Transcription factors, microRNAs and extracellular vesicles at the crossroad of right and left heart failure

CHAPTER 7

“Summary and General Discussion”

The health system has been tremendously affected by COVID-19 and its associated deaths in the last years. Despite this, the nominator for one of the largest causes of death remains constant: cardiovascular diseases (CVDs). CVDs include a broad group of diseases that affect the heart and vessels that, when left untreated, can progress to heart failure (inability of the heart to pump enough blood) and eventually death.

The impact of Hand2 in RVF and LVF – different roads to the same destination

Heart failure (HF) is an end-stage of many CVDs, which are commonly divided HF into left ventricular (LVF) or right ventricular failure (RVF). The differences in LVF and RVF can be partly explained by each ventricle's intrinsic and specific characteristics, and their responses to damage are distinct, therefore requiring specific treatment. Despite RVF's severity, to date, studies on RVF are significantly less compared to LVF. RVF is a complex condition that requires accurate knowledge of its mechanisms and effective treatment. **Chapter 2** investigated the molecular mechanisms underlying RVF and studied the contribution of the transcription factor Hand2 to RV remodeling in response to pressure overload injury. Specific cardiac Hand2 depletion was associated with severe cardiac dysfunction under conditions of RV pressure overload. Hand2 depletion sensitized the RV to cardiac injury, as observed in animal models that developed more severe cardiac hypertrophy and dysfunction. Higher expression levels of HAND2 were also observed in RV samples from human heart samples of patients with pulmonary hypertension, usually associated with RVF. Although inhibition of Hand2 expression can prevent cardiac dysfunction under conditions of LV pressure overload, the same could not be valid for conditions of RV pressure overload. Hypertrophy is a common ground factor for both LV and RV responses to pressure overload. However, the pathways that activate and withstand hypertrophy may be different. Initially, pressure overload leads to calcineurin activation in both ventricles, but in LV, NFAT is dephosphorylated and translocates to the nucleus; it activates the expression of hypertrophic genes such as RCAN1.4 [23]. In the RV, it is suggested that myocyte enhancer factor 2 (MEF2) transcription factor is responsible to promote the transcription of hypertrophic genes [65].

Triggering the exact same injury in both ventricles might be challenging comparing previous work [1] with **Chapter 2**, different types of needles and time endpoints are used and thus, there is a possibility that the extent of injured LV and RV is different. Additionally, the baseline differences in pressure in the LV and RV may also explain the different responses to stress [2].

Other reports on humans with PAH, show that, initially, the ventricular pressure in RV leads to a stretch on the right ventricular wall followed by an increase in muscle mass. Sustained pressure overload is not supported by the RV as it cannot maintain an "adaptive hypertrophy" and will ultimately enter into a dilated phase [3, 4]. Later stages of the disease showed a decline of RV function and a decrease in RV stroke volume, but also a deformation of RV/LV septum, which is shifted towards the left, resulting in a reduction of LV filling volume and altogether contributes to decreased cardiac output [4, 5]. **Chapter 2** reported an increase in cardiac cell size and deterioration of RV function parameters but preserved cardiac output, suggesting that the used model did not reach an advanced disease stage like observed in severe PH, which is usually associated with RVF. Consequently, the results observed in **Chapter 2** are possibly valid for a mild form of RVF and PH. There is a possibility that Hand2 has different roles and significances during the different stages of PH and RVF. Nevertheless, the results of Chapter 2 suggested that there are multiple pathways to HF and also multiple pathways to avoid HF, remarking the importance of looking at HF both as a whole and individually at RVF and LVF.

