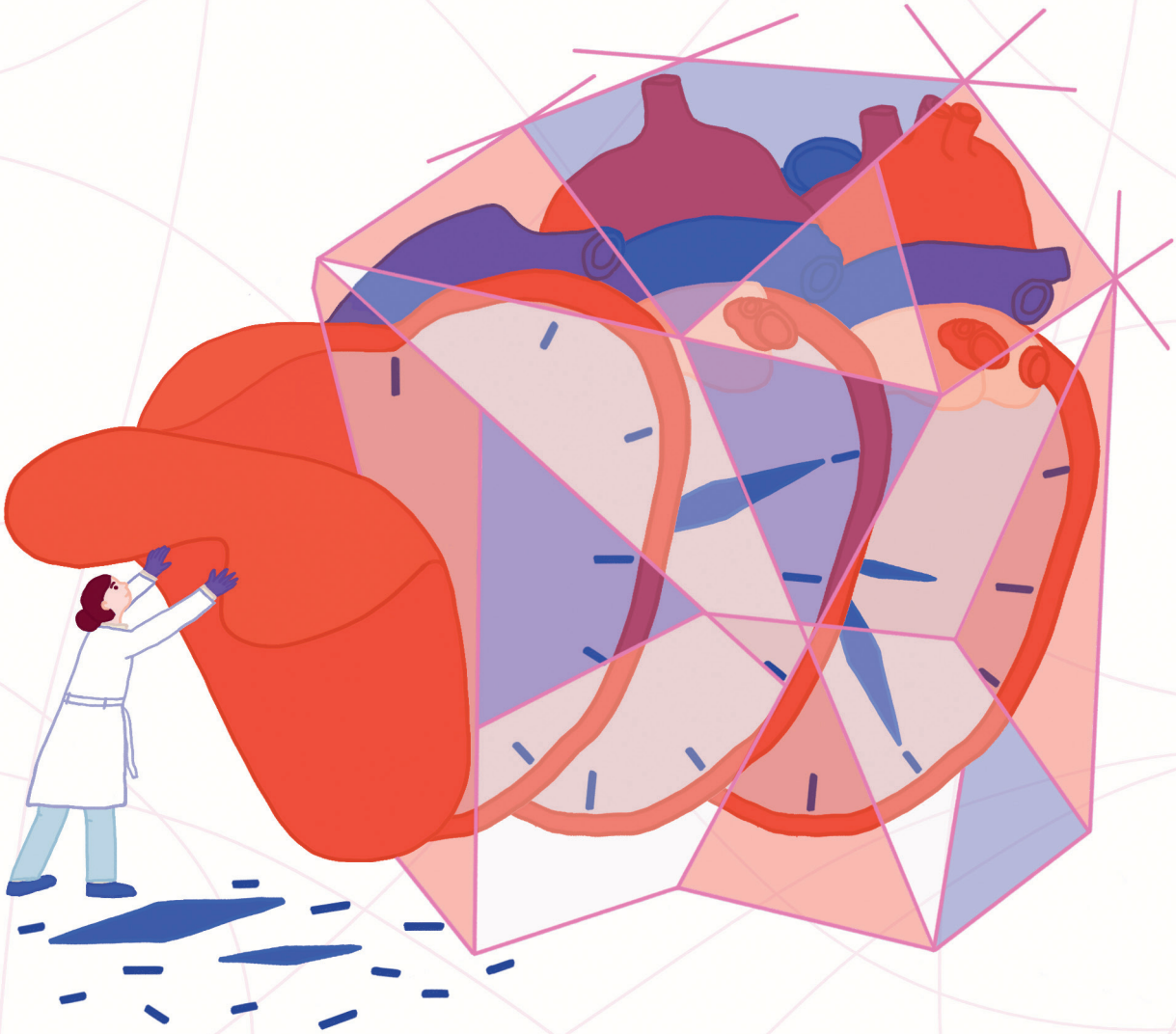
THROUGH THE MULTIDIMENSIONAL LOOKING GLASS:

Novel perspectives on heart failure



Sandra Crnko

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Through the multidimensional looking glass: *Novel perspectives on heart failure*

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(met een samenvatting in het Nederlands)

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Sandra Crnko

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Za moju femili.

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CHAPTER 1

Introduction and Thesis Outline

General introduction

Heart failure is a deadly chronic cardiovascular disease which affects millions of people worldwide. It is defined as a complex clinical syndrome caused by any structural or functional cardiac disorder that impairs the ventricular filling or ejection of blood, resulting in a reduced cardiac output and/or elevated intracardiac pressure at rest or during stress.[1,2] Heart failure is accompanied by a characteristic hemodynamic, renal, neural and hormonal response.[3] Sustained neurohormonal activation, namely activation of the sympathetic (adrenergic) nervous system (SNS) and the renin–angiotensin–aldosterone system (RAAS), underlies the disease progression by exerting deleterious effects on the heart, kidneys and peripheral vasculature.[4] Based on the ejection fraction (EF), heart failure can be classified into heart failure with reduced EF (HFrEF; EF < 40%), heart failure with mid-range or mildly reduced EF (HFmrEF; EF between 40 and 49%), and heart failure with preserved EF (HFpEF; EF > 50%).[5] HFrEF is characterized by the inability of the left ventricle to contract properly (systolic dysfunction), while in HFpEF the left ventricle cannot relax properly and be sufficiently filled with blood (diastolic dysfunction). Despite recent treatment advances[6], heart failure remains a fatal disease with no readily available cure. And while heart transplantation remains the only solution for curing end-stage heart failure, there is a severe shortage of available donor organs.[7] Given the devastating 5-year prognosis of overall 52.6% mortality[8], heart failure can be considered more "malignant" than many common cancers.[9,10] In view of this, novel approaches to understand and tackle this disease are warranted.

