure were not significantly different to healthy volunteers and P-Selectin did not modify after implantation of LVAD and in 3 and 12 months-follow up. P-Selectin was not able to predict thrombotic events in our population. Consequently, according to our results, P-Selectin is not a good predictor of thrombotic events in patients supported with Jarvik LVAD.

Similar results have been found by other authors. Nascimbene et al (118) analysed the association between cell-derived microparticles including P-selectin and adverse events in patients supported with nonpulsatile left ventricular assist device HeartMate II LVAD. The authors found no difference in the levels of P-Selectin before LVAD implantation, after LVAD implantation and 3-months follow up. The authorsdid not detect a significant difference in the levels of these markers between patients who had adverse events and those who did not.

In our population P-Selectin showed an interesting inverse significant correlation with miR-96. MiRNA-96 has been associated with a reduction in high platelet reactivity (79) and P-Selectin with an increase in platelet reactivity, so increased levels of miR-96 and low levels of P-Selectin.

Despite both P-Selectin and miR-223 has been reported to be released by activated platelets, and inverse correlation was found between levels of P-Selectin and levels of miR-223 in our study. MiR-223 regulates the expression of the functionally important platelet purinergic P2Y12 adenosine diphosphate receptor (80).

Despite miRNA-378 has not been described to be associated to thrombotic diseases before (119), it was the only miRNA able to predict thrombotic events (p 0.01) in our population.

F) Final remarks:

Our study presents several limits that could condition the lack of significance of the results. The main limit is the small size of our population due to some pre-analytical difficulties and considering that only patients who were supported for more than a year and that performed their follow up at Udine were able to be recruited. Another limit is that most miRNAs (such as miR-223) are ubiquosly expressed and this could influence the results when plasmatic and not tissue miRNA expression is analyzed. Circulating stead of cardiac tissue miRNA were analyzed so it is possible that the undetectable levels of some miRNAs such as miR-1202 where associated to the efficacy of miRNA extraction procedure and cDNA synthesis. Moreover, the hemodilution of the miRNA in whole plasma could lead to an analysis of too low concentrations of miRs for reliable statistical analysis. Another limit could be the low sensibility of the current real time instrument. Maybe a comparison with a myocardial tissue sample could be a control of adequate method of analysis, but only an invasive procedure such as endomyocardial biopsy could afford to obtain tissue at 6 days, 3 moths and 12 months of LVAD support.

The high prevalence of idiopathic dilated cardiomyopathy (80%) in our population could influence the low modification of some miRNAs such as miR-21 during LVAD support.

Despite all these limits, this preliminary study has allowed us to refine the methodology being concern regarding the difficulties of miRNAs detection specially in a so particular population such as LVAD supported patients that is burdened by a series of pre-analytical limitations.

Currently, the analysis of plasmatic miRNAs compared with tissue miRNA has been less studied and only small studies have been published in literature. This remains still a new method to be refined and further large studies are necessary to completely understand the potential diagnostic, prognostic and therapeutic role of miRNAs in end-stage heart failure. But our study and other studies in this field lay the foundations for a future framework, leading to an innovative diagnostic and therapeutic approach to a so severe pathology still burdened by high mortality. In addition, in the future this approach could help to identify which are the right medical and mechanical therapies for each specific patient in a social context of limited resources**.**

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V. LIST OF FIGURES

VI. LIST OF TABLES

VII. **Appendix I: INTERMACS classification**

INTERMACS® 1: Critical cardiogenic shock describes a patient who is "crashing and burning", in which a patient has life-threatening hypotension and rapidly escalating inotropic pressor support, with critical organ hypoperfusion often confirmed by worsening acidosis and lactate levels.

INTERMACS® 2: Progressive decline describes a patient who has been demonstrated "dependent" on inotropic support but nonetheless shows signs of continuing deterioration in nutrition, renal function, fluid retention, or other major status indicator. Patient profile 2 can also describe a patient with refractory volume overload, perhaps with evidence of impaired perfusion, in whom inotropic infusions cannot be maintained due to tachyarrhythmias, clinical ischemia, or other intolerance.

INTERMACS® 3: Stable but inotrope dependent describes a patient who is clinically stable on mild-moderate doses of intravenous inotropes (or has a temporary circulatory support device) after repeated documentation of failure to wean without symptomatic hypotension, worsening symptoms, or progressive organ dysfunction (usually renal). It is critical to monitor nutrition, renal function, fluid balance, and overall status carefully in order to distinguish between a patient who is truly stable at Profile 3 and a patient who has unappreciated decline rendering him a Profile 2. This patient may be either at home or in the hospital.

INTERMACS® 4: Resting symptoms describes a patient who is at home on oral therapy but frequently has symptoms of congestion at rest or with activities of daily living. He or she may have orthopnea, shortness of breath during activities such as dressing or bathing, gastrointestinal symptoms (abdominal discomfort, nausea, poor appetite), disabling ascites or severe lower extremity edema. This patient should be carefully considered for more intensive management and surveillance programs, which may in some cases, reveal poor compliance that would compromise outcomes with any therapy.

INTERMACS® 5: Exertion Intolerant describes a patient who is comfortable at rest but unable to engage in any activity, living predominantly within the house or housebound. This patient has no congestive symptoms, but may have chronically elevated volume status, frequently with renal dysfunction, and may be characterized as exercise intolerant.

INTERMACS® 6: Exertion Limited also describes a patient who is comfortable at rest without evidence of fluid overload, but who is able to do some mild activity. Activities of daily living are comfortable and minor activities outside the home such as visiting friends or going to a restaurant can be performed, but fatigue results within a few minutes of any meaningful physical exertion. This patient has occasional episodes of worsening symptoms and is likely to have had a hospitalization for heart failure within the past year.

INTERMACS® 7: Advanced NYHA Class III describes a patient who is clinically stable with a reasonable level of comfortable activity, despite history of

previous decompensation that is not recent. This patient is usually able to walk more than a block. Any decompensation requiring intravenous diuretics or hospitalization within the previous month should make this person a Patient Profile 6 or lower.

(Stevenson LW, Pagani FD, Young JB et al. INTERMACS profiles of advanced heart failure: the current picture. J Heart Lung Transplant 2009;28:535-41)