**Socio-economic impact**

Cardiovascular diseases (CVD) have been the leading cause of mortality over the last 30 years, affecting nearly 523 million people worldwide in 2019 [1]. Herein, ischemic heart disease (IHD) accounted for 182 million cases and 9.14 million deaths (49.2 % of total CDV deaths). The currently available treatments, including angiotensin-converting enzyme inhibitors, beta-blockers and anticoagulants have greatly improved the survival rate after an ischemic event [2] but they do not account for different disease etiology among patients with similar symptoms, limiting the long-term prognosis. Accordingly, disability-adjusted life years (DALYs) have also been rising which increases the demand in the healthcare system and the economic burden of the disease, through direct medical costs and indirect lack of productivity due to long-term morbidity. For example, it has been projected that in the United States alone, the total costs of CVD will rise from $555 billion in 2015 to $1,117 billion dollars in 2035 [3]. This highlights the urgent need for effective therapies that address the underlying mechanisms of the disease and minimize disease recurrence.

The traditional “one-size-fits-all” therapeutic approach focused on symptom relief is outdated. Contrarily, precision medicine holds great promise to stratify patients according to the mechanistic traits of their disease and to directly treat the underlying condition, reducing morbidity and mortality.

The approved therapies rely on the targeting of proteins implicated in the most common disease symptoms, such as high systolic blood pressure and platelet aggregation. Given that less than 2% of the genes encode for proteins and, among these proteins, only 10-14 % contain druggable binding sites, targeting RNA and particularly ncRNA (which account for more than 80% of the genome), could greatly extend the druggable genome and therapeutic options [4, 5]. According to the most recent GENCODE release (version 39), there are miRNAs 1879 genes or transcripts and 17755 IncRNA genes that are transcribed into 51306 transcripts. Owing to their small size and easy synthesis, miRNAs have been thoroughly explored in pre-clinical studies and clinical trials with great success. Despite promising pre-clinical data on the role of IncRNAs in heart diseases, difficulties to study IncRNAs, including their large size and 3D conformation, have been delaying their therapeutic translation. In fact, less than 50 clinical trials are currently listed to identify disease altered IncRNAs and, so far, none using IncRNAs or a IncRNA inhibitor as therapeutic agents. While the recognition that one miRNA can affect the expression of several target mRNAs and each mRNA can be targeted by distinct miRNAs make miRNA potent drugs by directly acting at different levels of the gene network, this same phenomenon may also translate into undesired effects. Contrarily, IncRNA therapies may potentially offer different mechanisms of action to activate or repress gene expression with high tissue and cell type specificity, limiting off-target effects. Accordingly, the interest in IncRNA therapies has been raising over the last years with 3 companies recently emerging that aim at targeting disease associated IncRNAs. One of them, HAYA Therapeutics, raised CHF 18 million seed financing in 2021 to develop IncRNA targeted therapies for fibrotic diseases such as non-obstructive hypertrophic cardiomyopathy. Due to current limitations in IncRNA synthesis and delivery, IncRNA therapies have mostly been focused on their knockdown. On the contrary, in this thesis, we highlight extracellular...
vesicles (EVs) as important carriers of ncRNAs of different sizes, including IncRNAs, that can be engineered as promising therapeutic tools for the cardiovascular system.

EVs have already reached clinical trials as a therapy for several diseases with an astonishing number of 38 interventional trials listed in clinicaltrials.gov, including mostly native EVs of different sources but also engineered EVs. Nonetheless, there is only one phase 1 clinical trial (NCT04327635) still in the recruitment phase to evaluate the safety of EV delivery in patients with acute myocardial infarction. The lack of clinical trials using EVs to treat cardiac diseases is most likely explained by the limited EV retention in the heart following intravenous administration. In this thesis, we propose a targeting strategy to improve EV delivery to the heart. This strategy is based on the loading of EVs on a transporter and could be applicable to a wide range of cardiac diseases. We envision that this technology could be used to target EVs to the heart or specific regions therein.

**Target groups**

The work presented in this thesis provides relevant and important insights for different stakeholders.

In chapter 2 we performed an extensive literature review of the most recent strategies to improve RNA delivery to the heart, highlighting their main advantages and caveats. Herein, we further dissected the current limitations underlying RNA therapies and put forward future steps to leverage this technology to clinical practice for cardiovascular applications. By providing a detailed, yet summarized overview of the field, this work can not only guide the scientific community to the unmet needs of the field but it also provides the medical community with information on pre-clinical testing ahead of clinical trials. Furthermore, we identified EVs as promising RNA therapeutic tools given their capacity to carry natural and synthetic RNAs of different sizes, though they do not naturally accumulate in the heart upon systemic administration.

Acknowledging that it is important to understand the loading of EVs with ncRNAs in order to use them for therapy, in chapter 3 we studied the production of IncRNA H19 by mesenchymal stem/stromal cells upon transfection and further incorporation in EVs. Admitting that splice variants of IncRNAs may have different functions in the cell and that their loading onto EVs has not been much explored, in this thesis we took a closer look at the incorporation of IncRNA H19 splice variants in EVs and their transfer to acceptor cells. This study demonstrated that splice variants may not be homogenously distributed in the EVs, even though they are produced at similar levels in the donor cells, a finding that is relevant both for their use as biomarkers by the medical community but also for the development of therapies by the pharmaceutical industry. Besides, this work raises the attention of the scientific community and industry for the importance of understanding the loading of the EVs with RNAs to guarantee standardization and avoid batch-to-batch variation which is vital for therapeutic efficacy.

To address the lack of EV retention in the heart upon intravenous administration, in chapter 4, we developed a novel non-invasive strategy for the targeted delivery of EVs to heart. This strategy uses a carriers for EVs and functions as a theranostic approach to monitor and deliver EVs to the heart,
which in humans may further allow the discrimination of the specific region inside the organ. Once intravenously injected, the targeting of this formulation is achieved remotely. Using this strategy, we were able to induce a 2-fold increase in EV retention in the heart, compared both to the untargeted formulation or free EVs. Importantly, the proposed formulation is produced using a straightforward method and which renders a quite stable (hours) formulation. Strikingly, due to its versatility, this technology is expected to accommodate both native and/or content-modified EVs and target them to the heart or other organ. These are important lessons for the scientific community working on delivery strategies to the heart but also to other organs such as the brain. Besides, by providing a simple production method, the results shown here are critical for the pharmaceutical industry.

Finally, patients and the general audience are important targets of the work portrayed here. By contributing to expand the knowledge on EV engineering and targeting to the heart, we are progressing towards their successful application for cardiac therapy which will hopefully have a great impact in the patients’ lives, reducing the socio-economic burden of CVD.

**Scientific dissemination and public outreach**

The findings reported in this thesis have been actively shared and discussed at international conferences of the European Society of Clinical Investigation, European society of Biomaterials and Tissue Engineering and Regenerative Medicine International Society (TERMIS) as well as at a more specialized audience of the Portuguese Network on Extracellular Vesicles. Furthermore, all the work presented in this thesis is under preparation for submission to peer reviewed journals actively seeking to share our findings with the broad scientific community.

Moreover, some concepts explored in this thesis have been presented and discussed with the general audience at several Portuguese public outreach events (European Researchers Night, Stem Cell Awareness Day and several visits to regional schools). In these events, targeted at children and adults, we aimed at contributing to accurate knowledge dissemination in an accessible and target-oriented language, instigating curiosity and promoting critical thinking among the general public.

**References**