A fundamental process in the development and progression of heart failure is fibrotic remodelling. Different types of cardiac fibrosis are present in HFrEF and HFpEF. Reparative or replacement fibrosis on the one hand is characterized by excessive deposition of extracellular matrix (ECM) proteins in response to an injury (e.g. myocardial infarction).[11] Although a beneficial response for preserving the structural integrity of the heart at first, it will excessively perpetuate, inducing adverse tissue remodelling and uneven cardiac muscle distribution, eventually leading to heart failure. The formed scar is thus a substitute for the lost cardiomyocytes. This type of cardiac fibrosis is mainly present in HFrEF, contributing to systolic dysfunction via different mechanisms, one of which is the loss of fibrillar collagen which subsequently impairs the transduction of cardiomyocyte contraction into myocardial force.[5] On the other hand, reactive interstitial fibrosis occurs due to the chronic maladaptive signalling (e.g. inflammation) during the pathological cardiac remodelling. It is characterized by the expansion of the ECM that surrounds the cardiomyocytes, without necessarily cardiomyocyte loss.[5,11] HFpEF is mostly characterized by

this type of fibrosis, where diastolic dysfunction and increased stiffness of the heart is caused by the excessive collagen deposition and a reduction of the more flexible collagen III.[5] Currently, no effective therapies for treatment or reversal of either type of cardiac fibrosis are available.

The rapid increase of new heart failure cases globally urges a necessary shift in the current approaches.[12] In this thesis, we offer two different dimensions from which heart failure can be tackled. First, we postulate that adding the dimension of time (4D) – in terms of circadian rhythms and daily fluctuations – should be considered when investigating heart failure. Circadian rhythms are defined as biological rhythms with a period of 24 hours which allow organisms to prepare for the changes brought upon by the daily light/dark cycle, and adapt their physiology and behaviour to it accordingly. With the overwhelming body of evidence regarding the importance of circadian rhythms in general, and in cardiovascular diseases (CVDs) in particular[13], we want to uncover the intertwined relationship between circadian rhythms and heart failure. Heart failure already provides us with hints of circadian influence, e.g. neurohormones relevant in heart failure have day/night rhythms[14]; patients with heart failure often suffer from insomnia and, vice versa, insomnia has been found to increase the risk of incident heart failure.[15] However, in spite of the growing realization that circadian rhythms are an important factor in heart failure, the characteristics of its involvement in the disease genesis, progression and treatment is yet unknown. Second, given the importance of fibrosis in the pathology of heart failure and the lack of adequate human models, we propose and develop a 3D *in vitro* model of human cardiac fibrosis. Although conventional monolayer (2D) cell-culture systems and animal studies gave us valuable information about cardiac physiology and pathology, translation of effective anti-fibrotic therapeutics to the clinical arena remains hampered.[11] The translational failure is due to the high complexity and heterogeneity of cardiac fibrosis and its underlying mechanisms. Furthermore, there is a lack of research platforms that can reliably reflect human cardiac tissue, necessary for a better understanding of species-specific mechanistic aspects of fibrotic remodelling. Instead, we suggest that the advanced 3D *in vitro* models can contribute to solving this issue by adequately recapitulating native human myocardium including its pathological states.

In this thesis, we aim to offer a unique multidimensional perspective on heart failure. After a general introduction in **Chapter 2**, where the importance of circadian rhythms in the context of cardiovascular diseases is outlined, we give a layered approach to the research of time as a fourth dimension in heart failure. First, in **Chapter 3** we show that minimally invasively obtained human peripheral tissues – blood, oral mucosa, hair/beard follicles – are suitable for

studying circadian rhythms-driven (patho)physiological processes. Profiling of peripheral clock gene expression is crucial for determining functional circadian rhythms, as well as for recognizing causes and consequences of deviating rhythms. Here, we also report our effort to investigate gene expression profiles of core clock genes isolated from the peripheral blood of heart failure patients and healthy age- and sex-matched controls. Then, in **Chapter 4** we utilize one of the described peripheral sources – peripheral blood – to characterize circadian rhythmicity in heart failure patients as reflected by the rhythmic expression of main endocrine products of the central clock: melatonin and cortisol. We further substantiate our findings in two animal models of heart failure: diurnal zebrafish and nocturnal mouse. The heart failure phenotype is induced by phenylhydrazine hydrochloride in zebrafish, and by ligation of the left anterior descending coronary artery and subsequent myocardial infarction in mice. There we investigate directly in heart tissue whether the gene expression of core clock machinery changes in heart failure. **Chapter 5** investigates biomarkers as another important biological feature possibly controlled by the circadian clock. We investigate if soluble ST2, a strong prognostic biomarker in heart failure, exhibits a 24-hour rhythm. This knowledge could bear important consequences for its diagnostic and prognostic usage in the clinic. In **Chapter 6** we draw closer to the clinical setting, seeking to determine the impact of time in daily pulmonary artery pressure measurements of heart failure patients by using the CardioMEMS HF System. Afterwards, we investigate the third dimension in heart failure. We focus more on the molecular background of heart failure by zooming into fibrotic remodelling as one of the fundamental processes in the development and progression of this disease. In **Chapter 7** we design a mechanically tuneable 3D *in vitro* model of human cardiac fibrosis to study its mechanistic properties, as well as for it to serve as a tissue-specific drug testing platform. By using human induced pluripotent stem cell-derived cardiomyocytes in this model, we offer an alternative to conventional 2D cell culture models, and open a window of opportunities towards a more personalized and humanized *in vitro* approach to tackling heart failure. In **Chapter 8**, we summarize the findings of all preceding chapters, placing them into context of current literature and future research.

