Chapter 1

Introduction and Aims
Heart Failure

Heart failure affects 1-2% of the adult population and is one of the leading causes of morbidity and mortality worldwide.[1] Heart failure is a clinical syndrome characterized by typical symptoms (such as dyspnea and fatigue) and signs (pulmonary rales, ankle edema, elevated jugular venous pressure), accompanied by structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress.[1] Heart failure is caused by either systolic or diastolic dysfunction of the heart. Diastolic dysfunction leads to inadequate filling of the heart caused by impaired relaxation, most commonly due to hypertension.[2] Systolic dysfunction is characterized by diminished contractile function and is usually the result of the loss of functional myocardial tissue, often due to myocardial infarction.[3,4]

The introduction of percutaneous coronary interventions in the 1980s led to a sharp decline in the mortality of myocardial infarction. However, even with a successful intervention, there is often some loss of myocardial tissue due to the temporary lack of oxygen supply to the heart. Consequently, many of the patients who have survived a myocardial infarction go on to develop heart failure. This trend, in conjunction with aging of the general population, has caused a heart failure epidemic. Currently, 20-30% of the individuals in the Netherlands above 40 years old will develop heart failure and this number is expected to increase even further.[5]

Another trend that has led to an increased prevalence of heart failure is the improved outcomes of cancer patients. The rise of new cancer therapeutics, together with improvements in prevention, early detection of tumors, and enhanced treatment schedules, have resulted in a dramatic increase in the number of cancer survivors.[6] However, many cancer survivors later present with cardiovascular complications of the chemotherapy. Results from long-term studies have now shown that indeed the risk of cardiovascular morbidity and mortality among survivors continues to increase even until decades later.[7]

The increasing burden of cardiovascular disease in cancer survivors has sparked the rise of cardio-oncology as a distinct clinical field.[8] However, a challenge in this field has been the lack of reliable indicators to predict which patients might suffer the most severe cardiovascular adverse effects after cancer therapy such as valvular defects, coronary artery disease or cardiomyopathy. Critically, our incomplete understanding of the biology of cancer therapy induced cardiotoxicity underpins the difficulty clinicians face in predicting which of their patients will suffer from cardiotoxicity after cancer treatment. This underscores the importance of illuminating the mechanisms by which chemotherapy induces cardiotoxicity. Moreover, models that accurately recapitulate the phenotype of chemotherapy induced cardiotoxicity are pivotal to allow for the study of these mechanisms.

In response to the increasing prevalence of heart failure, a massive research effort has been undertaken during the past few decades to provide effective treatment options for this syndrome. The stepwise introduction of beta-blockers, ACE inhibitors, angiotensin II receptor blockers, mineralocorticoid receptor antagonists, and most recently the combination of angiotensin receptor blockers with neprilysin inhibitors, has led to incremental improvements in the prognosis of systolic heart failure patients.[9] Next to medication, the development of implantable cardioverter-defibrillator (ICD) and cardiac resynchronization therapy (CRT) devices has improved the survival of
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Despite these improvements in treatment, the prognosis of heart failure remains very poor. Patients who are diagnosed with heart failure have a one-year mortality rate of 7-17% [10] and a disconcerting five-year mortality rate of 41-60% [3], depending on sex and other clinical parameters. If patients are hospitalized for heart failure, five-year mortality rate increases even further to 75%.[11] In conclusion, due to its high prevalence and very poor prognosis, heart failure represents a major unmet need for patients. Heart failure also places a major financial burden on healthcare systems worldwide. In 2012 the estimated total cost of heart failure was estimated to be a staggering $1.6 billion in the Netherlands alone.[12] This cost is projected to increase due to the aging population.

At present, heart failure typically progresses with a gradual but unrelenting decline in cardiac function that ultimately results in end-stage heart failure and death. Due to the non-regenerative nature of the human heart, any cardiomyocytes that were lost during a myocardial infarction are not replaced. Subsequently, pathophysiological remodelling of the heart leads to cardiomyocyte dysfunction and further progression of the disease.[13] A process of maladaptive remodelling is also present in heart failure that is caused by a sustained increase in hemodynamic load beyond the heart’s contractile reserves, such as with hypertension or valvular defects.

