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Extracellular Vesicles Mediate of Cardiac Fibrosis in Heart Failure With Preserved Ejection Fraction

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Dissertação

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ABSTRACT

Cardiovascular diseases (CVDs) represent the leading cause of death across the globe. Among the different types of CVDs, heart failure with preserved ejection fraction (HFpEF) has a prevalence of up to 5% on the general population and prognosis is very poor. Presently, effective therapies for this syndrome are a major unmet clinical need. Both aging and chronic systemic inflammation have been advanced as important triggers of myocardial stiffening and diastolic dysfunction, but underlying mechanisms are largely unknown.

Extracellular vesicles (EVs) are important carriers of biological content and have been explored as mediators of long-distance intercellular communication in the pathophysiology of various systems, including the heart. Hence, one can hypothesize that circulating EVs may contribute toward the development of cardiac fibrosis in the context of HFpEF. To test this hypothesis, blood samples were collected from HFpEF and control patients with matched age, sex and comorbidities. Then, human cardiac fibroblasts were exposed to serum from the two patient cohorts. Circulating factors from the blood of HFpEF patients were able to induce fibroblast activation and increased expression of pro-fibrotic markers when compared with the control group. These results demonstrate that the systemic environment of HFpEF patients may contribute to fibroblast activation in the heart. To evaluate whether EVs mediate this effect, size-exclusion chromatography combined with ultrafiltration were used to successfully isolate EVs from these patients. The EV-enriched preparations were characterized and no differences were found in terms of EV integrity, size and concentration across the two groups. Following EVs characterization, human cardiac fibroblasts were exposed to EVs, inducing significant upregulation of pro-fibrotic genes when compared with EVs isolated from control patients. These findings advocate that EVs may carry fibrogenic molecules from the circulation to the heart and contribute to the development of interstitial cardiac fibrosis in HFpEF; further studies are needed to dissect the underlying mechanisms.

RESUMO

As doenças cardiovasculares representam a maior causa de morte em todo o mundo. Entre os diferentes tipos de doenças cardiovasculares, a insuficiência cardíaca com fração de ejeção preservada (HFpEF) tem uma prevalência até 5% na população e o prognóstico é muito fraco. Atualmente, há uma grande inexistência de terapias eficazes para esta síndrome. Tanto o envelhecimento como a inflamação crónica sistémica têm sido apontados como fatores importantes no desencadeamento da rigidez do miocárdio e disfunção diastólica, mas os mecanismos que levam ao seu desenvolvimento ainda são bastante desconhecidos.

As vesículas extracelulares (EVs) são transportadores de conteúdo biológico muito importantes e têm sido explorados enquanto mediadores de comunicação intercelular de longa distância na patofisiologia de vários sistemas, incluindo o coração. Desta forma, é possível formular a hipótese de que EVs circulantes podem contribuir para o desenvolvimento de fibrose cardíaca no contexto de insuficiência cardíaca com HFpEF. Para testar esta hipótese, foram colhidas amostras de sangue de pacientes com HFpEF e de um grupo controlo com idade, sexo e comorbidades semelhantes. Depois, fibroblastos cardíacos humanos foram expostos a soro dos dois grupos de pacientes. Fatores circulantes presentes no sangue de pacientes de HFpEF induziram ativação nos fibroblastos e aumentaram a expressão de marcadores de fibrose em comparação ao grupo controlo. Estes resultados demonstram que o ambiente sistémico de HFpEF pode contribuir para a ativação de fibroblastos no coração.

Para compreender se as EVs são responsáveis por este efeito, as EVs foram corretamente isoladas através de uma combinação entre cromatografia de exclusão por tamanho e ultrafiltração a partir destes pacientes. As preparações enriquecidas em EVs foram caracterizadas e não foram encontradas diferenças em termos de integridade, tamanho ou concentração das EVs entre ambos os grupos. Seguidamente à caracterização, fibroblastos cardíacos humanos foram cultivados com EVs, que induziram um aumento na expressão de genes pro-fibróticos em EVs isolados de pacientes com HFpEF em comparação a EVs de pacientes controlo. Estas descobertas demonstram que as EVs podem transportar moléculas indutoras de fibrose existentes em circulação até ao coração, e contribuir para o desenvolvimento de fibrose cardíaca intersticial; serão necessários estudos subsequentes para a disseção destes mecanismos.

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ÍNDEX

LIST OF ABBREVIATIONS

ACEi -angiotensin-converting enzyme inhibitors
AFM – Atomic Force Microscopy
ARB – Angiotensin receptor blockers
Asc-2P - 2-phospho-L-ascorbic acid
BCL-2 - B-cell lymphoma-2
BMI – Body-mass index
BNP – B-type natriuretic peptide
BSA- Bovine serum albumin
CD - Cluster of differentiation
CFs – Cardiac fibroblasts
CRP – C reactive protein
CTRL –Comorbidity-matched control group
CVDs – Cardiovascular diseases
Da - Dalton
DAPI- 4',6-diamidino-2-phenylindole
DLS – Dynamic Light Scattering
DMEM- Dulbecco's Modified Eagle's Medium
ECM - Extracellular matrix
ESC – European Society of Cardiology
ESCRT- Endosomal sorting complexes required for transport
EVs- Extracellular vesicle
FAP - Fibroblast activation protein
FBS – Fetal bovine serum
FGF- Fibroblast growth factor
FGM- human fibroblast growth medium
H - Hour
hCFs- Human cardiac fibroblast
HF – Heart failure
HFmrEF – Heart failure with midrange ejection fraction
HFpEF – Heart failure with preserved ejection fraction
HFrEF - Heart failure with reduced ejection fraction
HSP- Heat shock protein
hsTnI – high-sensitivity troponin I
ICAM - Intercellular adhesion molecule
IL - Interleukin
ISEV - International Society for Extracellular Vesicles
LAVI – Left atrial volume index
LV – Left ventricle
LVEF – left ventricular ejection fraction
MAPK- Mitogen-activated protein kinase
MCP - monocyte chemoattractant protein
MI- Myocardial infarction
min - Minutes
miR- microRNA
MISEV - Minimal information for studies of extracellular vesicles
MMP- Matrix metalloproteinase
MPs - Microparticles
MRA - Mineralocorticoid receptor antagonists
mRNA- messenger RNA
MVs - Microvesicles
n.a. – non-available
NO – Nitric oxyde
ns- Non-significant
NTA - Nanoparticle tracking analysis
NYHA – New York Heart Association
P/S - Penicillin-streptomycin
PASP – Pulmonary artery systolic pressure

PBMC - Peripheral blood mononuclear cell

PBS - Phosphate buffer saline

PKG - Protein kinase G

ROS - Reactive oxygen species

RT - Room temperature

RT-PCR - Real time-polymerase chain reaction

SD - Standard deviation

SDS - Sodium dodecyl sulphate

SEC - Size-exclusion chromatography

secs - Seconds

SEM - Standard error of the mean

SPM - scanning-probe microscopy

SPR - surface plasmon resonance

TEM - Transmission electron microscopy

TGF-ß - Transforming growth factor-ß

TNF-α - Tumor necrosis factor-α

UC - Ultracentrifugation

VCAM - Vascular cell adhesion molecule

WHO - World Health Organization

α-SMA - α-Smooth muscle actin

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CHAPTER I – INTRODUCTION

1.1. Epidemiology and Risk Factors of Cardiovascular Diseases

Cardiovascular diseases (CVDs) are a group of conditions that involve the heart and/or the blood vessels, such as heart failure (HF), ischemic heart disease, cerebrovascular disease, and others. It is estimated by the World Health Organization (WHO) that these diseases represent the number one cause of death worldwide, accounting for approximately 17,8 million deaths in 2017 [1] which represents 31% of the total world mortality (**Figure 1**) [2]. Due to the noticeable increase in life expectancy and the growing number of the global population, the number of CVDs is expected to rise to more than 23,6 million deaths by the year 2030, which constitutes an alarming estimate [2]. Apart from the genetic influence that may contribute to the occurrence of these diseases, the most relevant risk factors for CVDs include male gender, aging, obesity, diabetes, high blood pressure, hypercholesteremia and others that rely on individual behavior such as low physical activity or sedentarism, high alcohol consumption, smoking habits and bad eating habits with high fat consumption [3-5]. Patients with these comorbidities have increased risk of suffering a cardiovascular event and, importantly, higher risk of mortality after a cardiac event [2].

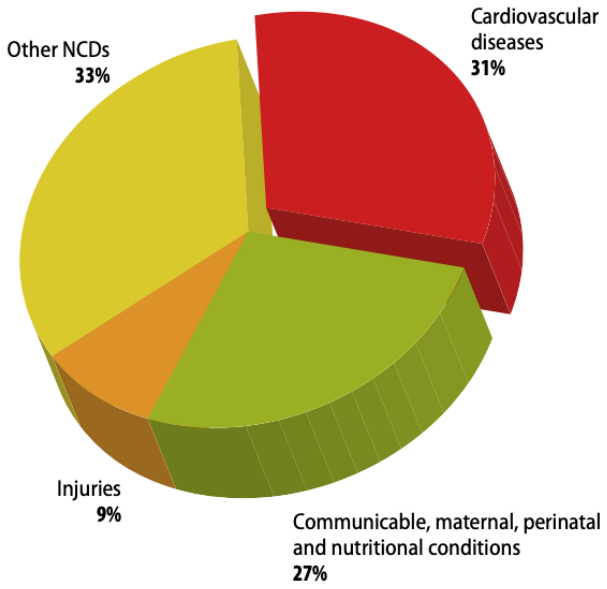


Figure 1 - Proportion of causes of death worldwide. Source: [2]. NCDs: non-communicable diseases

Among the different types of CVDs, HF constitutes a major health concern for patients and health care systems across the globe, affecting more than 64 million patients worldwide [6].

1.2. Heart Failure: from definition to diagnosis and treatment

According to the European Society of Cardiology (ESC), HF is a clinical syndrome caused by structural and/or functional heart abnormalities that result in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress [6].

The identification of the type of HF involves all levels of care, including general practitioners, internists, general cardiologists, HF specialists and invasive cardiologists. HF can be broadly classified, accordingly with the measurement of the left ventricular ejection fraction (LVEF), in heart failure with reduced ejection fraction (HFrEF) or systolic HF (LVEF < 40%), heart failure with preserved ejection fraction or diastolic heart failure (HFpEF) (LVEF ≥ 50%) [7] and heart failure with mid-range ejection fraction (HFmrEF) (LVEF 41-49%) (**Table 1**) [7]. Although, HFmrEF may progress into HFrEF or HFpEF, the pathophysiological mechanisms underlying these two subsets of HF are completely different. Several pre-clinical and clinical studies had proven that in HFrEF there is an inherent incapability of the left ventricle (LV) to contract and empty correctly, leading to increased diastolic volume and pressure and decreased ejection fraction. Usually, there are perturbations on calcium handling, intracellular energy supply and utilization and electrophysiological functions increasing and cardiomyocyte loss [8, 9]. Contrastingly, in HFpEF the LV is not efficiently filled since the heart becomes less compliant and unable to efficiently relax leading to increased LV filling pressures at rest and during exercise. With time, the patient will present orthopnea, ankle swelling, reduced exercise capacity and fatigue; symptoms that have a high impact on patient's quality of life [10].

Table 1 – Definition of the three types of heart failure according to the ESC guidelines (2016). Adapted from [6]

Type of HF		HFrEF	HFmrEF	HFpEF
CRITERIA	1	Symptoms ± Signs	Symptoms ± Signs	Symptoms ± Signs
	2	LVEF <40%	LVEF 40-49%	LVEF≥50%
	3	-	1. Elevated levels of natriuteric peptides; 2. At least one additional criterion: a. Relevant structural heart disease (LVH and/or LAE), b. Diastolic dysfunction	1. Elevated levels of natriuretic peptides; 2. At least one additional criterion: a. Relevant structural heart disease (LVH and/or LAE), b. Diastolic dysfunction

Typically, all HF subsets are associated with several comorbidities such as diabetes, obesity, hypertension, and aging [6, 11]. However, the prevalence of hypertension and coronary heart disease have been more associated to HFrEF [12], whereas comorbidities such as obesity and diabetes are more strongly related HFpEF development and mortality/hospitalization [13,

14]. Also, women present higher HFpEF prevalence than men and the contrary is observed in HFrEF patients (**Figure 2**) [11].

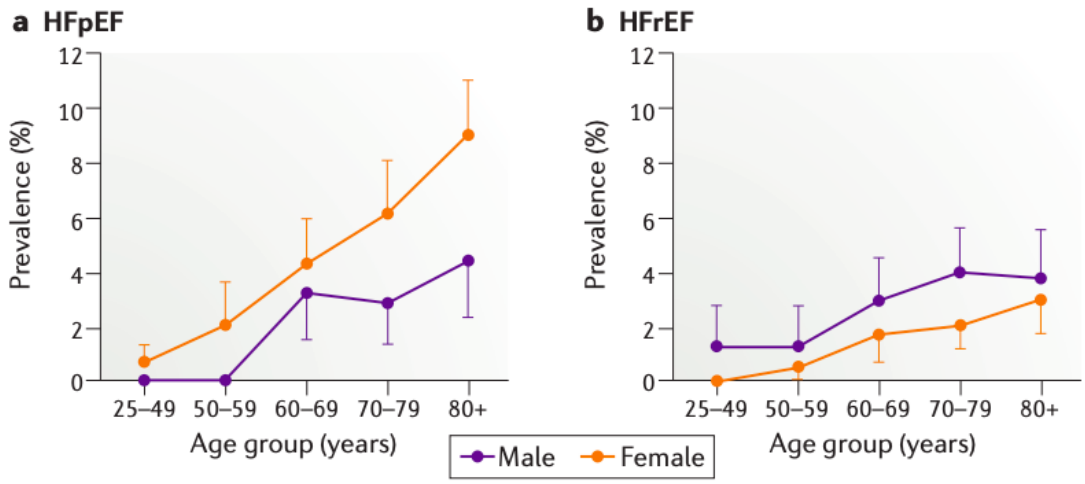


Figure 2 - Prevalence of HFpEF and HFrEF by age and sex in a southwestern European community-based cohort. Source: [11]

Due to changes in population demographics, the prevalence of hypertension and coronary heart disease is expected to decrease modestly and consequently the incidence of HFrEF is expected to decrease. Also, the outcome for HFrEF patients is improving due to effective therapies such as angiotensin-converting enzyme inhibitors (ACEIs) [15], mineralocorticoid receptor antagonists (MRAs) [16] and beta-blockers [17].

In the opposite direction, the prevalence of HFpEF is expected to continue to rise in aged societies [18-21]. HFpEF is commonly associated with a vast number of comorbidities (such as diabetes, obesity, hypertension, and aging), which translates into a multifactorial disease, with difficult and complex diagnosis. According to the ESC guidelines (2019) [10], the diagnosis is made in four steps [10]. In the pre-test assessment (step 1) patients with other cardiovascular alterations such as HFrEF or heart valve disease are excluded. This is followed by a second step, with detailed cardiac structure and functional measurements. For the intermediate phenotypes a step 3 is done to assess the exercise capacity of the cardiovascular system in stress. In the last step, it is important to characterize the etiology according to the patient's risk factors and comorbidities, to further advance in targeted therapies that are specific for each individual.