References

1. Yancy, C. W. et al. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. *Circulation* **128**, e240-327 (2013).
2. Ponikowski, P. et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC)Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur. Heart J.* **37**, 2129–2200 (2016).
3. Davis, R. C., Hobbs, F. D. R. & Lip, G. Y. H. History and epidemiology. *BMJ* **320**, 39–42 (2000).
4. Hartupee, J. & Mann, D. L. Neurohormonal activation in heart failure with reduced ejection fraction. *Nat Rev Cardiol* **14**, 30–38 (2017).
5. Simmonds, S. J., Cuijpers, I., Heymans, S. & Jones, E. A. V. Cellular and Molecular Differences between HFpEF and HFrEF: A Step Ahead in an Improved Pathological Understanding. *Cells* **9**, 242 (2020).
6. Levin, R. et al. Reverse Remodeling Induced by Sacubitril/Valsartan. *J Am Coll Cardiol* **73**, 702 (2019).
7. Fuchs, M. et al. Does the heart transplant have a future? *Eur J Cardiothorac Surg* **55**, i38–i48 (2019).
8. Benjamin, E. J. et al. Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. *Circulation* **135**, e146–e603 (2017).
9. Mamas, M. A. et al. Do patients have worse outcomes in heart failure than in cancer? A primary care-based cohort study with 10-year follow-up in Scotland. *Eur. J. Heart Fail.* **19**, 1095–1104 (2017).
10. Stewart, S., MacIntyre, K., Hole, D. J., Capewell, S. & McMurray, J. J. More 'malignant' than cancer? Five-year survival following a first admission for heart failure. *Eur J Heart Fail* **3**, 315–322 (2001).
11. de Boer, R. A. et al. Towards better definition, quantification and treatment of fibrosis in heart failure. A scientific roadmap by the Committee of Translational Research of the Heart Failure Association (HFA) of the European Society of Cardiology. *Eur J Heart Fail* **21**, 272–285 (2019).
12. Savarese, G. & Lund, L. H. Global Public Health Burden of Heart Failure. *Card Fail Rev* **3**, 7–11 (2017).
13. Crnko, S., Du Pré, B. C., Sluijter, J. P. G. & Van Laake, L. W. Circadian rhythms and the molecular clock in cardiovascular biology and disease. *Nat Rev Cardiol* **16**, 437–447 (2019).
14. Sole, M. J. & Martino, T. A. Diurnal physiology: core principles with application to the pathogenesis, diagnosis, prevention, and treatment of myocardial hypertrophy and failure. *J. Appl. Physiol.* **107**, 1318–1327 (2009).
15. Laugsand, L. E., Strand, L. B., Platou, C., Vatten, L. J. & Janszky, I. Insomnia and the risk of incident heart failure: a population study. *Eur. Heart J.* **35**, 1382–1393 (2014).

CHAPTER 2

Circadian Rhythms and the Molecular Clock in Cardiovascular Biology and Disease

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Abstract

The Earth turns on its axis every 24 h; almost all life on the planet has a mechanism — circadian rhythmicity — to anticipate the daily changes caused by this rotation. The molecular clocks that control circadian rhythms are being revealed as important regulators of physiology and disease. In humans, circadian rhythms have been studied extensively in the cardiovascular system. Many cardiovascular functions, such as endothelial function, thrombus formation, blood pressure and heart rate, are now known to be regulated by the circadian clock. Additionally, the onset of acute myocardial infarction, stroke, arrhythmias and other adverse cardiovascular events show circadian rhythmicity. In this Review, we summarize the role of the circadian clock in all major cardiovascular cell types and organs. Second, we discuss the role of circadian rhythms in cardiovascular physiology and disease. Finally, we postulate how circadian rhythms can serve as a therapeutic target by exploiting or altering molecular time to improve existing therapies and develop novel ones.

Introduction

In 2017, Jeffrey C. Hall, Michael Rosbash and Michael W. Young received the Nobel Prize in Physiology or Medicine for their discovery of the molecular machinery underpinning the biological clock[1-4] (**Box 1**). Their discovery was the start of a period in which the important role of circadian rhythms in physiology and disease was elucidated.

Circadian rhythms are biological rhythms with a period of approximately 24 h that allow organisms to prepare for the daily fluctuations brought on by day– night cycles, aligning internal biological functions with environmental changes. In mammals, circadian rhythms are regulated by circadian clocks. These clocks can be divided into a central or primary clock, comprising around 20.000 neurons located in the suprachiasmatic nucleus of the hypothalamus[5], and peripheral clocks, which can be found in almost every tissue[6]. Light cues, the main clock input signal (synchronizer), are received through the retina and transmitted to the master clock, which consequently synchronizes peripheral clocks throughout the body via various neurohumoral signals[7]. In addition to central clock signalling, peripheral clocks respond to tissue-specific synchronizers (for instance, food intake and exercise)[8-10]. When natural synchronizer input is present, 24-h rhythms are officially referred to as diurnal (or nocturnal) rhythms, and some claim the term circadian should be reserved for processes that persist under constant environmental conditions. In practice, however, almost all diurnal rhythms are found also to be circadian[11]. For the sake of clarity, we use the term circadian in this Review for all 24-h oscillatory processes, regardless of external input.

Circadian rhythms are important regulators of cardiovascular physiology and disease. Peripheral clocks are present in each of the cardiovascular cell types[12-16] (**Figure 1**), regulating various (patho) physiological functions, such as endothelial function, blood pressure and heart rate[17,18], as well as the onset of acute myocardial infarction and arrhythmias[19-21]. In this Review, we discuss the role and importance of circadian rhythms in the cardiovascular system and provide an overview of the latest advances in the understanding of circadian cardiovascular physiology. Furthermore, we describe how circadian rhythms are related to cardiovascular disease and elaborate on the importance of maintaining a healthy circadian rhythm. Finally, we discuss chronotherapy and other translational aspects of circadian rhythms, a novel approach to improve existing therapies and develop new ones.

Key points

- Molecular clocks are found in all cardiovascular cell types.
- Various cardiovascular functions, including endothelial function, thrombus formation, blood pressure and heart rate, are regulated by the circadian clock.
- Disruption of 24-h rhythms leads to cardiovascular disease, including heart failure, myocardial infarction and arrhythmias.
- 24-h rhythms are present in the development, risk factors, incidence and outcome of cardiovascular disease.
- Cardiovascular disease leads to disrupted circadian rhythm and sleep problems.

Circadian cardiovascular function

Molecular clock: loops and beyond

Virtually all mammalian cell types have a functional circadian clock[6]. On a molecular level, this clock consists of complex autoregulatory transcription-translation feedback loops whose interplay results in a rhythmic expression of clock-controlled genes, eventually leading to oscillations in proteome and cell function[22]. Although the core clock pathway is preserved among tissues, rhythmic transcription of clock-controlled genes is tissue-specific[6]. Approximately 10% of genes within each mammalian tissue are regulated by the circadian clock[23,24].