The maladaptive cardiac remodelling is associated with several changes in the phenotype of cardiomyocytes, such as the reactivation of a fetal gene program, a switch to a fetal-like metabolism dependent on glucose instead of fatty acids, sarcomeric protein isoform switches, and hypertrophic growth.[13-16]

The progression of heart failure is influenced by various risk factors and comorbidities such as obesity, smoking, hypertension, and diabetes. Another factor that is related to a worse prognosis is iron deficiency.[17-20] Iron deficiency is present in 40% of patients with chronic heart failure, even in non-anaemic patients.[1,23-26] In addition to having a worse prognosis, iron-deficient heart failure patients also have an impaired exercise capacity and reduced quality of life.[17-22] In addition to its key role in oxygen uptake and transport as a part of hemoglobin, iron has an important role in cellular oxygen storage and metabolism, redox cycling and as an enzymatic cofactor. Therefore, maintaining a normal iron homeostasis is crucial for cells that have a high energy demand such as cardiomyocytes. Iron deficiency impairs functional status in heart failure patients independently of hemoglobin levels.[27] The effect of iron deficiency on cardiomyocytes is unknown as to date, no studies have assessed the consequences of iron deficiency in human cardiomyocytes.

Notably, the drugs that have been developed for heart failure in recent decades are mostly focused on unloading the heart by reducing volume overload and targeting the pathological activation of the neuro-hormonal axis. There are currently no medications available that directly target the maladaptive changes observed in cardiomyocytes. In large part, this is due to the fact that the underlying mechanisms are only partly understood. For the development of new treatments that can improve outcome, a better understanding of the biology of heart failure is crucial. In this light, human stem cell derived cardiomyocytes have emerged as an attractive model to study cardiovascular diseases in vitro.
Pluripotent Stem Cells

Human embryonic stem cells were first derived in 1998.[28] These cells showed the potential to differentiate into cells of all three germ layers of the embryo. Importantly, pluripotent stem cells can be maintained in culture indefinitely and thus provide a limitless source of starting material from which the somatic cell type of choice can be created. Reports of the differentiation of human pluripotent stem cells towards cardiomyocytes soon followed.[29] Over time, an improved understanding of the pathways involved in natural cardiac differentiation has informed the systematic improvement of differentiation protocols to generate human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs).[30,31] Currently, hPSC-CMs are routinely cultured by many research groups. When combined with methods like lactate selection for isolation of CMs from other cell types, this results in > 99% pure cardiomyocyte populations.

hPSC-CMs have been particularly useful for the investigation of human cardiovascular disease because the alternative options require invasive biopsy of human cardiac tissue samples, which also cannot be maintained in prolonged culture. Moreover, in the rare case that cardiac tissue samples are available, they are usually obtained within the setting of end-stage heart disease. Until 2006, hPSCs could only be derived from human embryos. Because of ethical concerns over the requirement for destruction of an embryo, some countries banned the use of government funding for research with these cells.[32] In 2006, Takahashi and Yamanaka made the groundbreaking discovery that differentiated murine cells could be reprogrammed to pluripotent cells capable of differentiating into cells of other germ layers.[33] Only a year later, it was shown that adult human cells, such as fibroblasts obtained from a skin biopsy, could also be reprogrammed into induced pluripotent stem cells (iPSCs).[34] Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have the advantage of retaining the genetic profile of the patient from which they were derived. This allows for the study of genetic or acquired cardiac diseases by relating the phenotype of the patient’s \emph{in vivo} phenotype to the \emph{in vitro} phenotype of hiPSC-CMs.[35]

hPSC-CMs have thus far been used to study many cardiovascular diseases. For example, long QT syndrome was modelled using hiPSC-CMs from an afflicted patient, demonstrating action potential prolongation and ion channel disturbances, as compared to hiPSC-CMs from a control patient.[36] Other cardiovascular diseases such as dilated cardiomyopathy, hypertrophic cardiomyopathy, and arrhythmogenic right ventricular cardiomyopathy have similarly been modelled using hiPSC-CMs.[37-39] The discovery of the palindromic repeat (CRISPR) approach to genome-editing now allows for the generation of disease-specific PSC lines with identical genetic backgrounds, allowing for even more precise investigations of the effect of genetic mutations on \emph{in vitro} phenotypes.[40]

However, a major limitation to the use of hPSC-CMs for cardiac disease modelling is their immaturity. hPSC-CMs display a fetal-like phenotype with respect to structure, sarcomeric organisation, force generation, electrophysiology, calcium handling, and metabolism. Significant improvements in the maturity of hPSC-CMs have been achieved using various approaches such as biochemical, electrical, and mechanical stimulation.[35,41] However, the goal of culturing fully mature human CMs from hPSC-CMs remains elusive and more studies are needed to unlock the full maturation potential of hPSC-CMs.
Because of the immaturity of hPSC-CMs, common assays to study adult CM function such as edge detection and sarcomere length measurements did not prove to be applicable to hPSC-CMs. Several other approaches to quantify the contractility of hPSC-CMs have been developed, including motion vector analysis combined with manual segmentation or block-matching algorithms.[42,43] These approaches, however, do not directly allow for the quantitative assessment of fractional shortening and force generation kinetics, key features of cardiomyocyte physiology. CM force generation has been assessed previously by a number of different methods, including fluorescent microsphere-based traction force microscopy, atomic force microscopy, flexible diving board deformation, and micropost deformation measurements.[44-47] These techniques are highly specialized, require advanced instrumentation, and cannot be easily combined with optical measurements of contractile kinetics, measurements of calcium cycling, or action potentials. This highlights the need for a method that allows for the integrated analysis of hPSC-CM function, irrespective of their developmental maturity.