Nevertheless, a uniform definition of HFpEF is still challenging. There is yet to be discovered a marker for the diagnosis of HFpEF. In addition to the heterogeneity of comorbidities in these patients, the fact that levels of B-type natriuretic peptide (BNP), a marker for HFpEF, are frequently below the threshold used for diagnosis makes it difficult to diagnose this disease. Of note, a validated gold standard method for HFpEF diagnosis or screening is crucial but still missing [10]. Unfortunately, these diagnostic criteria of HFpEF exclude stages of the disease at which patients can display asymptomatic systolic or diastolic

LV dysfunction, enhancing the importance of understanding the progression of HFpEF in order to act before symptoms appear and the quality of life and life expectancy diminishes.

In addition to the challenges in diagnostics, to date there is still no evidence of effective therapies that show a reduction in HFpEF morbidity or mortality. HFpEF has a year mortality rate worldwide of 75,7% (**Figure 3**) [22]. Current approaches for these patients include the administration of diuretics for symptom attenuation and the treatment of associated comorbidities, neglecting the treatment of the disease itself [23].

As such, HFpEF is the most prevalent form of HF in the elderly and the number of hospitalizations has been increasing over time [22, 24], thus turning HFpEF into one of the biggest unmet needs in current cardiology where outcome-modifying treatments are a large unmet clinical need [25].

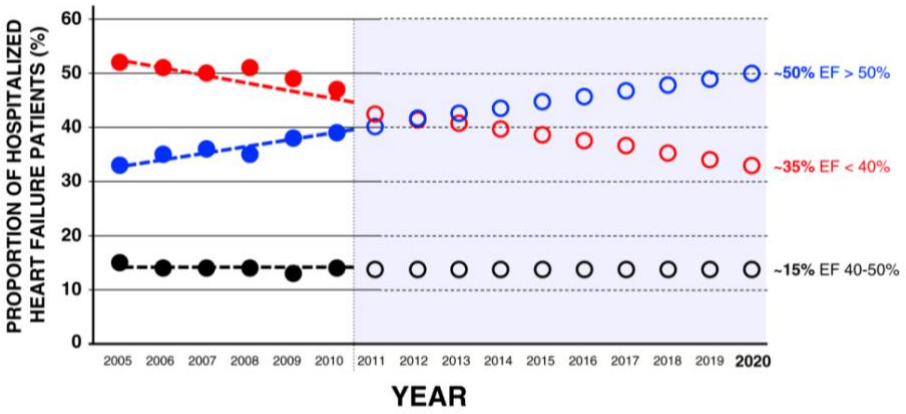


Figure 3 - Projected development of the different types of HF over the years and clinical outcome in hospitalization rate (2010). Source: [6]

1.3. Pathophysiology of Heart Failure with Preserved Ejection Fraction

The pathophysiology of HFpEF is not completely known, nonetheless, in the last years a huge effort has been made to improve the knowledge of the events that culminate in this disorder [12, 13, 23]. HFpEF is broadly characterized by impaired myocardial relaxation that leads to diastolic dysfunction, while the systolic function is preserved[26]. Although HFpEF was initially described as a left ventricular (LV) dysfunction [27], various studies over the years have highlighted the systemic nature of the HFpEF pathology [28], providing a more expanded view on this syndrome that involves other organ systems. Studies have demonstrated that HFpEF patients display within the heart elevated arterial stiffness [29] and consequent increased arterial loading, LV remodeling, fibrosis, and diastolic dysfunction [30]. On the other hand, small vessel vasodilatation is impaired [29], which combined with poor cardiac output, weakens

tissue oxygenation [31]. Pulmonary complications are also related with pathophysiological processes of HFpEF. Evidence shows that the compromised LV diastole results in elevated ventricular filling pressures at rest, contributing to the development of pulmonary hypertension and lower functional capacity [32, 33], contributing to high hospitalization and mortality rates. Moreover, complications in the renal system have also been demonstrated to be related with HFpEF, particularly with chronic kidney disease being a common pathology in HFpEF patients [34], hence suggesting the importance of the bidirectional crosstalk between the heart and the kidney. HFpEF patients often suffer from skeletal muscle sarcopenia, increased muscular fat and abnormal mitochondrial content [35], contributing to the reduced aerobic capacity of HFpEF patients (**Figure 4**) [36]. However, the trials for pharmacological intervention in HFpEF have incomprehensively failed [37-39].

Although the pathophysiological mechanisms driving the disease are poorly understood, the present perspective is that HFpEF-associated comorbidities and aging contribute to HFpEF appearance and progression by promoting the formation of a systemic pro-inflammatory milieu (described by high levels of several circulating molecules such as interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and C-reactive protein (CRP)) [40, 41]. These factors drive microvascular endothelial dysfunction where, through the production of reactive oxygen species (ROS) and decrease of nitric oxide (NO) bioavailability [42], protein kinase G (PKG) activity is reduced, therefore contributing to titin hypophosphorilation and increased cardiomyocyte stiffness [43, 44]. In addition to this, local monocyte infiltration, activation and subsequent differentiation into macrophages contribute to the exacerbation of the inflammatory environment via secretion of the inflammatory cytokine interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) [45]. Studies have proposed that the crosstalk between these macrophages and local cells result in cardiac fibroblast (CFs) activation, leading to extracellular matrix (ECM) production via collagen deposition [46, 47]. This collagen deposition and cardiomyocyte hypertrophy then contribute to the stiffening of the myocardium [48, 49], that becomes unable to relax efficiently during diastole [43]. Of note, in patients with symptomatic HFpEF, diastolic dysfunction is more associated with ECM deposition than to cardiomyocyte stiffness [44].

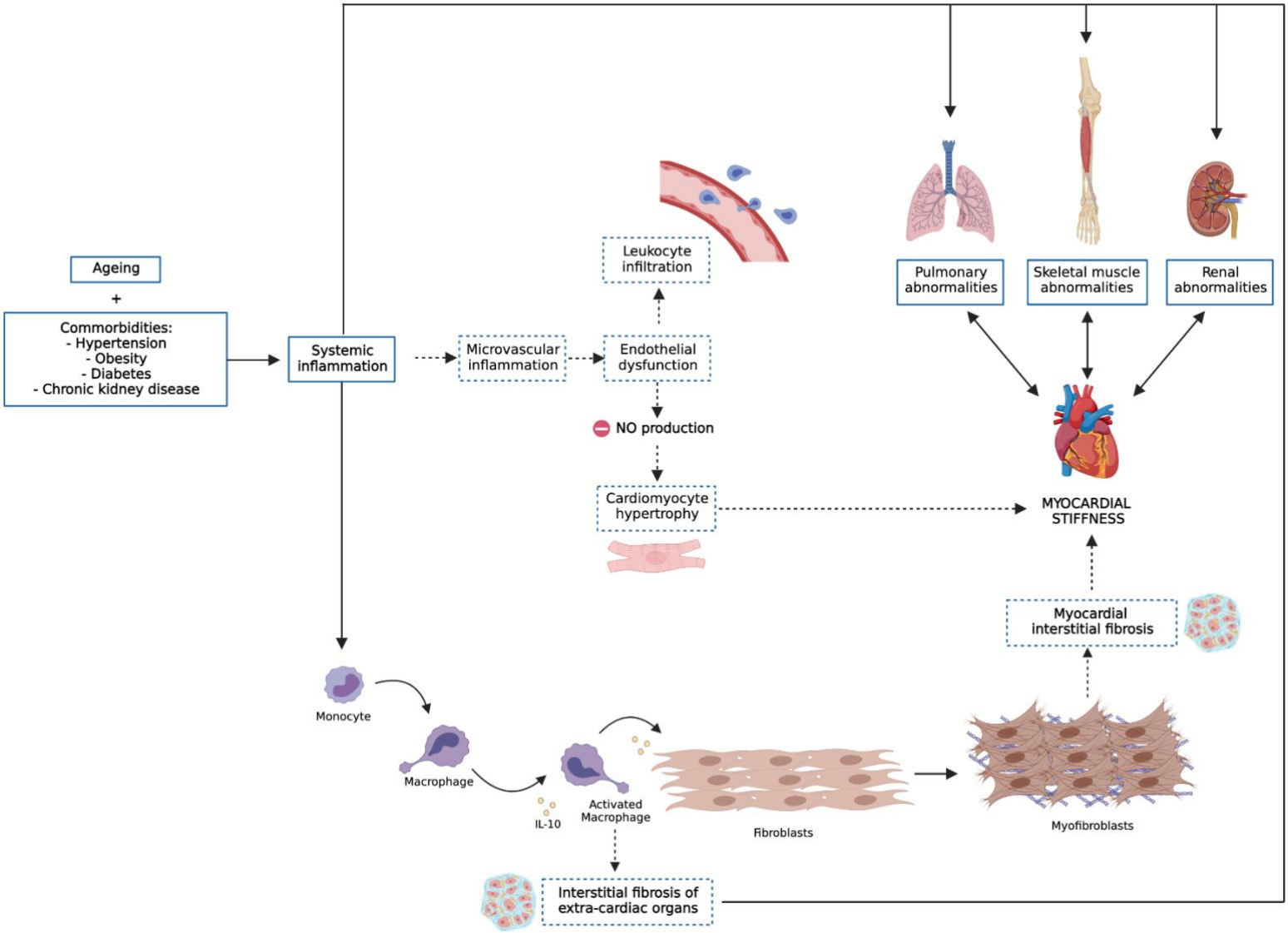


Figure 4 - Schematic representation of the proposed molecular mechanisms underlying HFpEF. Adapted from [50].

1.4. Overview of myocardial fibrosis

Most forms of CVDs, including heart failure, are associated with myocardial fibrosis, characterized by exacerbated production of extracellular matrix that contributes to adverse remodeling and progressive functional decline of the heart [44, 51]. Hence, early detection, prevention and reversion of cardiac fibrosis are key targets to advance HF management. However, reliable diagnostic biomarkers for early and non-invasive fibrosis detection and therapies able to modify scar properties for patient benefit are two unmet clinical needs.

There are two main types of myocardial fibrosis: replacement fibrosis and reactive fibrosis [52]. The first, is typically observed in cases of myocardial infarction, where voids left by cardiomyocyte death are replaced by fibrotic tissue that restore the physical integrity of the myocardial wall. Although replacement fibrosis is crucial to substitute the tissue defect, excessive fibrosis contributes to progressive functional decline [53, 54]. On the other hand, reactive interstitial fibrosis is a mechanism that occurs in the absence of extensive cardiomyocyte death. This type of cardiac fibrosis involves the deposition of collagen fibers in the interstitial space between cells (interstitial fibrosis) and/or in the perivascular region (perivascular region), usually associated with abnormal loading conditions [55, 56] or pro-fibrotic systemic conditions [57, 58].

Myocardial interstitial fibrosis contributes to left ventricular dysfunction and is normally present years before the onset of heart failure [59]. In fact, the extent of fibrosis and cardiomyocyte hypertrophy are strongly correlated with the duration of HF symptoms. As previously mentioned, this fibrillar ECM accumulation is often described in patients with HFpEF and contributes significantly to impaired LV filling [60]. In 2017, a study conducted by Schelbert et al. [60] investigated whether cardiac fibrosis is equally prevalent in patients with diagnosed with HFpEF and those at risk of developing this pathology, as well as with disease outcome and severity. They demonstrated that patients at risk of developing HFpEF and those with the established disease demonstrated similar extent of myocardial fibrosis and worse clinical prognosis when compared with patients without HFpEF [61]. Additionally, fibrosis is shown to be strongly correlated with increased arrhythmias, hospitalization rates and mortality in HFpEF patients [51, 62]. Taken together these finding demonstrate the importance of unraveling the mechanisms that regulate fibrotic deposition in HFpEF, which are largely undisclosed.

Different cardiac cell subsets contribute either directly or indirectly to the establishment of myocardial fibrosis [80] but resident CFs are a master orchestrator of this process [62, 80]. Upon stimulation with inflammatory cytokines and chemokines (TNF-α), monocyte chemoattractant protein (MCP-1) [83], interleukins (IL-3, IL-4, IL-5, IL-10 and IL-17a) [84, 85]) or after mechanical stress [86, 87], CFs become activated and differentiate into non-proliferative myofibroblasts. The latter are characterized by increased cell size, prominent stress fibers [62, 89] and production of α-smooth muscle actin (α-SMA), granting these cell contractile ability [90-92]. Also, myofibroblasts secrete high amount of ECM proteins such as fibronectin and MMPs but also and production of fibrillary collagens, such as collagen types I and III [80, 88]. At a less extent, myofibroblasts can also be originated from endothelial cells by endothelial to mesenchymal transition [81, 82]. Moreover, activated CFs also secrete cytokines to the surrounded environment affecting other cardiac cells leading to the increased hypertrophy, exacerbation of inflammatory response and problems of contraction [60, 93].

Although the cellular and molecular mechanisms leading to myocardial fibrosis are well established in HFrEF, these are yet largely undisclosed in HFpEF. The only mechanistic study

addressing the latter showed that IL-10 inflammatory molecules produced by resident cardiac macrophages have promoted cardiac fibrosis [49]. IL-10 induced fibroblast activation and myofibroblast proliferation and deposition of extracellular matrix components. The authors further speculated that the increase of IL-10 production by resident heart macrophages resulted from the pro-inflammatory milieu promoted by aging. In line with this perspective, the systemic environment of HFpEF patients contains several molecules, cytokines and other factors that are thought to impact on the heart [63]. Recently, sub-groups with different phenotypes of HFpEF were obtained using machine learning algorithms with a particular one with a distinctive inflammatory/profibrotic signature [64]. In line with this perspective, in the following sections we will explore the communication between the other organs to the heart, with particular emphasis on the role of extracellular vesicles (EVs) as mediators of long-distance communication.

1.5. Extracellular Vesicles-Mediated Communication in Cardiovascular Diseases

1.5.1. Overview of Extracellular Vesicles (EVs)

EVs are small (20 nm to 2 μm) structures enclosed in a phospholipid bilayer that are released through exocytic budding of the plasma membrane of all cells and that carrying biological content such as proteins, lipids and genetic material [65]. Even though EVs were first reported as structures responsible for waste removal from cells [66], they are nowadays considered key mediators of intercellular communication, both in physiological and pathological conditions [67]. These structures are released by virtually all cells and mediate communication with neighboring cells but also circulate in the organism delivering molecular cargo to distant cells [65].

Based on different biogenesis and size, EVs were classified as exosomes (50 to 150 nm), microvesicles (MVs) (150 to 500 nm) and apoptotic bodies (500 to 5000 nm)[65]. Exosomes are formed in the endosomal network [68]; microvesicles (MVs) are produced through outward budding of the plasma membrane v [69]; and apoptotic bodies are secreted by outward budding of the cell membrane of apoptotic cells [70]. As revised in Samanta et al. (2017)[68], the content and surface markers of the different EVs commonly overlap. Exosomes, for example, are rich in micro-RNAs (miR), mRNA and membrane and cytoplasmatic proteins [71, 72], and have transmembrane proteins that serve as biomarkers in their surface such as CD5L [73], tetraspanins (CD9, CD63, CD81), transferrin receptors (CD71)[74], endosomal sorting complexes required for transport (ESCRT) proteins, and several heat shock proteins such as HSP70 and HSP90 [71, 72]. The usual content in MVs is similar to the ones found in exosomes [72] and also

share some surface markers (e.g CD81, CD9) [65], however they can be distinguished through the presence of annexin V, integrins and CD40 ligand [72]. On the other hand, apoptotic bodies usually transport nuclear fractions, cell organelles and DNA due to their apoptotic nature, and their surface biomarkers also consist of annexin V and phosphatidylserine[72].