The main feedback loop consists of core clock components: aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL; also known as BMAL1), circadian locomotor output cycles protein kaput (CLOCK), cryptochrome 1 and 2 (CRY1 and CRY2, respectively) and period circadian protein homologue 1, 2 and 3 (PER1, PER2 and PER3, respectively)[25]. Additional components, such as tyrosine-protein kinase transmembrane receptor ROR1 and ROR2 and Rev-erbα (also known as NR1D1), form secondary feedback loops[26].

At the start of a 24-h cycle, BMAL1 and CLOCK proteins form a complex (heterodimer). BMAL1–CLOCK heterodimers bind to the enhancer box elements (E-boxes) of various clock genes, including *PER* and *CRY*, activating their transcription and translation (positive feedback). PER and CRY accumulate in the cytoplasm, also form a heterodimer and translocate to the nucleus to inhibit the transcriptional activity of BMAL1–CLOCK and, therefore, the transcription of their encoding genes (negative feedback). The whole process results in a negative feedback loop with a period of approximately 24 h[25]. Core elements of the clock, such as BMAL1 and

CLOCK, function not only to keep the clock turning but also to bind to the E-boxes of clock-controlled genes to realize the rhythmic activation of a large part of the genome, consequently leading to 24-h variations in bodily functions.

BMAL1–CLOCK heterodimers also activate ROR and Rev-erbα. These components compete for the ROR response element (RORE), a binding site located in the promoter region of *BMAL1*. This binding leads to either activation or inhibition of *BMAL1* transcription, depending on the bound protein. ROR1 and ROR2 act as transcriptional activators, whereas Rev-erbα represses *BMAL1* transcription[27]. This secondary feedback loop is necessary for a rhythmic expression of *BMAL1*[28].

Complementary to these feedback loops, circadian rhythms are regulated by various other processes[29]. In brief, on the post-transcriptional and post-translational levels, circadian rhythms are modulated by methylation, polyadenylation, histone modifications and non-coding RNAs, such as antisense RNAs and microRNAs. All these epigenetic and translational processes are necessary for the precision and robustness of the circadian oscillations. Details of their exact role and function are beyond the scope of this Review, but they have been reviewed previously[30].

Development of the cardiac clock

The developing circadian system can be observed through in utero emergence of circadian rhythms and during differentiation from cultured stem cells to different cellular lineages. The molecular clock develops gradually during differentiation and maturation, with circadian oscillations in the expression of core clock genes starting around the middle to the end of gestation. Maternal synchronizers, mainly melatonin, can pass through the placenta and influence fetal circadian rhythm. After birth, changes in period, amplitude, mesor and phase occur, aligning with the outside environment[31].

Circadian rhythms have also been observed during (stem) cell development and differentiation. Rhythmic expression of clock genes emerges during cardiac differentiation, although oscillation is not present in the undifferentiated embryonic stem cells beforehand[32]. Expression levels of the core clock genes *BMAL1*, *PER2* and *CLOCK* in human embryonic stem cells gradually increase during cardiac differentiation, with robust oscillations in more mature embryonic-stem-cell-derived cardiomyocytes after 45 days in culture. A similar effect was observed for the neuronal lineage[33]; clock genes were expressed in embryonic stem cells but started oscillating only after the addition of retinoic acid, which induced neuronal differentiation. Interestingly, these rhythms were lost and then regained with induction of de-differentiation

and re-differentiation, respectively[34]. This finding points to the early development of the clock and its link to the differentiation status of the cell. In contrast to embryonic stem cells, adult stem cells, which can differentiate only into specific cellular lineages, show functional circadian clocks[35]. Overall, the emergence of circadian rhythms on a cellular level can be traced from embryonic and adult stem cells to adult, aged cardiovascular cell types, which are described in the next section.

The clock in adult cardiovascular cells

The molecular clock has specific roles within each type of cardiovascular tissue[36]. Rhythmic activation of clock-controlled genes ultimately leads to an oscillation in various functions of endothelial cells, vascular smooth muscle cells, fibroblasts, cardiomyocytes and cardiac progenitor-like cells.

In blood vessels, the circadian clock was first discovered in mouse aortas, when they were isolated at different time points in a period of 24 h[37]. These findings were later confirmed in an *ex vivo* study, in which rhythmic PER1 luciferase activity was measured in veins and arteries cultured from transgenic rats[36]. Subsequent studies showed that all cell types of the major blood vessel layers have a functional circadian clock. In the inner endothelial layer, the existence of circadian rhythms was confirmed by synchronizing haemangioendothelioma and human umbilical vein endothelial cells[13]. Vascular smooth muscle cells, situated in the middle layer of vessels, also showed substantial oscillations of core clock components[38,39]. Finally, robust oscillations were found in cultured fibroblasts, which reside in the outer wall of the vessels[40].

In addition to the vasculature, circadian rhythms have been found in other cells residing in the heart, including cardiomyocytes, myocardial stromal fibroblasts and cardiac progenitor-like cells. Rat cultured cardiomyocytes have an intrinsic circadian clock mechanism with oscillations that persist for ≥60 h when synchronized with a serum shock, similar to the circadian oscillations seen in the rat intact heart[41]. Further studies found robust, cell-autonomous oscillations in isolated neonatal mice cardiomyocytes[12] as well as in human embryonic stem cell-derived cardiomyocytes[32]. Sato and colleagues investigated the roles of *Bmal1* and *Smad3* in myocardial stromal fibroblasts and found that both genes were expressed in a circadian manner in mouse hearts[42]. Our group also found circadian oscillations in SCA1 (also known as ATXN1)-positive cardiac progenitor-like cells from human fetal and adult hearts[14]. In addition to transcriptional and translational oscillations of core clock elements, substantial circadian variation existed in cell functions, including proliferation, stress tolerance and paracrine factor secretion.

The cardiovascular clock in context

The clock-controlled oscillation of cellular function causes variation in many cardiovascular processes throughout the day. Peripheral clocks have an important role in the cardiovascular system by ensuring daily variation in its physiological functioning. Most knowledge on the circadian clock in cardiac physiology is gathered using animal models with disrupted clock gene expression (**Table 1**).