MicroRNAs and EVs as Mirrors of Cardiac Condition

Rescuing the heart from HF is now possible by inducing reverse remodeling (RR) through cardiac therapy and medical treatments [6]. MicroRNAs (miRs) are a class of small non-coding RNAs found both in cellular and extracellular spaces. Due to their ability to mirror cardiac conditions, they have recently emerged as potential therapeutic tools and biomarkers [7]. MiR expression is altered during cardiac pathological conditions and identification of miR expression profiles within extracellular fluids could potentially facilitate CVDs' diagnosis [8-10]. Moreover, circulating miRs are an attractive class of potential biomarkers owing to their high abundance and stability in the blood and other biologic fluids [11]. The intrinsic complexity of CVDs and their diverse mechanisms toward pathological conditions limit the use of a single biomarker for a correct diagnosis. To understand the current position of miRs and other non-coding RNAs **Chapter 3** summarized up-to-date research on the use of ncRNAs, including miRs, in human blood, plasma, and serum samples as biomarkers of CVDs, also discussing their weaknesses and potential. Many papers described a direct association between miR expression profiles and CVDs, including aortic-related diseases, myocardial infarction, right ventricle dysfunction, and also cardiac RR, with some ncRNAs such as muscle-specific miR-1 and lncRNA HOTAIR being reported in different CVDs [12-17]. However, several obstacles should be overcome, namely the small sample size of the study, with some possible cofounders associated (region, ethnicity, age, sex) and short-term follow-up, which might contribute to the general lack of reproducibility [18]. There is still a long road until miRs eventually reach clinical practice. While **Chapter 3** reviewed and concluded on the potential of miRs as biomarkers of CVDs, and their ability to mirror the cardiac condition, it did not critically assess the origin of these miRs. This gap was filled by **Chapter 5**, which reported on the source, vehicle, and destination cell of ncRNAs, including miRs, upon cardiac pathological remodeling. **Chapter 5** reviewed the current research on the role of ncRNAs in intercellular communication that use extracellular vesicles (EVs) as vehicles. EVs are double-layer vesicles secreted by cells to mediate intercellular communication [19] and may help maintain cardiac homeostasis. EVs transport cargo as ncRNAs coming from the host cell and may be functionally active on target cells. When the cardiac injury occurs, EV ncRNAs expression is altered and EVs-ncRNAs can act on recipient cells to promote remodeling processes such as cardiac hypertrophy, fibrosis, capillary rarefaction, inflammation, and others, leading to heart failure [20-23]. Therefore, modifications on EV ncRNA content contribute to and reflect the current cardiac condition. It has been suggested that modulation of ncRNAs can successfully prevent and even reverse cardiac maladaptive remodeling, however, so far, miR-122 is the only ncRNA that has reached a phase II clinical trial [24]. These findings highlight that pathological conditions imply complex crosstalk between different cell types extensively mediated by EVs. Analysis of the miR content of EVs or even miRs alone can contribute to understanding the heart condition at a specific time point.

MicroRNAs as Biomarkers for Reverse Remodeling in Aortic Stenosis -a dead-end road

MiRs have a pivotal role in regulating gene expression at a post-transcriptional level, but a new door was opened after discovering miRs in extracellular fluids namely plasma and serum [11]. Their features are particularly attractive due to the easy and minimally-invasive access to body fluids and rapid identification. Furthermore, extracellular miRs are remarkably stable in body fluids and, as previously discussed, they acutely mirror the present cardiac condition. The reason underlying miRs stability is still unknown; however, it is hypothesized that they are resistant to RNase A activity due to either EV encapsulation, being bound to high-density lipoprotein (HDL), or complexed with the AGO protein family [11]. Despite cardiac symptoms and the available biomarkers such as natriuretic peptides and troponins may indicate pathological cardiac conditions, more specific and sensitive

markers are still urgently needed [25]. In this regard, **Chapter 3** demonstrated that miRs are promising biomarkers and may increase the diagnostic capacity of cardiac pathological conditions if combined with other factors. Not only CVDs are associated with changes in miR levels but medical interventions, including aortic valve replacement (AVR), manifested alterations in miR expression profiles [26, 27]. Therefore, miRs have proven to be of diagnostic and prognostic value, possibly helping to predict the outcome (and consequently the type of RR) of a cardiac intervention such as AVR. Simultaneously, it has been observed that patients respond differently to therapy and it is necessary to predict the outcome of RR to improve the time of intervention, thus, aiming for personalized medicine strategies [6]. **Chapter 4** focused on identifying the expression of plasma miRs that emerged as potential biomarkers of complete RR after AVR surgery in a case study of a Portuguese sample. MiR-133a-3p and let-7b-3p were found significantly decreased in plasma samples from patients with incomplete RR (patients who do not show improvement in cardiac function and an unfavorable clinical response) compared with patients with complete RR (patients with recovered cardiac function). Qualitatively, the diagnostic accuracy of let-7b-3p is "very good", while miR-133a-3p is rated "excellent". Thus, our data suggested miR-133a-3p and let-7b-3p as potentially good biomarkers of complete RR after AVR surgery. Previously, miR-133a was also reported by *Nistal et al*. as a good predictor of LV RR after AVR in patients with aortic stenosis, which corroborates our results [27]. Our results also suggested that let-7b-3p could potentially be a new marker to recognize the RR type of remodeling and identify patients who benefit from SAVR. Given the exploratory nature of this project, the results obtained were not sufficient to recommend the use of miR-133a-3p and let-7b-3p in clinical practice but are strongly suggested to be further tested in larger cohorts. Despite the large potential of miRs to be used as biomarkers tools not only for CVD diagnosis but also prognosis, the standardization of plasma collection (fasting vs postprandial status, current sickness, physical activity) and storage (time and temperature) remains important, as well as miR isolation and detection methods. While no clinical trials with the appropriate study designs and strict timepoints to reach reproducible results are made, miRs will not reach clinical practice and continue on this “dead end” road that was reached years ago.