EVs produced by different cell types and circumstances transport different cargo and impact differently on recipient cells [65, 75]. These are internalized by endocytosis, phagocytosis or fusion of the membranes and their content is released in the recipient cell, and they can interact with target cells via ligand-receptor interactions [65]. According to Panagiotou et al. (2018) [76], EVs can impact on target cells in at least three different ways: i) mRNA translation within the recipient cell, ii) post-transcriptional regulation of gene expression through miRNA released by EVs, and iii) direct action of proteins released by EVs on recipient cells.

The number of scientific publications assessing the biological effect of EVs in physiological and pathological conditions has been growing fast over the years. However, being an emergent and recent field of research, isolation and characterization protocols varied greatly across studies, precluding effective comparisons. In response to this, and aiming at promoting standardization, the International Society for Extracellular Vesicles (ISEV) elaborated and released a series of recommendations of different methods for the isolation/separation, characterization, and usage of these biological structures in functional studies [77, 78].

1.5.1.1. Isolation of EVs from biological samples

The first important step to isolate EVs is to collect an EV-containing fluid, such as plasma or cultured media. This procedure is crucial to obtain a good EV preparation and parameters such as the experimental procedure and storage conditions must be taken into consideration. Currently, even though there is no single optimal method for EV isolation, there are different methods capable of achieving a preparation that is pure and enriched in these structures [78]. These separation methods include differential ultracentrifugation (UC), density gradients, filtration, precipitation, size exclusion chromatography (SEC), precipitation kits and immune isolation, and can be classified according to their recovery and specificity rates as shown in **Figure 5** [78].

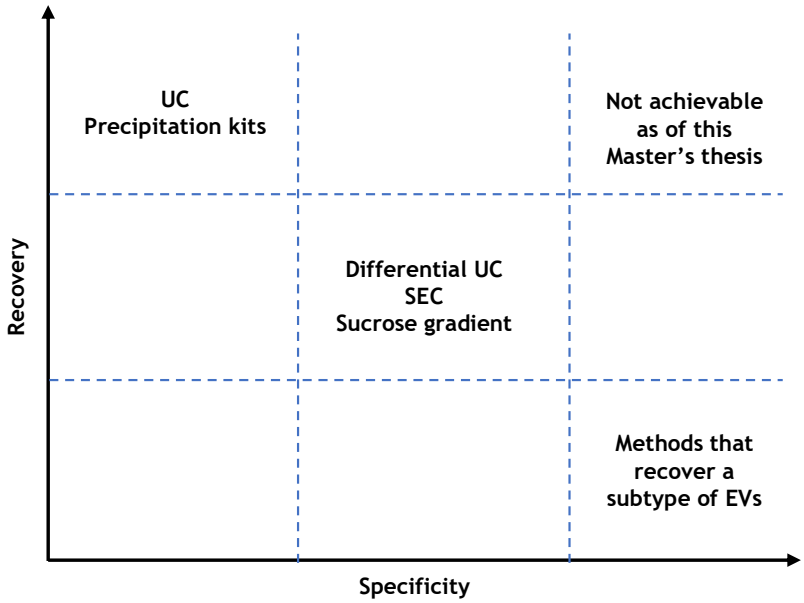


Figure 5 - Different isolation methods of EVs according to recovery and specificity rates. UC – ultracentrifugation; SEC – size-exclusion chromatography

At the end of 2015, a worldwide ISEV survey demonstrated that differential ultracentrifugation was the most used technique for EV isolation [79]. In this method the subcellular components are separated according to their density by centrifugation. This is a cost-effective EV isolation method that does not introduce additional chemicals to the samples. Initially, particles with high buoyant densities such as cells, cell debris, apoptotic bodies and aggregates are sedimented to reduce contamination using a series of centrifugations with acceleration rates that range from 300 to 10000 x g. Then, EVs are sedimented by ultracentrifugation at 100 000 to 200 000 x g, allowing most non-EV proteins to be separated (supernatant) from EVs (pellet). Then, EV-enriched fractions are subjected to additional steps of washing and microfiltration to increase purity [80-82]. The low RNA yield and damage of exosomes associated to ultracentrifugation, the complexity of the protocol, the specificity of the equipment and low reproducibility associated with the type of rotor, force and sample viscosity are the main disadvantages of this technique [83].

Another common technique of EV isolation is filtration, where the components present in a certain biological sample are separated according to their size [84]. These include ultrafiltration [85], hydrostatic dialysis [86] and size exclusion chromatography (SEC), the latter being widely used for the separation of biopolymers, protein complexes and lipoproteins, as well as EVs from blood plasma and urine [87-89], based on the hydrodynamic radius of these particles. The number of studies using SEC for EV isolation have been increasing as an alternative to ultracentrifugation since it produces EV preparations with similar purity whilst EVs integrity is better preserved [89, 90]. In SEC, the samples run through a previously prepared

column that contains a porous matrix (usually sepharose). The fact that the protocol is very high throughput and multiple samples may be processed simultaneously represents a great advantage. When compared with ultracentrifugation, Western Blot assays of EV markers after SEC display a higher number of markers with less non-EV content [91], further supporting that this method is efficient, rapid, and with high reproducibility rates. Considering that EVs, especially exosomes, have a larger hydrodynamic radius than lipoproteins and protein complexes, SEC is an effective way of separating these components. However, since some chylomicrons have a similar size and are not as easy to separate from exosomes, they are often present in SEC isolates [91]. The main disadvantages of this method are the expensive chromatographic sorbents and the fact that the obtained EV-containing solution is rather diluted and might require subsequent concentration steps [84]. A possible alternative for this process is centrifugation with a linear sucrose gradient, using the floatation density of the EVs for separation from other components. However, this method still has some disadvantages as it may fail to separate vesicles with similar sedimentation rates [92-94]. It has been suggested that the combination of different methods or even the repetition of the same one may decrease the levels of lipid contaminants, however, there is a significant decrease in the yield and also result in damage to the vesicles [91].

In addition to ultracentrifugation, differential centrifugation and SEC, there are other EVs isolation methods that take advantage of the differences in solubility and aggregation parameters of these particles, by precipitation with hydrophilic polymers [95], protamine [96], sodium acetate [97] and organic solvents [98]. Even though these procedures are often simple and cost-effective, the use of additional chemicals may interfere with the vesicular structures of the exosomes and the high rates of non-EV contaminants also contribute to the low rates of specificity of these methods. Lastly, other techniques that are based on affinity interactions are also used. Typically, EVs –associated antigens (e.g. tetraspanins, heat shock proteins) are used to isolate EVs through covalent binding to magnetic beads [26, 99], highly porous monolithic silica microtips [100], cellulose filters [101] or membrane affinity filters [102]. The use of antigens for EV isolation may provide high purity and selectivity rates but nonspecific binding and high cost of antibodies represent the two major disadvantages of this method [103].

Overall, among all these methods, sucrose gradient centrifugation, ultracentrifugation and SEC are the mostly used and show a good balance between recovery and specificity, however, a gold-standard EVs isolation method that works efficiently across different biological samples is yet to be described.

1.5.1.2. Characterization of EVs

EV-enriched preparations may also contain carryover of other components such as small organelles, lipids, cholesterol, and other types of microparticles, which may have biological activity and impair discrimination of the EV-mediated effect. Thus, EV-enriched preparations should be analyzed and characterized to access EV quantity and integrity but also for the presence of possible contaminants.

For a detailed characterization, firstly, a quantitative description of the source of EVs should be provided. This includes, the number of cultured cells (in cases of cell culture derived EVs), total starting volume of biofluid (blood plasma or urine, for example) or weigh/volume/size of tissue at the time of collection should be indicated for each experiment [78]. Also, a quantitative analysis on the EV-enriched preparation should also be provided regarding total particle number, protein amount or lipid quantification. To assess the number of particles, light scattering technologies can be used such as nanoparticle tracking analysis (NTA) [104-106], quantitative flow cytometry (standard for larger EVs and high resolution for smaller EVs)[107], surface plasmon resonance (SPR) or other techniques with similar functionalities. Another important characteristic that needs to be addressed is the integrity of the EVs, especially when downstream studies include protocols to determine the biological effects of the isolated EVs *in vitro* assays. EVs are not stable at room temperature, and storage at -80ºC has proven to have a negative impact on size and content. Therefore, to preserve the structure and content of the EVs and minimize the damage, they should be analyzed immediately after isolation [108] and freeze—thaw cycles should be avoided [109].

One important recommendation of the MISEV2018 is the assessment of EVs-associated markers as shown in **Table 2**. For being considered EVs, the EVs preparation should have a population that marks for at least one protein of categories 1, 2 and 3. The analysis of proteins of category 4 is only required in cases of specific analysis of small EVs vs large EVs, and of category 5 to document functional activities in the identification of a functional soluble factor in EVs [78]. Various methods can be used to quantify and identify proteins inside or on the surface of EVs, with Western blotting being the most commonly used. Flow cytometry of EVs attached to beads [110] is also used in this context, as well as fluorescence scanning [111], SPR [112, 113] and mass spectrometry [114].

An extensive characterization on the samples of EVs is of extreme importance, including, whenever possible, distinction between different sub-types of vesicles. These parameters are important because exosomes, MVs and apoptotic bodies differ in RNA and protein composition, therefore their biological purpose may differ as well. For example, experimental studies have previously reported that serum exosomes contain a smaller amount of miRNAS when compared with MVs [107], which may be relevant when assessing the role of

these types of structures in pathophysiological contexts. Also, the backtracking of each EV to its site of origin is of great importance, since it can contribute to unraveling the role of EVs in pathophysiological conditions[115-117]. In line with this, antigens on the surface of EVs or surface markers can be analyzed to identify their cells of origin [107, 118, 119].

Table 2 - Protein content-based EV characterization according to the MISEV2018 categories. Adapted from [78]

Category	Description	Uses	Examples
1	Transmembrane or GPI-anchored proteins associated to plasma membrane and/or endosomes	All EVs	Tetraspanins (CD9, CD63, CD81), transferrin receptors (CD71), integrins, MHC class I, cell/tissue specific proteins (CD37, PECAM1)
2	Cytosolic proteins recovered in EVs	All EVs	ESCRT-I/II/III, TSG101, ALIX, Flotillin-1 and -2, Hsp70, GAPDH
3	Major components of non-EV co-isolated structures	All EVs for purity control	APOA1/2, APOB, APOB100, Albumin
4	Transmembrane, lipid-bound and soluble proteins associated to other intracellular components than PM/endosomes	Subtypes of EVs and/or pathological/atypical state	Histones, Cit C, Calnexin, Grp94, ATG9A, ACTIN1/4
5	Secreted proteins recovered with EVs	Functional components of EVs	Cytokines, growth factors, adhesion and ECM proteins

Lastly, after the global quantification and characterization of the EV isolates, it is necessary to provide information on the individual EVs. For this characterization of single vesicles, the most common techniques include transmission electron microscopy (TEM) [120, 121], scanning-probe microscopy (SPM) [122], cryo-EM [121], AFM [122] and super-resolution microscopy[123]. Considering the rapid development of novel technologies, other techniques are currently being developed that may contribute to a better comprehension of these particles.

1.5.2. Extracellular Vesicles in Age-Related Cardiovascular Diseases

As mentioned before EVs transport a series of bioactive molecules, representing an important mechanism of intercellular communication and influencing target cells through the modulation of gene expression, cell phenotype and molecular pathways. These structures play significant roles in both physiological and pathological conditions, which in the heart translates into maintaining normal cardiac function and changing their composition under pathological

conditions, intermediating alterations that culminate in the development of CVDs [124]. Indeed, a growing number of studies support that exosomes, microvesicles and other types of EVs are key messengers in the development of age-related cardiovascular pathologies [125, 126], mainly through the transport of miRNAs and proteins and other bioactive molecules [127].

Different studies have demonstrated that the levels of circulating EVs are elevated in patients with cardiovascular risk factors and in patients with CVDs [116, 128], and that there is a correlation between the number of EVs and CVDs, reflecting that activated cells show an increase in the secretion of EVs in pathological contexts [129, 130]. A study in mild and severe hypertensive individuals showed that the level of circulating EVs is significantly increased compared to normotensive subjects [117], and in patients with HF and atherosclerosis these levels are elevated as well [131]. Accordingly, other works showed that the circulating levels of EVs was increased in patients with endothelial dysfunction [130] and in atherosclerosis [129], and the levels of plasma-derived EVs are also increased in patients with cardiovascular risk factors or myocardial diseases when compared to healthy subjects [116, 132]. In this case, endothelial microparticles were used as biomarkers of the pathological condition and, after treatment, the levels of microparticles decreased to the levels found in healthy patients [116]. Nevertheless, conclusions on the potential use of EVs/MPs as prognostic/diagnostic biomarkers and the biological relevance of this increase awaits further investigation.

Of interest, unveiling the cellular source of the EVs may be important to fully understand important disease mechanisms. Leukocyte-derived EVs isolated from the plasma of CVD patients were shown to have an impact on the development of atherosclerotic plaques, supporting a clear relationship between high amounts of circulating EVs with the establishment of a pro-inflammatory environment and increased cardiovascular risk [174]. In addition to leukocytes, monocyte-derived EVs have also been studied and proven to increase NO production in endothelial cells [175]. Platelet-derived EVs are also directly involved in the development of atherosclerotic lesions in older individuals with multiple comorbidities by anchoring to the endothelium [173]. This mechanism facilitates leukocyte recruitment and adhesion to the injured vessel, therefore creating an age-related pro-inflammatory state that is characteristic of the development of HFpEF.

In addition to the impact of circulating EVs on the heart, resident subsets myocardial cells are also producers of EVs that impart the development of cardiovascular pathologies[133-135]. In this setting, EVs are also involved in tissue remodeling and fibrosis. Studies have demonstrated that TGF-ß-containing exosomes can promote the differentiation of fibroblasts in myofibroblasts [136] and induce fibrotic responses in the renal system [137]. Following the same line, several works demonstrated that in the context of disease cardiac cells release EVs containing miR-21-5p, which promotes fibroblast activation and fibrosis [138]. Moreover, injured cardiac muscle cells in post-MI conditions secrete EVs enriched in miR-208a that induce profibrotic results in the heart [139]. On the other end, EVs can also avoid extensive and

irreversible changes in the myocardium by promoting cardioprotective responses in the same conditions through the transport of miR-133a, that suppresses the deposition of fibrotic tissue in the heart [140]. In addition, in diabetic rats, studies have shown that exercise promotes the secretion of anti-fibrotic miRs transported by exosomes that target ECM remodeling through the downregulation of MMP-9[141].

Overall, there is clearly growing interest in EVs as mediators of intercellular information during the development of CVDs. As described in this section, most studies addressing this subject involve EVs isolated from the plasma of CVDs patients, mainly produced by platelets, endothelial cells and immune cells. However, the knowledge is narrow regarding the impact of these EVs on cardiac cells on cardiac fibroblasts, which are master regulators of myocardial fibrosis. **Table 3** reviews key studies stressing the importance of EVs as mediators of cellular communication in the diseased heart. Collectively, these findings support the premise that EVs may play an important role in the development of HFpEF namely by regulating pathological processes such as myocardial fibrosis.