Within the vasculature, the circadian clock is involved in signalling of residing cells, thrombus formation, and vascular function and tone[13,43]. The most well-known example is blood pressure, with higher values in the wakefulness and activity periods than during sleep or rest. This connection with observed daily fluctuations can be illustrated by the example of diurnal humans and nocturnal rodents, which each have active and inactive phases during different times of the day. In humans, blood pressure rises before wakening early in the morning, peaks in the mid-morning and then decreases towards the night[44], whereas in nocturnal rodents, blood pressure has a diametrically opposite pattern[45]. These observed daily fluctuations are linked not only to sleep and wake cycles but also to daily fluctuations in intrinsic blood vessel properties[46-48].

Moreover, circadian clocks reside in the heart, with confirmed oscillations of core clock components found in rodent[49] and human[50] tissue as well as in cultured cardiomyocytes[41]. Many excellent review articles have been published previously[51-53] that highlight the importance of the physiological roles of the circadian clock in the cardiovascular system. Briefly, heart rate has long been recognized to vary throughout the day[44], which was attributed to central, neurohumorally mediated circadian input. By using cardiomyocyte-specific *Clock*-mutant mice, circadian rhythms in heart rate have also been shown to be regulated by the cardiomyocyte circadian clock[54]. In these mutant mice, circadian rhythmicity is disrupted only in cardiomyocytes, whereas the neurohumoral system and the central clock in the suprachiasmatic nucleus are not affected. Nevertheless, cardiomyocyte-specific *Clock*-mutant mice showed a significant reduction in heart rate compared with wild-type mice. Physiological studies on these mutant mice further showed that cardiac metabolism of non-oxidative fatty acids and glucose, the responsiveness of the heart to fatty acids, contractility and cardiac function (output) are also regulated by the cardiomyocyte circadian clock. In summary, circadian rhythms regulate some of the major features of heart physiology, including heart rate, cardiac metabolism, responsiveness to various extracellular signals, contractility, signalling, and heart growth and regeneration[55].

Box 1 | **Discovery of circadian rhythms and the molecular clock**

24-h changes in nature have been studied throughout history. One of the first notions of circadian rhythms dates back to the 18th century when French astronomer d'Ortous de Mairan observed a rhythmic behaviour of *Mimosa pudica*. The plant unfolded its leaves during the day and folded them again at nightfall. Surprisingly at the time, this behaviour continued under conditions of constant darkness. Described and published in the *Proceedings of the Royal Academy of Paris*[165], the observation launched interest and research in the circadian field. Important discoveries that followed involved research in *Drosophila* conducted by Seymour Benzer and Ronald Konopka in the late 1960s. Their main finding resulted in the identification of a gene that, when mutated, led to disruption of the circadian rhythm. The unknown gene was named *period* (*per*) and its existence raised additional questions about the molecular milieu underlying the rhythms[166]. The mystery of *per* began to unfold in the 1980s when Michael Rosbash, Jeff Hall and Michael Young isolated and characterized the gene and its associated protein (Per). They showed the 24-h oscillating pattern of Per levels, with a higher concentration during the night and lower during the morning[1-4]. In 1995, Michael Young discovered another clock component in *Drosophila* encoded by *timeless* (*tim*) and demonstrated a negative feedback loop required for the functioning rhythm[167]. Furthermore, he showed that the double-time (Dbt) protein, encoded by *dbt*, was necessary to tune the 24-h oscillation by delaying the accumulation of Per[168]. Other discoveries followed soon after and painted a clearer picture of clock machinery and its regulatory loops as well as the environmental influences that could synchronize the clock. However, these scientists provided us with crucial and fundamental insights into circadian rhythms and enabled the future research that would ultimately link these rhythms to human physiology and disease.

Circadian cardiovascular disease

Circadian rhythms are an important component of human physiology and are linked to almost all diseases, including cancer[56], infection[57] and those of the nervous system[58]. In cardiovascular disease, knowledge gathered from animal and human studies led to the association between circadian rhythms and the development, incidence and outcome of disease.

From disturbed rhythm to pathologies

Circadian rhythm disruption in animal studies is achieved either by genetically modifying the molecular circadian clock that orchestrates rhythmicity or by desynchronizing external stimuli with the internal clock; for example, housing animals in always-light or continuously changing light conditions.

When circadian rhythms in rodents and their environment are out of

synchrony, animals can develop cardiomyopathy, cardiac fibrosis and systolic dysfunction, which can lead to cardiovascular death[59,60]. After the onset of disease, disrupted environmental rhythms lead to further progression of disease and a worse outcome[61]. Disruption of the molecular circadian clock in rodents shows similar effects and causes cardiomyopathy — specifically, thinning of the myocardial walls, dilatation of the left ventricle, altered sarcomeric structure and decreased cardiac function[62]. Other animal studies link the molecular clock to cardiac arrhythmias. In one study, for example, clock disruption led to a diminished 24-h rhythm in repolarization variation and, as a result, occurrence of ventricular arrhythmias[63].

In addition to the heart, disruption of the molecular clock causes atherosclerosis, insulin resistance, dampening of blood pressure rhythmicity, and a reduced production of vasoactive hormones and neurotransmitters[47,48]. Of note is a study by Martino and colleagues, who studied a combination of environmental and genetic rhythm alterations. Rodents in this study were genetically modified to shorten their molecular clock to a 22-h period, which under normal 24-h light–dark schedules led to cardiomyopathy, extensive cardiac fibrosis and severely impaired cardiac contractility[60]. When the animals with a 22-h clock were housed in a 22-h light–dark schedule, thereby restoring synchrony, no cardiac dysfunction was observed. These data show that both disruption of rhythms and desynchronization of external, central and peripheral clocks lead to cardiovascular disease.

Young and colleagues studied pathological characteristics of circadian dysfunction in the heart in more detail using genetic models. In addition to dampening of the oscillation in physiological functions, cardiomyocyte-specific *Clock*-mutant mice were found to have an altered metabolism, with an increase in oxygen consumption and fatty acid oxidation[54]. Cardiomyocyte-specific *Bmal1*-knockout mice have an even worse phenotype[64]. In addition to metabolic, histological and functional changes, these animals develop severe dilated cardiomyopathy that leads to a reduced lifespan.