The Cre Lox-P system- a new road to track EVs in a 3D model

Increasing evidence has placed EVs at the center of CVDs since they have a significant role in mediating intercellular communication and, consequently, maintaining cardiac physiology [28, 29]. EVs are secreted from numerous cardiac cell types and alteration in their content or route could have a tremendous impact on heart homeostasis. Recent preclinical work on the ischemic heart using EVs as therapeutic tools generated promising results at a preclinical stage [30]. Some EV-based treatments evolved and are now at a clinical trial stage [31]. However, most current studies of EVs are based on the isolation of vesicles from cell cultures and therefore do not truly mimic the physiology of the human heart while retaining track of EVs behavior. Hence tracking EVs in a cardiac context is crucial to gain insights into their intricate biogenesis, release, biodistribution, timing, and trafficking. To better understand these aspects of EV biology, in **Chapter 6,** we proposed a new viral method using a 3D modified cardiac muscle-engineered human myocardium (EHM) model to monitor the intercellular transfer of vesicles by color change in recipient cells. The Cre-LoxP method uses two viral constructs previously reported by *Zommer et al*. [32] to help visualize how cardiomyocyte-derived EV-mediated cellular transfer occurs. An adeno-associated virus 6 (AAV6) harboring a cyan fluorescence reporter protein (CFP) and Cre 25nt that indicates cell secretion via EVs and a lentivirus harboring the reporter protein DsRed immediately followed by a stop codon, flanked by two Lox P sites and followed by an enhanced green fluorescence protein (eGFP) were used. Upon EV release by cardiomyocytes, recipient cells, namely stromal cells, can incorporate EV cargo, specifically the *Cre* recombinase. If EV cargo is

functionally active, recombination occurs and a change of color from red to green will be observed in recipient cells. Results reported in **Chapter 6** suggested that this Cre-LoxP method works in the cardiac context and could be a pilot proof towards a more complex *in vivo* EV tracking in the heart. It also elucidated whether up EVs cargo is guided to degradation by the endolysosomal system or if cargo is functionally active on recipient cells. The use of EHM allows the study of EVs transfer in a "human" HF setting and, therefore, contributes to understanding how cardiac communication via EVs is altered under HF conditions, particularly the miR composition within EVs. Since EHMs are composed of different types of cells, such as cardiomyocytes and stromal cells, the study of EVs using the EHM HF model facilitates research on how HF can compromise cardiac function, namely through cardiac fibrosis and cardiac hypertrophy, limiting events for cardiac recovery and RR. These findings could be further used to investigate the role of miRs on cardiac pathological remodeling. Simultaneously, the use of different cell types on EHM elucidates the uptake affinity of cardiomyocyte-derived EVs by different cell types. At last, **Chapter 6** described whether promising models such as EHMs can be used to study EV cardiac communication. Despite the preliminary nature of these data, the results obtained are very encouraging to continue more research based on the Cre-LoxP method beyond EHMs, perhaps *in vivo*, and use EHMs as a systematic model to study EVs transfer. Overall, we present a new tracking method for cardiac EVs biodistribution and understanding of their fluctuation throughout time according to different pathologies.

Challenges and Future Perspectives:

CVDs comprise a diverse group of cardiac pathologies and, it is important to perceive them as complex processes. The lack of efficient treatments for CVDs might be partially explained by our limited understanding of the mechanisms driving HF, often looked at by the effect of a "tunnel vision". Although relevant, the findings in this thesis may suffer from several limitations. In **Chapter 2** we used mice models that although currently accepted and used in basic cardiac science, still lack human translation. In contrast, in **Chapter 6**, we employed a human model. However, since it is an *in vitro* model composed of a hydrogel matrix, it cannot precisely recapitulate the human cardiac environment. Another major challenge presented in **Chapter 4** is the absence of direct evidence that the circulating miRs were initially released from cardiac cells. Transversal to all chapters, there are constraints on research design or methodology, for example, the low number of subjects. However, even the smallest progress in basic science may contribute to advances in clinical treatments. Being so, advances in plasma miR profiling and their use in clinical practice before mechanical interventions, as suggested in **Chapter 4**, may lead to personalized patient profiles and therefore, allow more targeted treatments. Moreover, this study reported the tracking of cardiac endogenous EVs by using the Cre-LoxP recombination system, a method that does not modify either EVs' biogenesis or morphology. EVs have tremendous potential in CVDs as biomarkers, therapeutic targets, and drug-delivery tools; therefore, it is crucial to understand the endogenous behavior of EVs but also standardize EVs isolation protocols, EVs administration routes, and EVs concentrations.

With this thesis, we worked on exploring the complexity of pathological cardiac remodelling at multiple levels ranging from basic mechanisms to diagnostic tools.

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