Table 3 - Collection of experimental studies addressing EVs in and their contribution to cardiovascular pathologies. n.a. – non-avaliable

Type of EV	Cargo	Organism	Secretory cell type	Target cell type	Physiological context	Main results	Reference
Extracellular vesicles	CD14; Serpin F2; Serpin G1	Human	n.a.	n.a.	Heart failure	Circulating EVs are directly associated with pathophysiological events of HF through Serpin G1.	[142]
Microparticles	n.a.	Human	Platelets	n.a.	Hypertension	The level of circulating platelet-derived MPs is significantly higher in mild and severe hypertense subjects, when compared to normotensive individuals.	[117]
Microparticles	miR-320b	Human (*in vivo* collection and *in vitro* HMEC1)	Platelets	Endothelial cells	Endothelial dysfunction	Platelet-derived MPs promote vascular dysfunction through release of miR-320b.	[143]
Microparticles	n.a.	Human	Platelets	Endothelial cells	Atherosclerosis	Activated platelets produce MPs that increase ICAM-1 expression and promote leukocyte adhesion to the endothelium. These leukocytes secrete inflammatory molecules, creating a damaging environment on the vasculature.	[144]
Microparticles	n.a.	Human	Platelets	n.a.	Atherosclerosis	Platelet-derived MPs of older individuals with multiple comorbidities are involved in the development of atherosclerotic lesion by anchoring to the endothelium.	[145]
Microparticles	n.a.	Human	Endothelial cells	Endothelial cells	Atherogenesis	The presence of CRP and IL-6 in healthy middle-aged men increase MP production in ECs. These MPs promote endothelial apoptosis.	[146]
Exosomes/ Microvesicles	GM-CSF; IL-6; IL-8; ICAM-1; CXCL-10; CCL5; TNF-α; TNFR	Human (*in vitro* HUVECs and THP-1)	Endothelial cells	Monocytes	Atherosclerosis	Inflamed ECs secrete EVs with immunomodulatory content that, once internalized by monocytes, promotes expression of pro-inflammatory markers IL-6 and IL-6.	[147]
Microparticles	n.a.	Human (*in vivo* MPs, *in vitro* HUVECs exposure)	Endothelial cells	Endothelial cells	Atherosclerosis	Inflamed ECs from atherosclerotic plaque secrete MPs that promote ICAM-1 expression and therefore monocyte adhesion to the endothelium.	[148]

Microparticles	n.a	Human	Endothelial cells	Macrophages	Coronary artery disease	When activated by inflammatory cytokines or in an apoptotic state, ECs produce MPs that bind to macrophages, promoting thrombus formation and inflammation.	[149]
Microparticles	n.a.	Human	Leukocyte	Endothelial cells	Atherosclerosis	Increased levels of leukocyte-derived MPs in the plasma of older patients of atherosclerosis correlated with increased cardiovascular risk.	[129]
Microparticles	n.a.	Human (*in vitro* THP-1 and HUVECs)	Monocytes	Endothelial cells	Atherosclerosis	Monocyte-derived MPs increase NO production in ECs.	[150]
Exosomes	miR-155; miR-146a	Human (*in vivo* collection and *in vitro* HUVECs culture	Monocytes	Endothelial cells	Endothelial dysfunction	Monocyte-derived exosomes promote NF-kB activation through miRNA content, contributing to endothelial dysfunction.	[151]
Microparticles	n.a.	Human (*in vitro* THP-1) and mice (*in vivo* exposure)	Monocytes	Endothelial cells	Atherosclerosis	MPs from monocytes promote monocyte and lymphocyte infiltration in the vessels of mice in a multimorbidity context.	[152]
Microparticles	IgG	Human	Macrophages	n.a.	Atherosclerosis	The collection of MPs from human carotid atherosclerotic lesions and subsequent proteomic and metabolomic analysis demonstrated that these MPs derive from macrophages.	[153]

CHAPTER II – PRELIMINARY DATA

Aging and chronic inflammation are associated with the development of heart failure with preserved ejection fraction (HFpEF). However, cellular senescence as a potential mechanistic link between both events and its pathophysiological and therapeutic role were yet unexplored. Here we show that ZSF1-obese rats, a model of cardiometabolic HFpEF, have exacerbated systemic inflammation and endothelial damage compared to ZSF1-lean littermates (**Figure 6**). In addition, ZSF1-obese rats accumulated immune and endothelial senescent cells in the peripheral blood and myocardium (**Figure 7**).

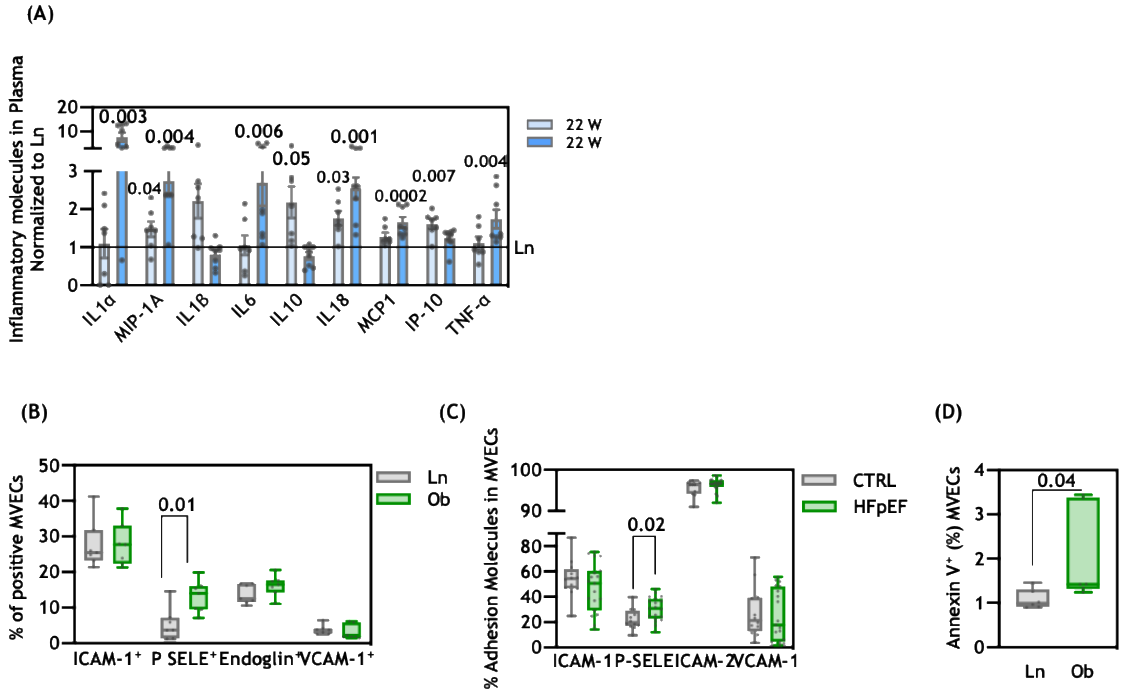


Figure 6 - **(A)** Profiling of inflammatory molecules in plasma of Ob and Ln rats with 22 and 26 weeks (n=7/group). Protein levels in ZSF1-Ob were normalized to Ln. **(B)** Human microvascular endothelial cells (MVECs) were exposed to plasma of Ln and Ob rats (n=6/group) and **(C)** plasma of HFpEF (n=19) and control (CTR) patients (n=12) for 24 hours and **p**ercentage of MVECs activation and **(D)** apoptosis was assessed by surface expression of adhesion molecules and detection of annexin V.

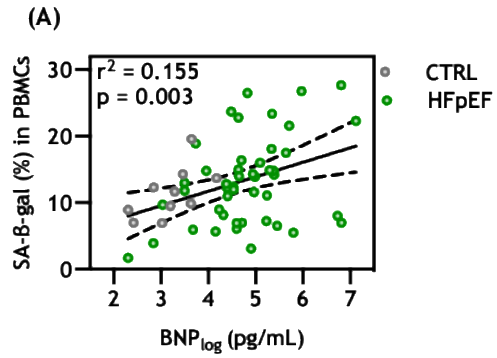


Figure 7 - Association between SCs and key analytical BNP log surrogates of cardiac overload and prognosticators in HFpEF. BNP log – B-type natriuretic peptide, log-transformed.

Accordingly, the frequency of circulating senescent leukocytes is associated with markers of disease severity in HFpEF patients (**Figure 8**). Notably, systemic treatment of ZSF1-obese rats with Navitoclax, a B-cell lymphoma-2 (BCL-2) family inhibitor, reduced senescent cell burden, decreased circulating B-type natriuretic peptide levels, and attenuated inflammation, vascular remodeling and cardiac fibrosis (**Figure 9**). Our findings advance cellular senescence as a key mechanistic pathway leading to HFpEF and provide proof-of-concept evidence that senolytics are a promising treatment for this disease.

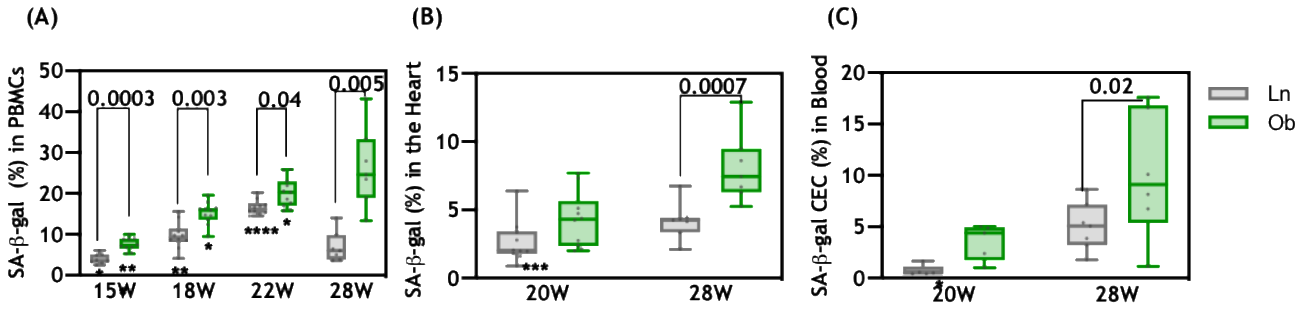


Figure 8 - Relative percentage of senescent-associated beta-galactosidase-expressing cells (SA-ß-gal) in **(A)** PBMCs, **(B)** heart cells and **(C)** endothelial circulating cells in ZSF1 OB and Ln animals (n=8/group).

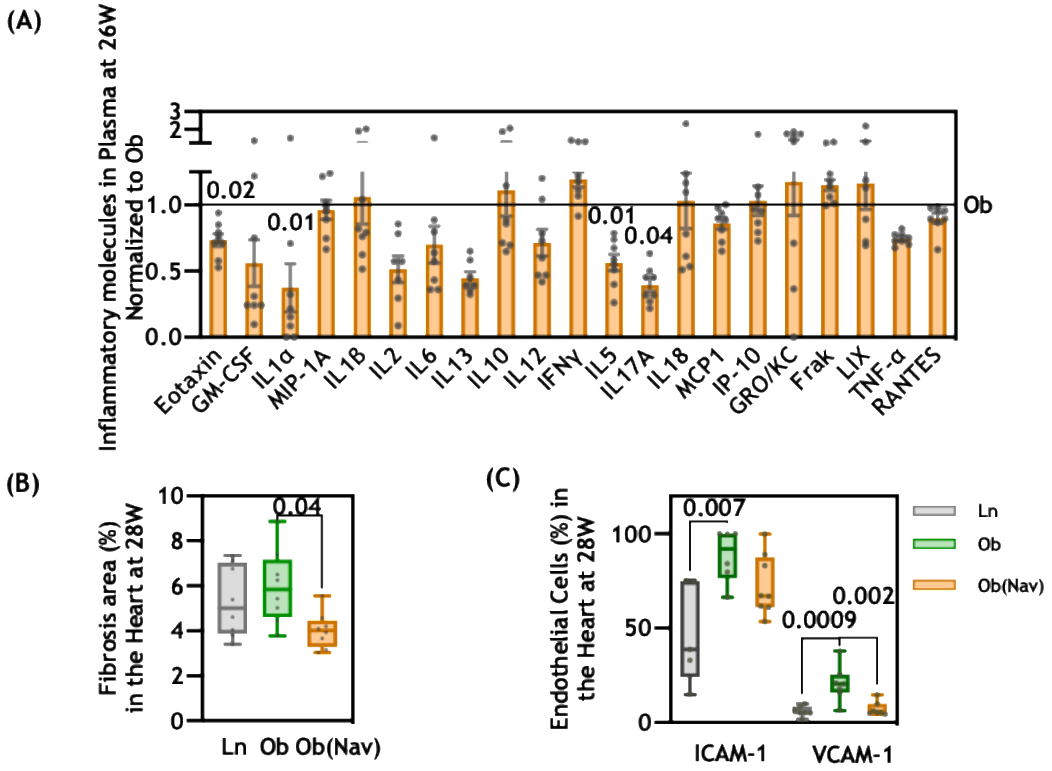


Figure 9 - **(A)** Profiling of inflammatory molecules in plasma of Ob (Nav) rats with 26 weeks (n=7/group), normalized to Ob levels. **(B)** Quantification of picrosirius red at 28 weeks (n=8/group). Scale bars 50 µm. **(C)** Percentage of cardiac endothelial cells expressing ICAM-1 and VCAM-1 (n ≥ 6/group).

These preliminary results evidenced that long-distance communication to the heart may be essential for the development of cardiac pathological alterations and may uncover important targets for therapy.

CHAPTER III – AIMS

Taking into consideration the preliminary data described and that EVs are important carriers of relevant cargo between distanced cells, we hypothesized that EV-mediated long-distance communication may be relevant in the context of HFpEF and specifically in the development of myocardial fibrosis. Hence, the broad aim of this project is to unveil the role of extracellular vesicles (EVs) in the development of cardiac fibrosis in patients with HFpEF.
To attain this main goal, we will:

- Evaluate whether the systemic environment of HFpEF patients promotes cardiac fibroblast activation *in vitro*;
- Isolate EVs from the plasma of a well characterized patient cohort;
- Characterize EVs-enriched isolates accordingly to MISEV2018 guidelines;
- Evaluate the effect of the EVs of the activation of cardiac fibroblasts *in vitro*;

This proof-of-concept study will pave the way for more thorough studies on the identification of EV-associated fibrogenic mechanisms in HFpEF, which will be essential for the development of novel therapies and remodeling biomarkers in HFpEF.

CHAPTER IV – MATERIALS AND METHODS

2.1. Sample collection and processing

Peripheral blood was collected from two patient cohorts: i) a control group, matched for age and main comorbidities with normal BNP (CTRL); and a HFpEF group, with HFpEF patients diagnosed according to the ESC guidelines of 2018[6]. Within two hours after blood collection, using EDTA as an anti-coagulant, plasma was separated from the buffy coat through centrifugation at 12000 x g for 30 minutes (min) at room temperature (RT), without brake and acceleration for posterior collection followed by a final centrifugation of 2500 x g for 30 minutes and storage at -80ºC.

2.2. Human EV isolation and concentration

EVs were isolated by SEC in sepharose (GE Healthcare). For column preparation, the sepharose was washed three times with PBS-sodium citrate 0.32% (w/v) and centrifuged at 300 x g for 10 min. The supernatant was discarded and the sepharose added to a syringe, filled with a stocking at the bottom. The sepharose was added on top of the column up to 10 mL and, when packed, a 3 mm paper filter was added on top to provide an even interface. Column was then washed twice with PBS-citrate buffer (0.32% (w/v)), followed by Triton solution (0.1%) and filtered PBS.

To isolate EV from plasma, 1-2 mL of plasma was diluted in an equal volume of filtered PBS. The plasma was filtered through a 0.8 µm filter (Millex®) and centrifuged at 12600 x g for 30 min at 4 ºC. Then, 1 mL of the supernatant was applied to the column and 1 mL fractions were collected from fraction 0 (before loading the sample) to 11. During the process of sample elution, filtered PBS was continuously added after the complete loading of samples in the column.