The cardiovascular system does not consist only of cardiomyocytes; other cells such as fibroblasts[16], vascular smooth muscle cells[15] and endothelial cells[13] have an important role in cardiovascular physiology. All these cell types have circadian clocks, but tissue-specific circadian knockout models are scarce. One study found signs of atherosclerosis (decreased luminal diameter and increased vessel wall) in mice with a (non-tissue-specific) disrupted circadian clock[65]. Transplantation of the blood vessels of these animals into wild-type littermates did not alter this process, indicating that circadian rhythms in the blood vessels mediate atherosclerotic changes.

Whereas animal studies have provided insights into the mechanisms of

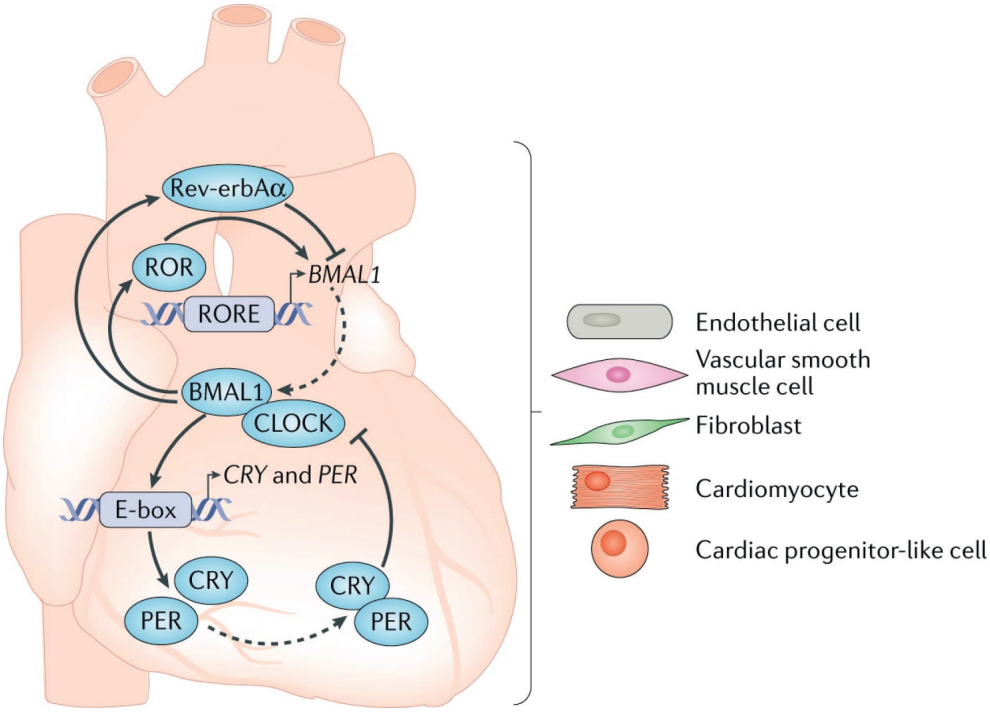

Figure 1 | **Overview of the core molecular clockwork within the cardiovascular system.** The core clock mechanism consists of intertwined positive and negative feedback loops, present in almost all cell types within the human body. Upon dimerization, aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL; also known as BMAL1) and circadian locomotor output cycles protein kaput (CLOCK) bind to the enhancer box elements (E-boxes) of *CRY* (which encodes cryptochrome) and *PER* (which encodes period circadian protein homologue), thereby initiating their transcription. CRY and PER then form a heterodimer, which inhibits the BMAL1–CLOCK complex[25]. Similarly, BMAL1–CLOCK activates tyrosine-protein kinase transmembrane receptor ROR and Rev-erbα (also known as NR1D1), which in turn activate or inhibit *BMAL1* transcription, respectively[26]. As a result of these transcriptional–translational interactions, clock-controlled genes display rhythmic activation, which leads to oscillating functions of various cardiovascular cells, including endothelial cells[13], vascular smooth muscle cells[15], fibroblasts[40,42], cardiomyocytes[12] and cardiac progenitor-like cells[14]. RORE, ROR response element.

circadian rhythms in both health and disease, most of the human studies to date have been observational. These studies demonstrate a clear relationship between circadian disruption and various cardiovascular risk factors and disease and vice versa[66-69]. Given that the molecular circadian clock is a well-preserved mechanism, genetic mutations that lead to complete clock dysfunction (often used in animal models) are very rare in humans.

Genome-wide association studies, however, have found several clock gene single nucleotide polymorphisms that are associated with metabolic syndrome, hypertension and diabetes mellitus[70-73].

A much more common cause of circadian disruption in humans is misalignment of external stimuli (day–night cycle) and the intrinsic circadian clock. Artificial light and technology (such as television, computer screens and smartphones) expose people to light in the evening, opposing and desynchronizing intrinsic clocks. Studies show that short-wavelength light (blue light and light-emitting diode (LED) light) is a particularly strong desynchronizer and is associated with the metabolic syndrome, psychiatric disorders, sleepiness and reduced quality of life, especially for particular chronotypes (morning types)[74].

More severe examples of circadian disruption are sleep disorders, jetlag and shift work. Patients with insomnia and sleep disorders have an increased risk of cardiovascular disease[75,76]. Shift work is associated with metabolic syndrome, myocardial infarction, ischaemic stroke and premature death[77-79]. In addition, shift workers experience more psychosocial stress, eat less healthily[80], have a higher blood pressure[81], have a greater incidence of diabetes[82], are more often overweight, smoke more, sleep less and generally have a lower socioeconomic status than their colleagues who do not work shifts[83,84]. Of note, some of these factors might also induce bias in epidemiological studies; therefore, causality is difficult to establish with this method. Another well-studied example of circadian disruption is the intensive care unit. Critically ill patients in the intensive care unit are often exposed to constant circadian input signals, such as parenteral feeding, constant light and no rhythm in activity or social interaction owing to bed rest or sedation. Cardiovascular functions with a 24-h rhythm, such as blood pressure and heart rate, as well as rhythms in core temperature, hormone secretion and activity, are disrupted in these patients[85]. Conversely, a reduction in physiological (cardiovascular) rhythms is a poor prognostic sign associated with organ dysfunction, delirium and an increased risk of death[86,87]. Moreover, an excessive circadian amplitude of blood pressure is independently associated with increased adverse cardiovascular events and could be a prognostic tool for clinical outcomes, regardless of other risk factors or blood pressure mesor[88].