The existing challenges in the phenotyping of hPSC-CM function as described above will be addressed in this thesis. Overcoming these challenges by developing a reliable and easily operated method will greatly empower studies that seek to better understand hPSC-CM biology in cardiac development, health and disease. For instance, an improved method for the analysis of hPSC-CM function could contribute to a deeper understanding of various pathophysiological mechanisms involved in cardiac disease like the impact of iron deficiency in heart failure and the cardiotoxic effects of chemotherapeutic agents. A thorough appreciation of the key differences between an in vitro model and the actual in vivo situation in patients is crucial for efficient translation of new insights from bench to bedside. In this light, the immaturity of hPSC-CMs is a major limiting factor in efficient translation of in vitro results obtained in hPSC-CM models towards new treatments that impact patient care. Importantly, although it is widely recognized that hPSC-CMs are immature in comparison to their native counterparts, a framework to gauge the level of maturity of hPSC-CMs does not exist at present. Therefore, the aims of this thesis are as follows.

Aims of this Thesis

1) Develop an easy to use method for the integrated analysis of hPSC-CM function that can be applied for cardiovascular disease modelling and cardiotoxicity screening.
2) Explore the effect of iron deficiency on hPSC-CMs using the method developed under aim 1.
3) Study the cardiotoxic effects of doxorubicin on hPSC-CMs using the method developed under aim 1.
4) Develop a framework for the comparison of the functional maturity of hPSC-CMs and adult human CMs.

The first part of this thesis describes the development of a method for the integrated analysis of hPSC-CM function. Chapter 2 lays out a method that allows for the quantitative analysis of contraction kinetics by analyzing the changes in cell morphology over time. A similarity matrix describing the
similarity between frames in movies of a contracting rod-shaped hPSC-CM is generated. From this matrix a signal of contractility is derived, as expressed in fractional shortening. This analysis of contractile kinetics is combined with a biomechanical model to concurrently calculate the CM force generated with each contraction as the CM deforms the flexible substrate on which it is seeded. Furthermore, a simultaneous application of this methodology with other single-cell physiological assays for the quantification of calcium cycling and action potentials is described. This integrated analysis is applied to study the effects of isoproterenol and verapamil on contractile kinetics, force generation and calcium handling simultaneously. Next, a demonstration of the value of integrated analysis is shown by identifying additional cardiotoxicity of dofetilide when simultaneous analysis of contractions and electrophysiology is applied. Additionally, this method for integrated analysis is validated for use in adult CMs, irregular shaped CMs, and clusters of hPSC-CMs. Chapter 3 sets out a protocol for the use of the method described in Chapter 2. A drawback of the methodology described in chapter 2 is the limited amount of rod-like CMs available for study. Therefore, Chapter 3 also describes a novel method for microcontact printing of proteins on a soft substrate. This allows for the generation of a large number of rod-like CMs or integrated anisotropic cardiac microconstructs. The second part of this thesis focuses on the application of the methods developed in the first part of this thesis for cardiovascular disease modelling. Chapter 4 examines the effect of iron deficiency on hPSC-CMs. It is demonstrated that iron deficiency directly affects human cardiomyocyte function, impairing mitochondrial respiration, and reducing contractility and relaxation. A rescue of this phenotype by restoration of intracellular iron levels is also shown. Chapter 5 examines the effect of different doses of doxorubicin on hPSC-CMs. It is concluded that the cardiotoxic effects are mediated by impaired contractility of cardiomyocytes caused by mitochondrial dysfunction and sarcomeric integrity. Chapter 6 describes a review comparing the maturation status of hPSC-CMs with in vivo cardiac maturation. Strategies that have been applied to mature hPSC-CMs are discussed. Next, a Maturation Score is proposed and used to assess the success of various strategies aimed at maturing hPSC-CMs. Finally, the findings and relevance of this thesis, as well as future perspectives pertaining to this, are discussed in the Discussion.
References