For EV concentration, fractions 3 and 4 were loaded on Vivaspin 6 centrifugal concentrator with a 10000 MWCO PES membrane (Sartorius) and centrifuged at 3000 x g, 4 ºC until approximately 200 µL of concentrated sample was obtained. As an internal control (Prot), fractions 9 and 10 were also concentrated using the same protocol. All samples were then either stored at -80 ºC or used for EVs characterization.

2.3. EV characterization

2.3.1. Protein quantification

DC™ Protein Assay (Bio-Rad) was used for protein quantification following manufacture instructions. Bovine serum albumin (BSA) and PBS were used to create a standard curve and as a blank control, respectively. Reagents were added to a 96-well plate though the following

order: 5 µL of the standards or samples in duplicate (EVs samples and respective internal controls diluted in filtered PBS, 1:5 and 1:10 respectively), 25 µL of reagent A and 200 µL of reagent B. After incubating in the dark for 15 min, the absorbance was read at 750 nm.

2.3.2. Dynamic Light Scattering (DLS)

DLS was used to profile the average size of the particles from each SEC fraction. For that, 100 µL of each fraction was added to a polysterine cuvette (ZEN0040, Malvern) with a 10 mm path length for DLS analysis using a Zetasizer Nano ZS (Malvern). The DLS measurements were performed at 25ºC following the incidence of a light beam at 633 nm in the cuvette and the back light scattered was recorded at an angle of 173º. The obtained DLS data is based on the Stokes-Einstein equation.

2.3.3. Transmission Electron Microscopy (TEM)

TEM was used to assess the morphology and size of the EVs. To prepare the samples form TEM, 5 µL of the EV suspension was mounted on Formvar/carbon film-coated mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA) and left for 2 min. The liquid in excess was removed with filtered paper. Then, 10 µL of uranyl acetate 5% was added on the grids and left for 10 secs, following removal excess liquid with filtered paper. The visualization was executed in a Joel JEM 1400 TEM at 120 kV (Tokyo, Japan), and the images were digitally recorded with a CCD digital camera Orious 1100W at the Histology and Electron Microscopy Unit.

2.3.4. Nanoparticle Tracking Analysis (NTA)

Each EVs suspension was diluted at least 200x in filtered PBS and injected into a Nanosight NS300 system (Malvern Instruments, U. K.) that captured 3 videos of 30 sec. The size was assessed taking in account the average of the tree measurements.

2.3.5. Western Blot (WB)

Twenty µg of protein from each EV sample was mixed with 1:1 Loading Buffer (Tris-HCl 1M, 5% SDS, 12% glycerol and 0,024% bromophenol blue) in H2O. Following heating at 95ºC for 5 min, samples were separated on a 10% Tris-glycine SDS-Page polyacrylamide gel for 1 h at 100 V. Proteins were transferred to a nitrocellulose membrane (AmershamProtran 0,45 NC, GE Healthcare) at 16 mV overnight (ON) in Tris/glycine transfer buffer (Bio-Rad) and 20% methanol.

The membrane was stained with Ponceau S (Sigma-Aldrich, Co.) to confirm efficient protein transfer. Then, to block the sample a Tris-buffered saline solution was used with 0,1% Tween-20 (Promega) (TBS-T) with 5% (w/v) non-fat dry milk (Molico) for 1h at RT. Then, the membrane was incubated at 4ºC, overnight with the primary antibodies (1:1000 anti-CD63, EXOAB-CD63A-1, System Biosciences; 1:1000 anti-Hsp70, EXOAB-Hsp70A-1, System Biosciences; 1:2000 anti-Albumin, sc-271605, Santa Cruz Biotechnology), prepared in blocking solution overnight. Further, they were washed 3 times in TBS-T during 10 min with agitation and incubated with the secondary antibodies (1:15000 IRDye 800CW goat anti-rabbit IgG secondary antibody, 925-32211, LI-COR; 1:20000 IRDye 680RD goat anti-mouse IgG secondary antibody, 925-68070, LI-COR) for 1 h at 4ºC. Finally, the membrane was washed 3 times in TBS-T for 10 min and, for signal detection, analyzed with Odyssey® Imaging System (LI-COR) and ImageStudio™ Software.

2.3.6. Flow cytometry

For flow cytometry analysis of EVs, a suspension was made with a 1:8:1 ratio of EVs, PBS citrate (PBS 1x in 0.32% sodium citrate) and previously diluted beads (1:25 latex aldehyde-sulfate 4% w/v 4 µm (Invitrogen) beads diluted in PBS). Then, 500 µL of BSA-BCB buffer (0.1% BSA, 0.01% NaN_3 in PBS 1x) was added to the suspension and incubated at RT in a rotation device overnight (ON). It was used as control, diluted beads with PBS. The tubes were centrifuged at 2000 x g for 10 min at RT and twice at 13000 rpm for 2 min. The supernatant was removed and the primary antibody (1:1000 CD71, Ab84036, Abcam) added. The sample was incubated for 30 min at 4ºC, protected from light and washed with 150 µL of BSA/BCB buffer for 10 min 2000 x g at RT, followed by incubation with the secondary antibody solution (1:500 anti-rabbit Alexa 488, A11008, Invitrogen in BSA-BCB buffer) (30 min, 4ºC, protected from light). Finally, it was washed twice with BSA-BCB buffer solution at 2000 x g for 10 min at RT.
The samples were acquired using BD Accuri Flow Cytometer.

2.4. Effect of human EVs on cardiac cells

2.4.1. Cell culture

Primary adult human cardiac fibroblasts (hCFs; Cell Applications, Inc.) were cultured in human fibroblast growth medium (FGM; Cell Applications, Inc.) and incubated in a HERAcell® 150 CO_2 incubator (Heraeus®) at 37ºC in a humidified atmosphere with 5% CO_2. The medium was changed every two days. Cells were passaged when they reached 70-80% confluency. For passage, cells were washed twice with filtered PBS, detached with 0,25% trypsin in PBS (Sigma

Aldrich, Co.), centrifuged at 300 x g for 5 min and then seeded with a constant cell density of 10000 cells/cm^2 in FGM.

2.4.2. EV internalization assay

The equivalent to 10^8 particles/mL of EVs were labelled using PKH26 (Sigma Aldrich, Co), a cell membrane binding dye, according to manufacturer instructions. To remove the excess of dye in solution, the labeled EVs were transferred to a spin column with 4 mL of PBS and centrifuged at 2500 x g for 30 min at 4ºC. The supernatant containing the EVs were resuspended in 5 mL of PBS and centrifuged once more at the same conditions until there were only 100 µL of EVs suspension in the spin column. Then, the labelled EVs were collected and stored at 4ºC overnight and until further use.

The hCFs were seeded as reported in 2.4.2.1. In the following day, hCF were exposed to 10^7 EVs labeled with PKH26 diluted in working medium (WM; DMEM supplemented with 0.1% depleted FBS (100000 x g, 4h, 4ºC), 1% P/S and 0.2 mM Asc-2P) during 12h, 37ºC. After the incubation period, hCFs were fixed with 4% paraformaldehyde in PBS for 12 min and a immunofluorescence assay was done as reported in 2.4.4. Samples were mounted with Fluroshield (Sigma Aldrich, Co.) and images were acquired in a Leica TCS SP5 II laser scanning confocal microscope and adjusted for brightness and contrast using Fiji software (ImageJ version 1.51n, NIH, USA).

2.4.3. HCF activation assay with hEVs

2.4.3.1. Culture of HCF with hEVs

The HCFs were seeded (day -3) at 36500 cells/cm^2 in a 96 culture well-plate in seeding medium (SM; Dulbecco's Modified Eagle's Medium (DMEM; Thermofisher) supplemented with 10% fetal bovine serum (FBS; Lonza), 1% Penicillin-Streptomycin (P/S; Biowest) and 0.2 mM 2-phospho-L-ascorbic acid (Asc-2P; Sigma Aldrich, Co.)). Following 24 hours (day -2), the medium was changed for working medium (WM; DMEM supplemented with 0.1% depleted FBS (100000 x g, 4h, 4ºC), 1% P/S and 0.2 mM Asc-2P). Two days later (day 0) the medium was exchanged for a media containing EVs at a concentration of 1x10^8 particles per mL in WM. Media exchange was performed every 48 hours until day 6. Cells were collected at day 7.

2.4.4. Immunofluorescence assay

For immunofluorescence assay, hCF were cultured clear bottom CellCarrier-96 well (PerkinElmer) in a protocol similar as 2.4.2.1. hCF were fixed with 4% paraformaldehyde (PFA)

in PBS pH 7.4 for 10 min at RT and washed twice for 3 min with PBS (3 minutes, RT). Further, they were permeabilized with 0.1% Triton X-100 in PBS for 10min at RT, washed twice with PBS for 3 min and, then, incubated with blocking solution (1% BSA and 4% FBS in PBS-Tween 0.1%) for 1h at RT. At the end of incubation period, the cells were incubated with primary antibody (1:400 anti-α-SMA mouse, A5228, Sigma Aldrich, Co.) in blocking solution overnight at 4ºC. In the following day, hCF were washed twice with PBS (3 min, RT) and incubated with secondary antibody (1:1000 donkey anti-mouse Alexa Fluor 594, A21203, Invitrogen) in blocking solution for 2h at RT. Cells were washed twice with PBS (RT, 3 min) and incubated with phalloidin 488 conjugate (1:5000 488 conjugate, sc-363791, Santa Cruz Biotechnology) in PBS for 20 min at RT. Followed this incubation, cells were washed twice for 3 min with PBST and incubated with DAPI 1:1000 for nuclei staining for 5 min at RT. After the last two washes with PBS for 3 min, the cells were kept in PBS protected from light until image acquisition using an IN Cell Analyzer 2000 (GE Healthcare Life Sciences, USA) with Nikon 20X/0.45 NA Plan Fluor objective and the 4,6-diamidino-2-phenylindole (DAPI), Texas Red and FITC filters. The number of cells per field of view was counted using Fiji software (ImageJ version 1.51n, NIH, USA).

2.4.5. Metabolic assay (Resazurin assay)

Resazurin (Sigma Aldrich, Co.) was prepared at a concentration of 0,1 mg/mL in PBS and stored at -20ºC until further use. At the desired timepoints (days 0, 2 and 6) a solution of 10% resazurin in WM was prepared and added to the cells and incubated for 2 hours at 37ºC, protected from light. Then, resorufin fluorescence was read using a microplate spectrofluorometer (λ_{exc} = 570 nm, λ_{em} = 590 nm).

2.5. RNA extraction and reverse transcription

RNA extraction from hCFs was performed using the TRIzol Reagent (Invitrogen) following manufacturer's protocol. The quantity of RNA was determined using a NanoDrop1000 spectrophotometer, and reverse transcription was performed to synthetizes complementary DNA using PrimeScript RT reagent Kit (Takara) according to manufacturer instructions as shown in **Table 4**.

Table 4 - Reverse transcription master mix components.

Components	Volume per reaction
5X PrimeScript Buffer	4
PrimeScript RT Enzyme Mix	1
Oligo dT Primer	1
Random 6 mers	1
Total volume	7 uL

2.5.1. Real-time (RT-PCR)

To quantify gene expression, RT-PCR was performed using iTaq™ Universal SYBR Green Supermix (Bio-Rad). A reaction mix for each primer was prepared without cDNA and added to a 96-well PCR low plate. Primer sequences are specified in **Table 5**.

Table 5 - Primer Sequences for qRT-PCR.

Gene	Primer sequence (5' to 3')
GAPDH	FW: CCTCCACCTTTGACGCT
	RV: GGACTCCCCAGCAGTG
COL1A1	FW: GAGGGCCAAAGACGAAGACATC
	RV: CAGATCACGTCATCGCACAAC
ACTA2	FW: GGCAAGTGATCACCATCGGA
	RV: GTGGTTTCATGGATGCCAGC
CCN2	FW: CTTGCGAAGCTGACCTGCAAGA
	RV: CCGTCGGTACATACTCCACAGA
FAP	FW: GCTACGATGGAGGCGCTAAT
	RV: CAGTGTGAGTGCTCTCATTGTA

Then, the cDNA was added to the corresponding wells and centrifuged at 1000 x g for 1 min. The wells were covered with a sealing tape to prevent the reaction mix from evaporation, and the plate was centrifuged again in the same conditions before being read in a CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad). The programmed PCR cycles are described in further detail in **Table 6**. Gene expression was calculated by the difference of the mean threshold cycle values between the reference (*GAPDH*) and the genes of interest ($2^{-\Delta Ct}$ method).

Table 6 - RT-PCR cycling conditions

Cycle	Number of cycles	Step	Time	Temperature
1	1	Activation	3:30	95ºC
2	40	Denaturation	0:20	95ºC
		Annealing	0:30	60ºC
3	81	Melt curve	0:10	55ºC

2.6. Statistical Analysis

Statistical testing was performed using GraphPad® Prism 8.0 Software. Normality tests (D'Agostino-Pearson) were performed between groups. Comparisons of two groups followed the non-parametric Mann-Whitney test, while comparisons of three groups used the Kruskal-Wallis test with Dunn´s post-hoc correction for multiple comparison. Outliers were excluded by ROUT analysis (GraphPad). Results are presented as Mean ± SEM and differences between groups were considered significant when $p<0.05$.

CHAPTER V – RESULTS

5.1. Systemic factors of HFpEF patients induce *in vitro* cardiac fibroblast activation

The mechanisms linking systemic inflammation and myocardial fibrosis in HFpEF are largely unknown. To address whether systemic factors directly influence resident myocardial fibroblasts in HFpEF, peripheral blood was collected from patients diagnosed with HFpEF (n=39, HFpEF group) according to ESC2016 guidelines and compared to a cohort of control patients (n=10, CTRL group) [6]. The two groups were similar in age, sex, body-mass index (BMI), and most cardiovascular comorbidities. Echocardiographic and analytical evaluation highlighted differences in the levels of left atrial volume index (LAVI), pulmonary artery systolic pressure (PASP), high-sensitivity troponin (hsTnl) and B-type natriuretic peptide (BNP) (**Table 7**). As reported in [154], our cohort of patients with HFpEF also display systemic and pulmonary alterations, presenting lung congestion and peripheral edema.

Human cardiac fibroblasts (hCFs) were stimulated with serum collected from both patient groups as represented in **Figure 10A**. After 48 hours of exposure, an immunofluorescence assay for the fibrotic marker α-SMA (**Figure 10B**) showed that cardiac fibroblasts stimulated with the serum of HFpEF patients showed an elongated morphology with prominent stress fibers (F-actin, arrow) and evident α-SMA, all typical features of myofibroblastic differentiation [155, 156]. Cells cultured in CTRL showed less evidence of these cardiac fibroblasts' activation features. Then, the expression of genes encoding proteins associated with cardiac fibroblast activation and differentiation in myofibroblasts was evaluated, namely, collagen type I (*COL1A1*), α-SMA (*ACTA2*), connective tissue growth factor (*CCN2*), fibroblast activation protein (*FAP*) were evaluated by qPCR (**Figure 10C**). Interestingly, the serum of HFpEF patients was able to induce a significant increase in the expression of *COL1A1* and *CCN2* when compared to CTRL patients whilst no differences were found in *ACTA2* and *FAP* levels.

Together, these results indicate that the serum of HFpEF patients contains circulating factors capable of inducing a fibrotic phenotype in cardiac fibroblasts *in vitro*.