Circadian rhythms in the incidence of disease. Circadian rhythms are involved in physiology and the development of cardiovascular disease. Many of these diseases have a 24-h rhythm in incidence and disease burden. Supraventricular and ventricular arrhythmias, stroke, aortic aneurysm dissection, pulmonary embolism and sudden cardiac death all occur more often in the early

morning[89-92]. The incidence of myocardial infarction also has a 24-h pattern[93]. Traditionally, these variations were ascribed to circadian variation in the autonomic nervous system[94]. Indeed, sympathetic activity, shear stress and cardiovascular risk factors such as blood pressure are increased in the morning, potentially triggering the onset of disease[95]. However, animal studies have shown that the peripheral clock has an important role[55]. Platelet aggregation and coagulation, ventricular repolarization abnormalities and many other relevant cardiovascular risk factors that are regulated by cellular (peripheral) circadian clocks logically contribute to peaks in the incidence of cardiovascular events at specific times of the day[63,96-98].

The circadian clock and outcome of disease. Circadian rhythms have an important role in the outcome of disease. A good example is myocardial infarction, in which outcome is related to both the time of onset of the infarction and clock disruption after the event[99-101]. If myocardial infarction occurs in the early morning, the resulting damage and cardiac dysfunction is worse than if a similar infarction occurs in the afternoon, although not all studies demonstrate the same findings[102]. Follow-up studies in animals showed that the 24-h rhythm in outcome is regulated by the cardiomyocyte circadian clock[102,103]. Young and colleagues linked the variation in outcome to the circadian rhythm in cardiac metabolism, whereas other researchers showed that rhythmicity in the immune response also contributes to the differences found[54,104]. Both time of onset and clock desynchronization after an infarction have a major influence on outcome. Studies from Alibhai and Martino showed that when mice are put in a circadian-disruptive environment after cardiac injury (myocardial infarction and pressure-overload-induced cardiac hypertrophy), outcome severely worsens[61,105]. Finally, myocardial infarction itself is associated with molecular clock alterations and dyssynchrony between the non-ischaemic and scar tissue[106]. A comprehensive summary of the intense interplay between circadian rhythms and myocardial infarction is provided in **Box 2.**

From pathologies to disturbed rhythm

Circadian rhythms are involved in the incidence, pathophysiology and outcome of cardiovascular disease. Vice versa, disease leads to disrupted circadian rhythmicity and sleep abnormalities. Development of cardiac hypertrophy in mice, either through a high-salt diet or aortic constriction, leads to decreased variation in molecular clock components including BMAL1, PER and CRY[107,108]. In human studies, cardiovascular risk factors (such as renal dysfunction) and heart failure are associated with blunted circadian

Box 2 | **Circadian rhythms and myocardial infarction**

Circadian rhythms are involved in every aspect of myocardial infarction (MI), including its pathogenesis, incidence and outcome. Vice versa, MI leads to disruption of circadian rhythms and sleep problems.

Clock disruption

- Single nucleotide polymorphisms in clock genes are associated with metabolic syndrome, hypertension and diabetes mellitus; these conditions increase the risk of MI[70-73].
- Shift work and insomnia are associated with the incidence of MI (risk ratio 1.23 and HR 1.45, respectively)[76,77].
- Patients exposed to constant synchronizers (such as lights always on or constant feeding) have an increased risk of delirium, which is associated with increased morbidity, incidence of MI and mortality[122].

Incidence

- The incidence of MI follows a 24-h pattern[93].
- Many risk factors for MI, such as elevations in blood pressure, have a 24-h rhythm; disruption of these physiological rhythms is associated with incidence of MI[85].
- Platelets are most active in the early morning, when most MIs occur[138].
- 24-h variation in the cardiac transcriptome and proteome leads to variation in cardiac response to coronary occlusion.

Outcomes

- Neutrophil recruitment to injured tissues is highest in the morning, leading to increased inflammation when an MI occurs in the morning[105].
- Outcome of MI is worse when internal and external rhythms are out of synchrony, such as when patients are exposed to constant light conditions after the coronary occlusion[105].
- Long-term outcomes after MI are substantially worse when MI onset is in the morning[100].
- After MI, cardiac remodelling is associated with disruption of circadian clocks[106]; pharmacological circadian modulators can partially prevent the harmful effects of cardiac remodelling in animal models[169].
- After MI, patients often experience sleep problems, which are associated with a worse outcome[170].

rhythms[109,110]. Arterial stiffness, for example, has a physiological circadian rhythm that is blunted in patients with dilated cardiomyopathy[109]. The classic

Table 1 | **Clock mechanism disturbance: insights from animal studies**

Method of clock disturbance	Effects	Refs
Bmal1-knockout mice	Loss of physiological rhythms in heart rate and blood pressure	48
Per2-knockout mice	Endothelial dysfunction	47
Csnk1e-mutant mice (rodents have a genetic (circadian) rhythm of 22 h living in 24-h environment)	Cardiomyopathy, cardiac and renal fibrosis, impaired cardiac contractility and renal disease	60
Cardiomyocyte-specific *Clock*-mutant mice (dominant negative overexpression of CLOCK specifically in cardiomyocytes)	Disruption of physiological variation in 10% of the transcriptome; bradycardia, disruption of normal heart rate and rhythm, and mitochondrial dysfunction	54
Bmal1-knockout mice and *Clock*-mutant mice	Vascular injury, endothelial dysfunction and pathological vascular remodelling	171
Dbp$^{-/-}$*Hlf*$^{-/-}$*Tef*$^{-/-}$ mice (knockout of three major transcription factors leading to clock dysfunction)	Cardiomyopathy, cardiac hypertrophy, left ventricular dysfunction and low aldosterone levels	172
Cardiomyocyte-specific *Clock*-mutant mice	Decreased tolerance to ischaemia–reperfusion injury	103
Cardiomyocyte-specific *Clock*-mutant plus cardiomyocyte-specific *Bmal1*-knockout mice	Drug-induced hypertrophic cardiomyopathy	173
Klf15-knockout mice (transcription of *Klf15* is regulated by the molecular clock)	Loss of physiological rhythm in ventricular repolarization duration and increased susceptibility to ventricular arrhythmias	63
Bmal1-knockout mice	Dilated cardiomyopathy (thinning of the myocardial walls, dilatation of the left ventricle and decreased systolic function)	62
Mice with inducible, cardiomyocyte-specific disruption of *Bmal1*	Bradycardia, prolonged QRS duration and ventricular arrhythmias	174
Cardiomyocyte-specific *Clock*-mutant mice	Disruption of physiological variation in 8% of the proteome	116
Cardiomyocyte-specific *Bmal1*-knockout mice	Disruption of physiological variation in 10% of the transcriptome; cardiac metabolism changes, dilated cardiomyopathy and premature death	64
Vascular-smooth-muscle-cell-specific *Bmal1*-knockout mice	Diminished 24-h rhythm in blood pressure and change in timing of blood pressure peak	175