Characteristic	CTRL (n=10)	HFpEF (n=46)	p-value
Median Age – years (IQR)	64 (57-73)	72 (66-79)	NS
Male sex – no. (%)	6 (60.0)	27 (58.7)	NS
BMI – Kg/m^2	30.5 ± 4.5	29.1 ± 4.9	NS
Cardiovascular Comorbidities			
Hypertension - no. (%)	8 (80.0)	44 (95.6)	NS
Diabetes - no. (%)	6 (60.0)	26 (56.2)	NS
Dyslipidemia - no. (%)	9 (90.0)	41 (89.1)	NS
Smoking - no. (%)	5 (50.0)	17 (36.9)	NS
Atrial Fibrillation - no. (%)	0 (0.0)	16 (34.8)	**0.03**
HF Signs and Symptoms			
Lung Congestion – no. (%)	2 (20.0)	27 (58.7)	**0.03**
Peripheral edema – no. (%)	2 (20.0)	33 (71.7)	**0.002**
NYHA functional classification - no. (%)			
I	-	19 (41.3)	-
II	-	23 (50.0)	-
III	-	4 (8.7)	-
Bioimpedance			
Median ECW/TBW - % (IQR)	38.1 (37.5-38.9)	39.4 (38.5-40.1)	**0.05**
Echocardiographic evaluation			
LVEF - (%)	62.8 ± 2.9	62.3 ± 3.9	NS
LAVI – (mL/m^2)	26.5 ± 6.1	36.6 ± 8.5	**0.002**
LVMI – (g/m^2)	126.7 ± 30.5	140.6 ± 28.7	NS
E/E'	8.1 ± 2.5	9.9 ± 2.9	NS
PASP – mmHg (IQR)	24 (21-29)	30 (25-40)	**0.05**
Analytical Evaluation			
Hemoglobin - g/dL	13.5 ± 1.6	12.6 ± 1.8	NS
Median eGFR - mL/min/1.73m^2	93.9 (82.6-109.8)	82.5 (49.8-112.9)	NS
Total Cholesterol - mg/dL	156.4 ± 36.6	145.2 ± 44.1	NS
Apolipoprotein B - mg/dL	81.0 ± 20.6	77.0 ± 19.8	NS
Median HbA1c - % (IQR)	6.2 (5.6-7.4)	6.1 (5.5-6.9)	NS
Median hsCRP - mg/L (IQR)	2.8 (1.1-4.6)	2.6 (0.7-6.4)	NS
Median hsTnI - pg/L (IQR)	3.1 (1.9-3.7)	6.6 (3.9-10.2)	**0.003**
Median BNP -pg/mL (IQR)	25.6 (17.2-37.7)	110 (80.1-210.8)	**<0.001**
Medication			
Beta-blocker – no. (%)	2 (20.0)	31 (67.4)	**0.01**
Loop Diuretics – no. (%)	1 (10.0)	31 (67.4)	**0.002**
ACEi/ARB – no. (%)	6 (60.0)	28 (60.9)	NS
Statin – no. (%)	6 (60.0)	37 (80.4)	NS

Table 7 - Functional characterization of the patient population. Plus-minus values are mean ± SD. IQR – interquartile range. 2-tailed t-test and Mann-Whitney test were used for variables with normal and non-normal distribution (Shapiro-Wilk test), respectively. **CTRL** - comorbidity-matched control group; **HFpEF** – heart failure with preserved ejection fraction. BMI – body-mass index; NYHA – New York Heart Association; ECW – extracellular water; TBW – total body water, LVEF – left ventricular ejection fraction; LAVI – left atrial volume index; LVMI – left ventricle mass index; PASP – pulmonary artery systolic pressure; eGFR – estimated glomerular filtration rate; HbA1c – glycated hemoglobin; hsCRP – high-sensitivity C-reactive protein; hsTnI – high-sensitivity troponin I; BNP – B-type natriuretic peptide; ACEi/ARB -angiotensin-converting enzyme inhibitors/angiotensin receptor blockers.

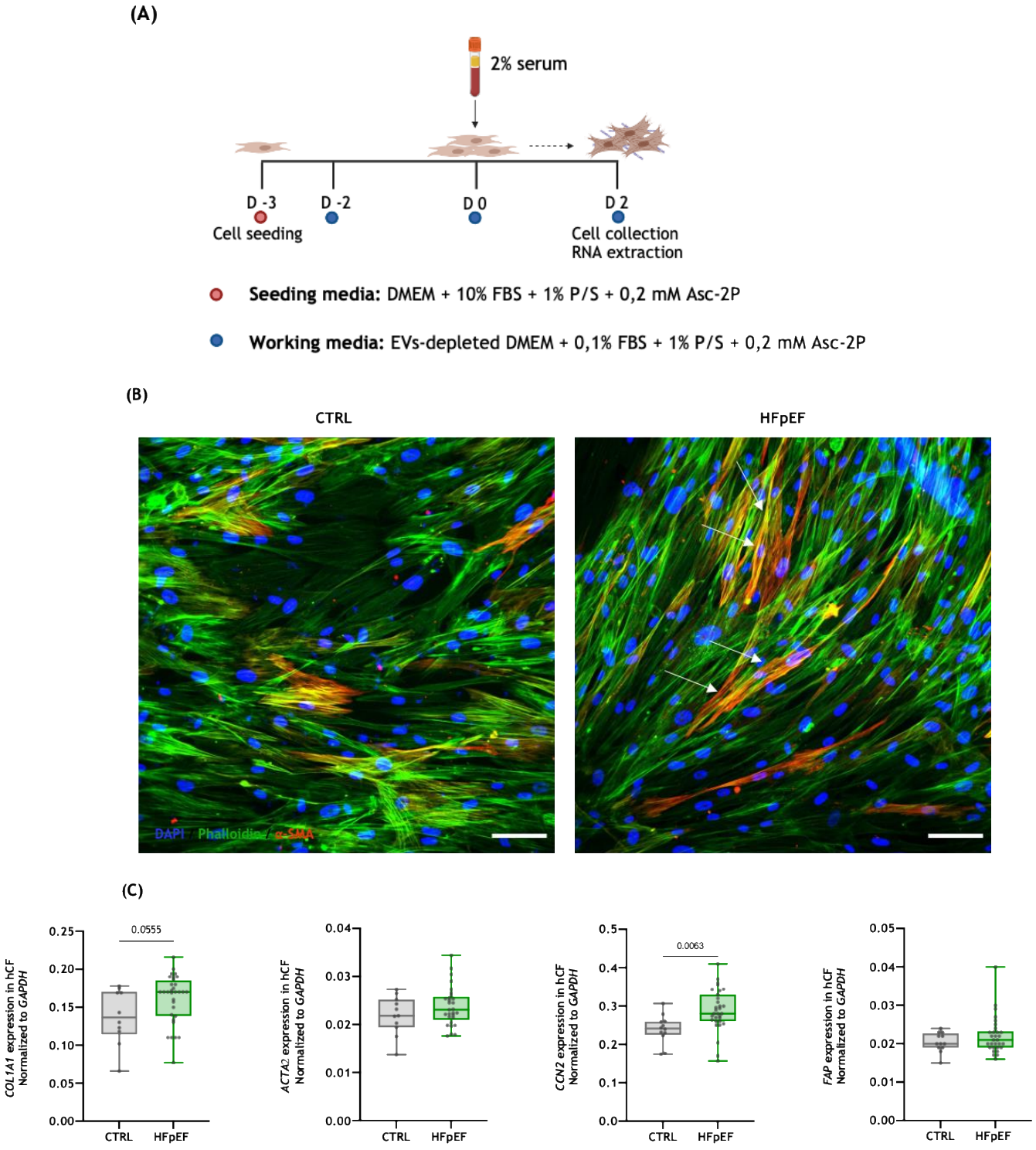
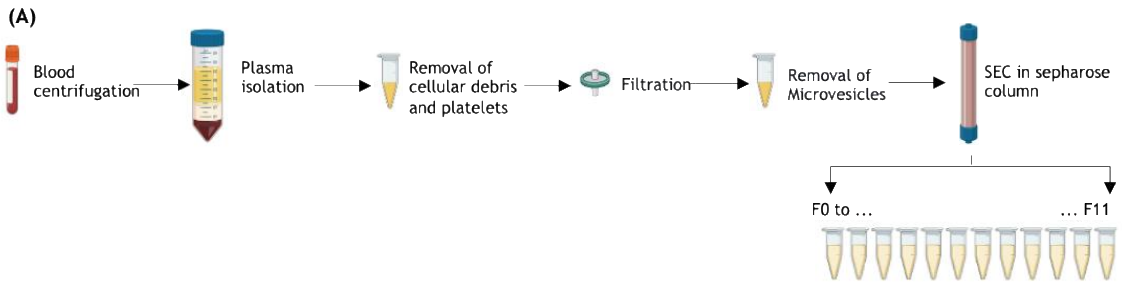


Figure 10 - Serum from HFpEF patients promotes human cardiac fibroblasts (hCF) activation *in vitro*. **(A)** Schematic representation of the experimental design. **(B)** Representative images of hCFs cultured with the serum of HFpEF and CTRL patients immunolabeled with phalloidin (green) and smooth muscle actin (red, arrow) (scale bar = 100 µm). **(C)** Expression of *ACTA-2*, *Col1α1*, *CTGF* and *FAP* in hCF exposed to 2% of serum from CTRL or HFpEF patients (n>11 (CTRL) and n>29 (HFpEF)). Data was normalized to GAPDH and presented as box and whisker plots in (C). Asc-2P – ascorbic acid-2-phosphate; DMEM – Dulbecco's Modified Eagle Medium; FBS – fetal bovine serum; P/S – penicillin and streptomycin.

5.2. Isolation and characterization of EVs present in the plasma of HFpEF patients

Extracellular vesicles (EVs) are key mediators of inter/intracellular communication in physiological and pathological conditions that carry bioactive molecules capable of modulating the phenotype of the recipient cell [65]. Thus, we hypothesized that EVs present in plasma of HFpEF patients may mediate the pathophysiological events that culminate in cardiac fibrosis in HFpEF. EVs were extracted from CTRL and HFpEF groups by SEC as demonstrated in **Figure 11A**. Fractions were further analyzed by dynamic light scattering (DLS) to determine which were enriched in exosomes (50 to 150 nm of diameter). In both studied groups, fraction 3-5 contained particles with a size compatible with exosomes (**Figure 11B**). Concomitantly, the total protein content was assessed to detect the presence of protein contaminants (**Figure 11B**). The protein content gradually increased until fraction 8, reaching a peak in the order of 9400 µg/mL and then decreasing until fraction 11. Of note, the overall size and protein concentration profile was consistent throughout all analyzed samples. Taken together, these findings suggested that fractions 3 to 5 were enriched in EVs, however, the beginning of the accentuated increase in protein content observed in fraction 5 indicated that this fraction is also enriched in protein contaminants, which is not advantageous for studies aiming to discriminate the biological effect of EVs from soluble proteins. To further strength the identification of EVs enriched fractions, bead-assisted flow cytometry for the EV marker CD71 [74, 157, 158] was performed (**Figure 11C**). The latter analysis further confirmed that EVs were more abundant in fraction 3 and 4 (**Figure 11C**). Based on these results, fractions 3 and 4 displayed a better EV/Protein ratio and were concentrated for further analysis. Fractions 9 and 10, that had low abundance of EVs and high concentration of proteins, were further selected to control (Prot) for the possible biological effect of protein contaminants in EVs suspensions in subsequent studies with cells.



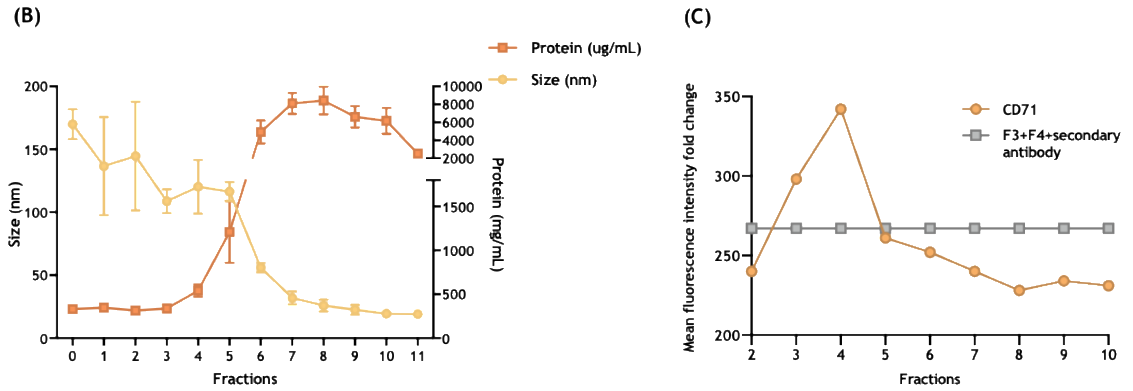
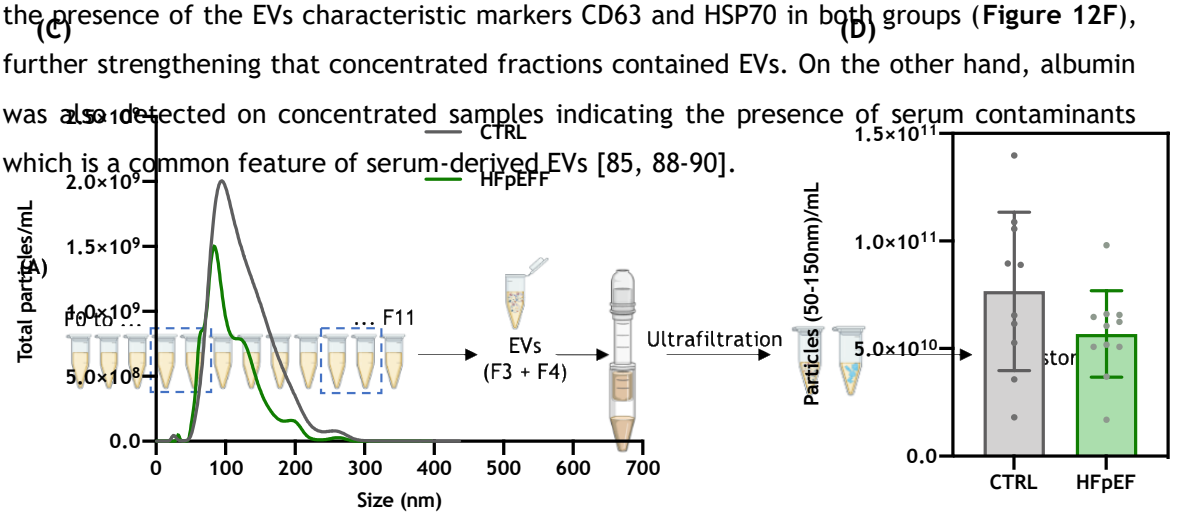


Figure 11 – Separation of plasma EVs from HFpEF and CTRL patients using SEC. (A) Schematic representation of EV separation through SEC. **(B)** Particle size distribution by DLS alongside protein quantification of fractions 0 to 11, collected during EV isolation (n=5). **(C)** Detection of the EV marker CD71 from fractions 2 to 10 by bead-based flow cytometry. F – fraction; SEC – size-exclusion chromatography.

Fractions 3 and 4 were further were then concentrated using ultrafiltration **Figure 12A** and EVs characterized accordingly to MISEV2018 guidelines[78]. Transmission electronic microscopy (TEM) demonstrated the presence of EVs limited by a lipid bilayer, with the typical cup-shaped morphology and size ranging from 50 to 150 nm in the concentrates from both patient groups (**Figure 12B**). NTA was used to obtain an accurate estimation of particle size and concentration (**Figure 12C**). The size mode of EVs by NTA was 90,3 ± 13,9 nm and 100,3 ± 10,9 nm in CTRL and HFpEF groups, respectively (**Figure 12D**). Although particle size was similar across the two groups, the CTRL group showed a tendency for higher concentration of EVs (7,73x10^{10} particles/mL), using a cut off size of 50-150 nm, compared to the HFpEF group (6,05x10 ^{10} particles/mL) (**Figure 12E**). Importantly, preliminary Western Blot analysis showed the presence of the EVs characteristic markers CD63 and HSP70 in both groups (**Figure 12F**), further strengthening that concentrated fractions contained EVs. On the other hand, albumin was also detected on concentrated samples indicating the presence of serum contaminants which is a common feature of serum-derived EVs [85, 88-90].



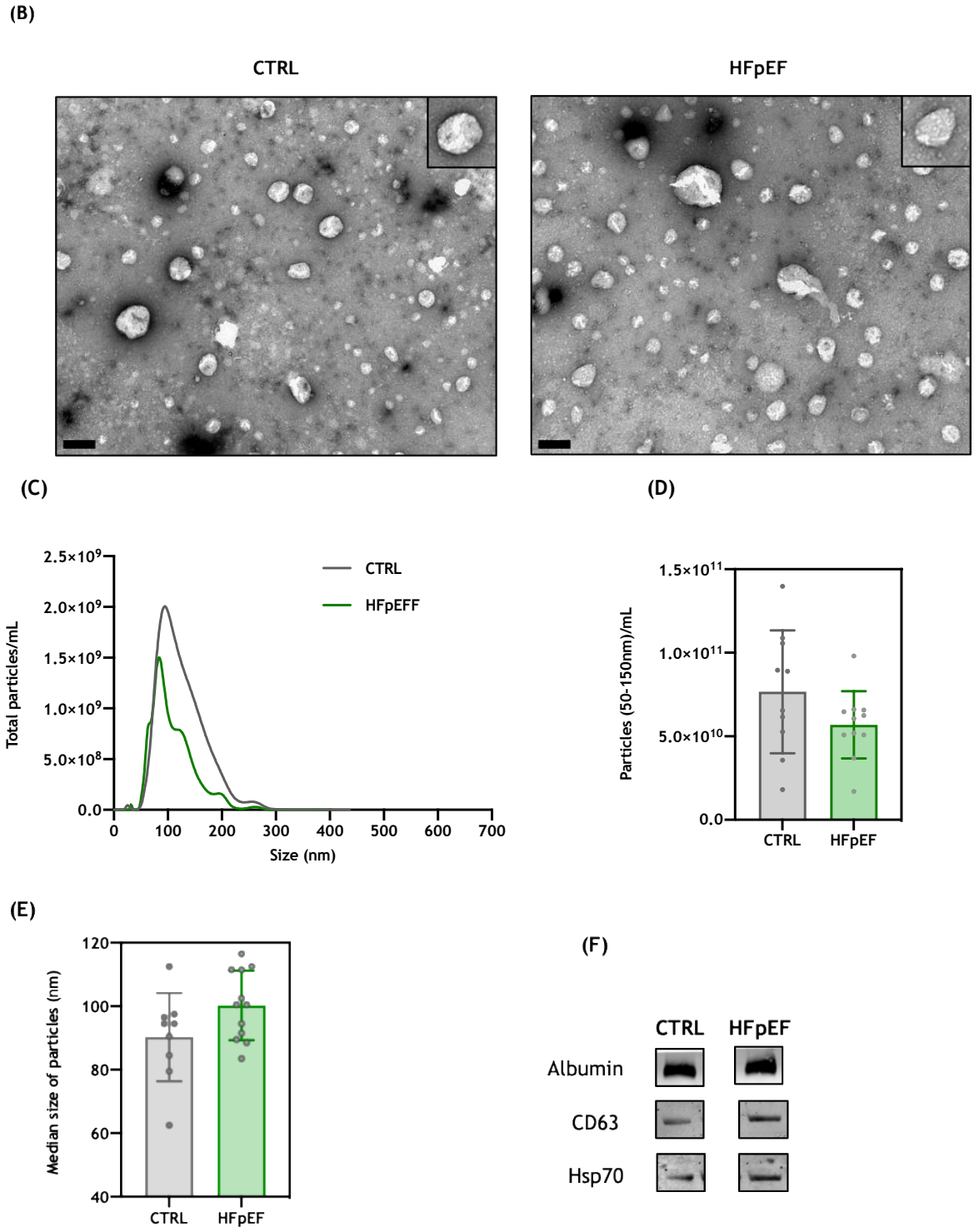


Figure 12 - EVs concentration and characterization. (A) Schematic representation of EV concentration through ultrafiltration. **(B)** Representative image of TEM of EVs concentrates of CTRL and HFpEF patients. (Scale bar = 200 nm). **(C)** Representative NTA of EVs collected from CTRL and HFpEF patients. (**D)** Mode and **(E)** EVs concentration of particles ranging from 50 nm to 150 nm by NTA (n=11 for CTRL and n=12 for HFpEF) **(F)** Representative Western Blot of common proteins found in EVs (CD63 and HSP70) or in the serum contaminants (albumin). Data are presented as histogram in (C) and mean ± SEM with points in (D) and (E).

5.3. Cardiac fibroblasts are able to internalize EVs derived from plasma of HFpEF and CTRL patients

Before testing the biological effect of serum-EVs on human cardiac fibroblasts, we performed a proof-of-principle assay to confirm that these cells were able to internalize EVs derived from plasma of HFpEF and CTRL groups. For that, hCFs were exposed to PKH26-labelled EVs and then observed by confocal microscopy. Of note, labelled EVs were found inside cells, confirming that EVs were effectively internalized by hCFs (**Figure 13**).

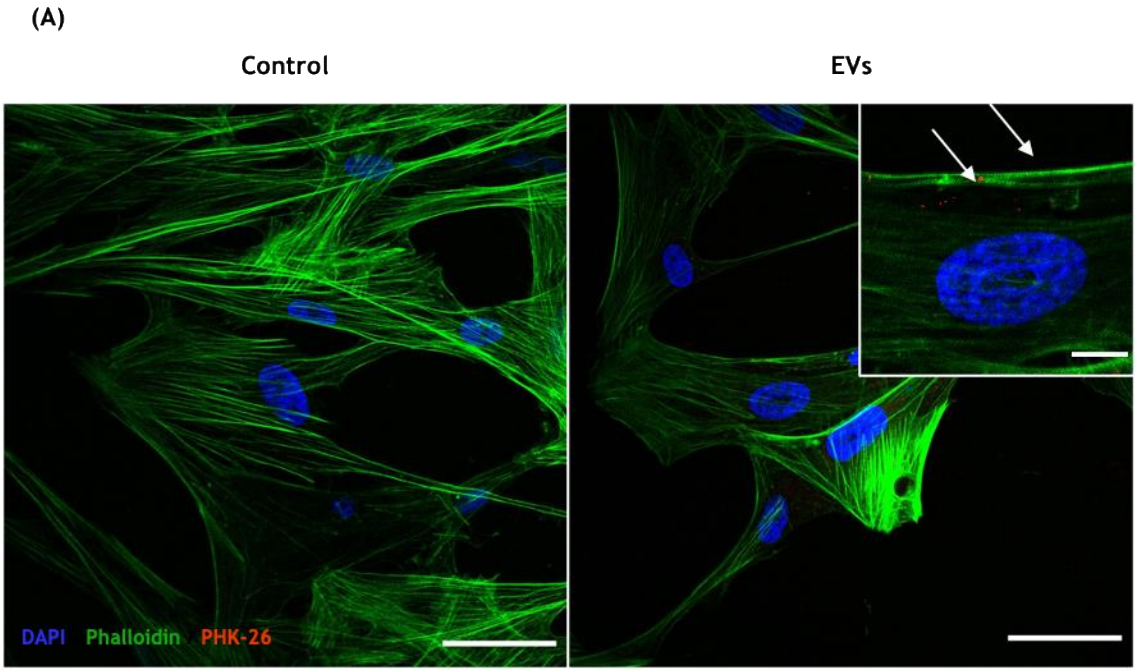


Figure 13 -Human cardiac fibroblasts are able to capture EVs from HFpEF and CTRL patients. **(A)** Representative images of hCF exposed to PKH26-stained EVs (in green) and respective controls (cells cultured with PKH26 and without EVs). Cells were immunolabeled with phalloidin (red) and DAPI (blue) (scale bar = 50 µm and 10 µm in the inset).

5.4. EVs induce a dose-dependent response in human cardiac fibroblasts

Following establishing that hCF are capable of internalizing EV, a dose-response assay was performed by exposing cells to different concentrations of EVs **Figure 14A**. Amongst the four different tested concentrations, the HFpEF and CTRL groups displayed the highest levels of *COL1A1* and *ACTA2* when exposed to an intermediate concentration of $1x10^8$ particles/mL (**Figure 14B**). Of note, at that concentration COL1A1 was significantly increased in HFpEF group when compared with CTRL patients. Interestingly, at the lowest concentration of $5x10^7$ particles/mL, the relative expression of both *COL1A1* and *ACTA2* showed a tendency to be

decreased in the HFpEF group, suggesting a possible protective effect from fibroblast activation at lower doses.

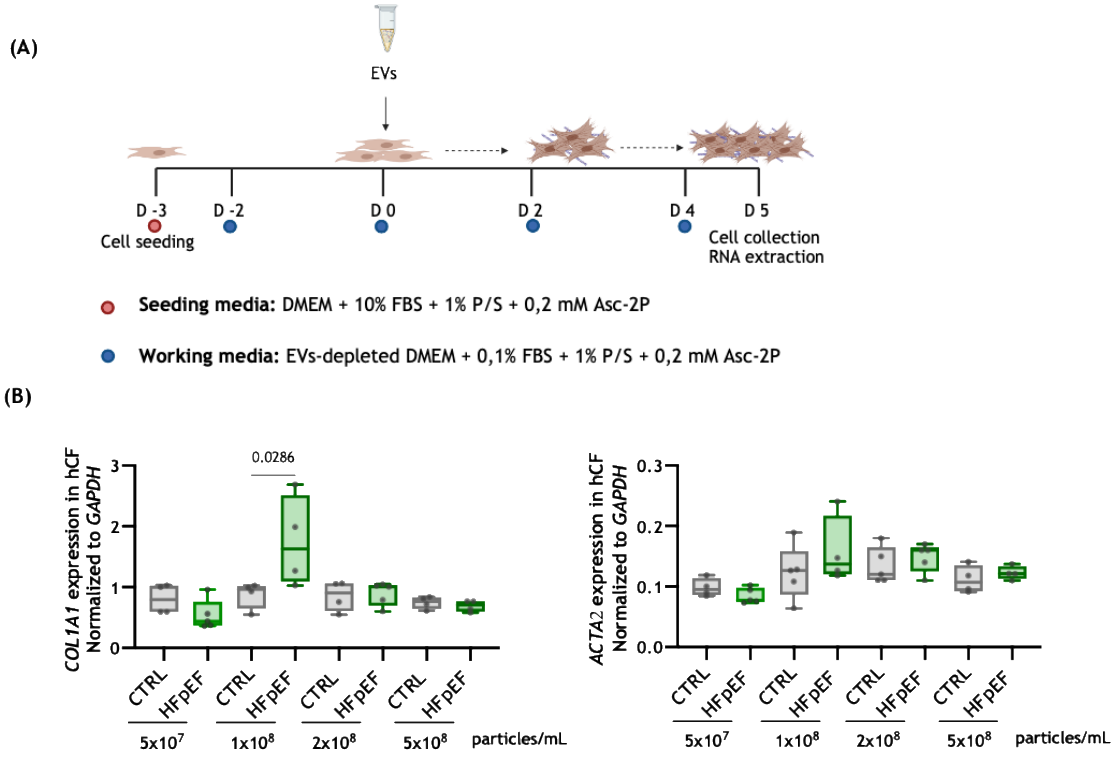


Figure 14 - Dose response assay of hCF exposed with EVs from HFpEF and CTRL patients. **(A)** Schematic representation of experimental design of human cardiac fibroblasts activation with EVs from CTRL and HFpEF patients. **(B)** Expression of *ACTA2* and *COL1A1* in hCF exposed with different concentrations of EVs isolated from CTRL and HFpEF patients (n=5 for CTRL and n=4 for HFpEF). Data normalized to GAPDH. Data presented as box and whiskers.

5.5. HFpEF-derived EVs promote the expression of fibrogenic-associated genes in cardiac fibroblasts when compared to CTRL

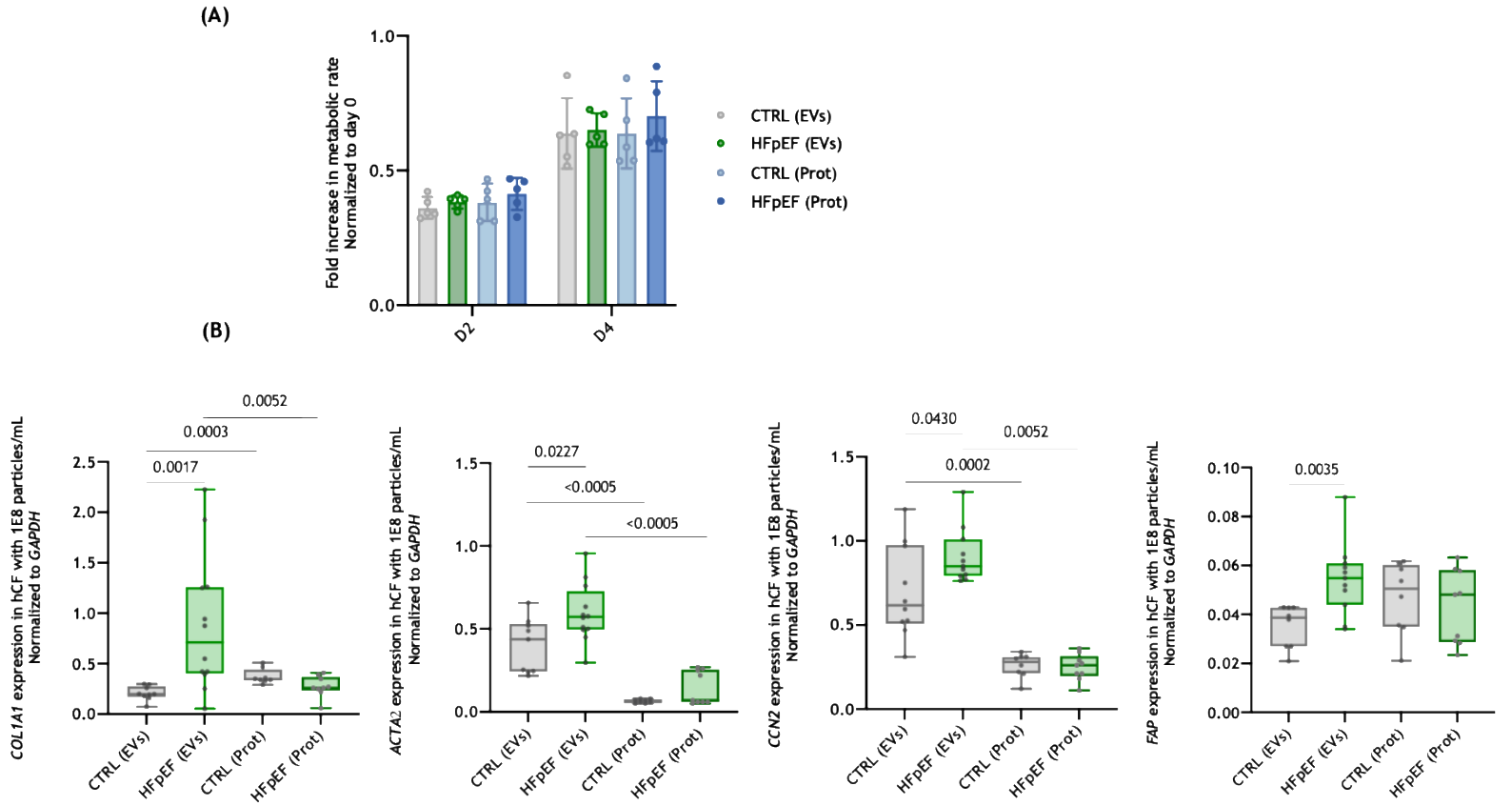
Having encountered the optimal concentration for EV administration (1x108 particles/mL) in biological assays, we repeated this protocol with an increased number of patients and additional fibrosis-associated molecules. To serve as internal controls, fractions 9 and 10 (Prot), which were enriched in protein and not in exosomes, were concentrated and administered to cells using the same amount of protein as the EV counterpart.

Firstly, a resazurin assay was performed to evaluate whether the administration of EVs and protein content to the hCFs had an effect on cell viability (**Figure 15A**). The results demonstrated that, all the conditions show a decrease in the metabolic rate in both timepoints when compared to the day 0. This decrease in metabolic activity was expected as cells were cultured in media containing 10% of fetal bovine serum (FBS) at day 0 whereas at day 2 and 4 cells were already in media with reduced (0,1%) amounts of FBS. Of note, no differences were

found across treatment groups supporting that EVs had no significant impact on cellular metabolic activity and/or survival.

At day 7 of activation, the expression of *COL1A1*, *ACTA2*, *CCN2* and *FAP* was evaluated by qPCR (**Figure 15B**), demonstrating a significant increase in all studied pro-fibrotic molecules in HFpEF derived EVs when compared to CTRL groups. Importantly, higher expression of most pro-fibrotic molecules, except for *FAP*, was found in EVs when compared to the respective protein controls (Prot) in both studied groups, supporting that the biological effect is specific of the vesicles.

Further immunofluorescence assay for pro-fibrotic marker α-SMA (**Figure 15C**) clearly showed that a subset of cardiac fibroblasts became activated in both groups, as demonstrated by strong expression of α-SMA, which co-localized with stress fibers (F-actin). An additional quantitative analysis of the immunofluorescence assay showed (**Figure 15D**) that cells stimulated with HFpEF and CTRL-derived EVs showed a similar percentage of α-SMA positive cells when compared with the CTRL group, indicating that following 7 days of incubation the two patient groups attained similar activation rates.



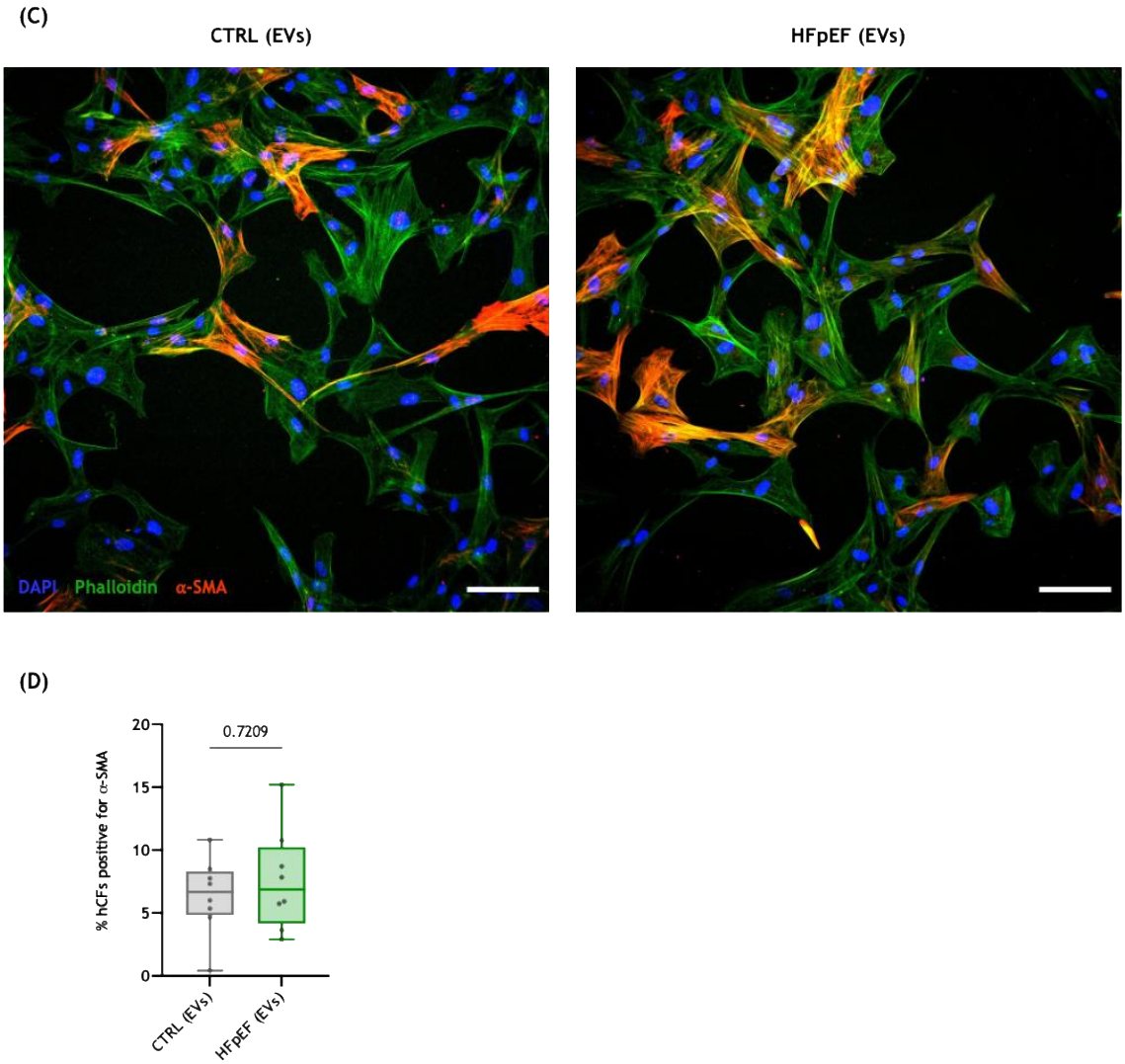


Figure 15 – EVs from HFpEF patients promote human cardiac fibroblasts activation. (A) Relative gene expression of *COL1A1*, *ACTA2*, *CCN2* and *FAP* in hCF exposed to EVs and respective protein (Prot) isolated from CTRL and HFpEF patients (n>8 for CTRL and n>9 for HFpEF). Data normalized to *GAPDH*. Data presented as box and whiskers. **(B)** Representative images of hCFs cultured with EVs and Prot immunolabeled with phalloidin (green) and α-SMA (red) (scale bar = 100 µm). **(C)** Percentage of hCFs positive for α-SMA (n=8 for CTRL and n=8 for HFpEF). **(D)** Metabolic activity of hCFs exposed to 1E 8 particles/mL of EVs from HFpEF and CTRL patients. Data normalized to day 0 of each respective condition. Data presented as box and whiskers in (A) and (D), and mean ± SEM in (B).

CHAPTER VI – DISCUSSION

HFpEF is a multifactorial disease, with late and insufficient diagnosis and poor treatment outcomes. One of the main difficulties in tackling this syndrome relies on the lack of knowledge on upstream mechanisms driving this disease. Although systemic inflammation is thought to be a key driver of cardiac pathological alterations, namely myocardial fibrosis, the underlying mechanisms remain largely unknown.

In the present work, serum collected from the HFpEF patient cohort was able to induce a pro-fibrotic phenotype in a primary cell line of cardiac fibroblasts, suggested by an immunofluorescence assay and confirmed by the up-regulation of the pro-fibrotic genes, *COL1A1* and *CCN2*. These findings support that circulating factors may constitute a potential source of fibrotic factors that can be targeted therapeutically to attenuate cardiac remodeling in HFpEF. Similarly, circulating factors from the serum of systemic sclerosis have been demonstrated to induce fibroblast activation in the lung, contributing to pulmonary fibrosis[159]. Also, circulating factors present in the plasma of patients with heart failure such as miR-130a was shown to have a pro-fibrotic effect and contribute to adverse cardiac remodeling [160]. However, the herein work is the first to address the cardiac fibrogenic effect of systemic factors in the context of HFpEF.

Previous studies have demonstrated the key role of EVs on long-distant communication in other cardiac pathologies [161-164]. Here, we hypothesized that EVs may mediate the crosstalk between different organs, namely the immune system (see **Chapter II**) with cardiac cells, contributing for the deposition of myocardial fibrosis in HFpEF. To investigate this, we used SEC to extract EVs from the plasma with subsequent ultrafiltration, obtaining samples enriched in EVs as confirmed by different techniques of characterization. Previous studies have demonstrated that SEC is an efficient method for EVs extraction due to the high yield and reproducibility rates, and good preservation of the EVs structure when compared with other methods such as ultracentrifugation [91]. Moreover, previous works that used SEC for the extraction of EVs from human plasma demonstrated to be easy to use, relatively fast and just requires low amount of sample for a sensitive and reproducible analysis when compared with, for instance, ultracentrifugation [87-89]. However, some chylomicrons (100 to 2000 nm [89]) and very low density lipoproteins (VLDL, 27 to 200 nm[89, 165]) are in the same size range of contaminants in the EV samples which may be separated with EVs with SEC [91]. In line with this, the evaluation of lipoprotein contaminants in the EVs preparations would constitute an important complementary characterization of this study. Nevertheless, and despite eventual lipoprotein and protein contamination, the presence of EVs-characteristic proteins herein confirmed through the presence of CD63 and CD71, and detection of vesicles with size ranging from 50 to 150 nm with a cup-shaped morphology. Still, considering that MVs present a size-range from 50 to 500 nm, further characterization with other exosome-specific markers (CD151, CD37, CD53) [65] and MVs-specific markers (phosphatidylethanolamine, CD82, CD40L) [65, 166] could help to define the type of EVs present in the isolated EV preparations.

A quantitative analysis of the isolated EVs showed similar concentration of EVs in the CTRL and HFpEF plasma. Interestingly, others have reported a correlation between the number of circulating EVs and CVDs [167-169]. In fact, the release of EVs has been demonstrated to be increased in post-myocardial infarction conditions [170, 171], arterial and venous thrombosis [167], atherosclerotic lesion [124], atherothrombotic disease [124], hypertension [172] and arterial fibrillation [168], and many of these works have managed to trace the origin of these vesicles to platelets [167, 170, 171], endothelial cells [124, 172-174], leukocytes [124] and erythrocytes [124]. In the context of HF, patients have higher levels of circulating EVs and increased number of endothelial-derived vesicles when compared to healthy subjects, which correlated with a stronger chance of cardiovascular events [173-175]. On the same line, our work would benefit from assessing the origin of the isolated EVs, by tracing their cell source, considering that our population of isolated vesicles may be enriched in exosomes derived from a particular cellular subset and represent interesting candidates for biomarkers of HFpEF.

In the present work, isolated EVs from HFpEF patients were able to promote higher up-regulation of pro-fibrotic genes and formation of stress fibers in hCFs than CTRL-derived EVs. Of note, this effect was not observed when cardiac fibroblasts were stimulated with the internal controls (i.e. protein-rich fractions) further corroborating EV-specificity. As carriers of biological content, EVs have different mechanisms of interacting with target cells [65]. Hence, the EV-mediated effect observed in the herein work may be a result of their interaction with molecules adsorbed to the surface of the EVs or the release of these molecules into these cells after uptake. The fact that EVs isolation methods are not completely efficient in the removal of non-EVs contaminants, these structures may have direct impact on target cells via biomolecules coating the EVs surface[65]. On the other hand, several studies have demonstrated that the uptake of miRNAS namely miR-130a [176] and miR-208a [177], may be delivered to cardiac fibroblasts via exosomes internalization, affecting the fibrotic response through the up-regulation of *COL1A1* and *ACTA2* genes. Accordingly, in our setting the genetic content inside our EVs may be responsible for the fibrotic response observed in hCFs. Moreover, cardiac fibroblasts-derived exosomes with miR-34 [178] and miR-27a [179] are also proven to contribute to myocardial dysfunction and inflammation in animal models of HF. Contrarily, other works have also identified several miRNAs that promote anti-fibrotic and anti-inflammatory characteristics, displaying a cardioprotective effect in the development of cardiac fibrosis [180-184]. A study conducted by Wang et al. identified miR-435 and miR-744-enriched exosomes as potential candidates for mediators cardiac fibrosis, and showed that these miRNAs act as protective regulators of cardiac fibrosis in the context of HF [185]. Hence, it would be interesting to further characterize the content of our isolated EVs, namely through RNA-sequencing to unveil the differential genetic content of the EVs between HFpEF and CTRL and identify the mechanism by which these EVs may induce cardiac fibrosis. This might provide

interesting information on the cellular and molecular mechanisms of HFpEF, which still represents a highly unknown field.

Previous data from our laboratory showed that hCF exposed to plasma from ZSF1-Ob animals treated with the senolytic drug navitoclax had decreased levels of pro-fibrotic genes *COL1A1* and *ACTA2* than ZSF1-Ob animals without any treatment. We hypothesized that cellular senescence may indeed trigger a change in the cargo of EVs towards a more fibrogenic one and thus contribute to cardiac fibrosis on HFpEF. Indeed, several works had demonstrated a close relationship between cellular senescence and fibrosis in several pathological conditions and have proven that EVs cargo change with senescence [186, 187]. Nevertheless, the intra/intercellular communication in this context is still poorly known. For instance, the discovery of microRNAs and other biomolecules that are specifically changed in HFpEF would be of extreme importance and might provide relevant information for the development of early diagnosis methods, biomarkers for screening in population and efficient treatments for this disease.

In summary, this study provides novel evidence for the etiology of HFpEF, indicating that EVs play a crucial role in the development of interstitial cardiac fibrosis in the context of this pathology.

CHAPTER VII – CONCLUSIONS AND FUTURE PERSPECTIVES

In summary, our study provides new evidence in pathological mechanisms underlying the development of HFpEF, namely that circulating EVs may contribute to the formation of cardiac fibrosis. To the best of our knowledge, this is the first study addressing the relevance of EVs in HFpEF. Importantly, these mediators of intercellular communication may represent a possible source for the discovery of new therapeutic targets and biomarkers for early diagnosis.

This study constitutes a solid foundation for future studies on the dissection of this long-distance communication, namely:

- Unveiling the involved molecular mechanisms by subjecting EVs to small RNA-sequencing and MS/MS to detect differences in miRNAs and proteins, respectively;
- Identify the producing cells source through the detection of specific cells markers on the EV by flow cytometry;
- Evaluate the impact of circulating EVs on other cardiac cell types, particularly on cardiomyocytes and endothelial cells;

We anticipate that these future studies will i) provide broad understanding on how these EVs contribute to the development of cardiac alterations in HFpEF and ii) generate new knowledge that may be important for the design of efficient therapies for this devastating syndrome.

CHAPTER VIII – REFERENCES

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