CLOCK, circadian locomotor output cycles protein kaput.

example is sleep apnoea, which is a sleep disorder caused by a combination of obesity and other risk factors for the metabolic syndrome. Patients suffering from sleep apnoea have increased risks of (pulmonary) hypertension, diabetes and cardiovascular disease, such as arrhythmias, stroke, heart failure and cardiovascular death[111]. Treatment of sleep apnoea leads to a reduction in cardiovascular disease[112].

Most studies investigating desynchronization or disruption of normal circadian rhythms in humans are observational. Therefore, causative conclusions about clock disruption and disease cannot be made. These studies do, however, convincingly show that cardiovascular disease is associated with disrupted 24-h rhythms. Conversely, these studies demonstrate that disruption and dyssynchrony of circadian rhythms, either intentional (shift work or jetlag) or as a result of disease, are also associated with cardiovascular disease. These associations are present in the incidence, pathophysiology and outcome of disease. Animals studies teach us that the molecular circadian clock is an important mediator in these rhythms and suggest that the correlations found in human studies might be causal.

Novel therapeutic approaches

Several strategies have been suggested to prevent clock disruption or desynchronization, to limit negative consequences when circadian rhythm disruption is inevitable and perhaps even to use circadian rhythms to cure patients (**Box 3**). A challenging but important first step is to monitor clock disruption or desynchronization in patients. For sleep quality and disruption, polysomnography is the gold standard. However, this technique is time consuming, and results are not easily interpreted. Alternatives, such as electroencephalography monitoring, are being tested but have not yet made it into the clinic for this purpose[113]. Another option is to measure hormones or (epi)genomic, proteomic or metabolic circadian markers that are known to fluctuate throughout the day[114-116]. These fluctuations diminish in disease states or during sleep or circadian disruption. A good example that is already available is melatonin, a hormone that fluctuates throughout the day, which can easily be measured and is a predictor of outcome after myocardial infarction[117]. Furthermore, with technological advances, variables such as activity, heart rate and blood pressure can now be measured noninvasively and automatically over a period of several days, providing useful (personalized) information on circadian disruption and/or response to treatment[118]. However, although these parameters are accessible and easily measured, some might become disturbed in disease states or upon administration of medication,

thereby potentially altering the correlation with circadian parameters in other organs. In the near future, a combination of traditional and novel markers will hopefully allow more accurate assessment of the circadian status of patients.

A second step is awareness that timing can be an important factor in the success or failure of therapy. Whereas other patient characteristics, such as age, sex and comorbidities, are increasingly taken into account, time is usually forgotten. In many preclinical and clinical cardiovascular studies, information about timing is not collected or reported. The routine collection and reporting of time of therapy as well as time of data collection as parameters in all medical research is necessary to assess potential clinical consequences for patients.

Studies have already shown that awareness of time of day is important in two cardiovascular scenarios. Troponin, a marker of cardiac damage, and corrected QT (QTc) interval, an electrophysiological maker of cardiac repolarization, both have a physiological 24-h rhythm[119,120]. Therefore, a rise or fall in troponin levels several hours after admission might not be indicative of an acute coronary syndrome (if within the normal oscillatory range) but instead might be a simple physiological phenomenon. QTc interval is monitored in patients receiving medication that can prolong QT duration, which would increase the risk of ventricular arrhythmias. However, comparing QTc intervals measured at different times of the day might lead to underestimation or overestimation of the QT-prolonging effect of the drug.

Environmental clock tuning

The most obvious way to deal with clock disruption is to prevent or minimize desynchronization, which can be done by exposing patients to normal 24-h input signals, high light intensity, activity and feeding during the day and darkness in the night. Studies in patients in the intensive care unit show that this strategy improves clinical outcomes. Bright intensive care rooms, especially with windows and visible daylight, reduce delirium and its complications[121,122]. Some studies suggest that critically ill patients might benefit even more from input signals with above-normal intensity, such as bright light in the morning[123]. Other input signals have not been studied thoroughly. Whether other environmental strategies, such as daytime feeding, or rhythmicity in activity or noise also improve outcomes is unknown.

Given that patients are less susceptible to physiological input signals, other strategies were tested to maintain circadian rhythmicity. A synthetic form of melatonin, a hormone produced by the central pacemaker, has produced beneficial results on clock physiology in blind patients[124]. In several clinical settings, melatonin reduced the incidence of delirium, either because of its anti-inflammatory or its clock-restoring effects[125,126].

When clock disruption is evitable, such as during shift work, strategies have been developed to minimize its harmful effects. Short naps during shifts, regular food intake, shift schedule (evening shift before night shift) and enough sleep in between shifts all partially prevent disease, although effects have been mainly studied in the short term in fairly small studies[127,128].

In the cardiovascular field, dialysis is a good example of environmental clock tuning. Many patients with kidney failure experience disruption of physiological rhythms in blood pressure, leading to nocturnal hypertension[129]. The use of nocturnal haemodialysis decreases (nocturnal) blood pressure and reduces left ventricular mass, potentially preventing cardiovascular disease[130].

Box 3 | **Circadian rhythm in future cardiovascular research and therapy**

Several aspects of the influence of circadian rhythms on the cardiovascular system should be taken into account in future research and therapy. Circadian rhythms can be observed from several angles — one being a tool to improve existing and to develop new therapies and another being a therapeutic target themselves. When testing possible therapies or utilizing existing ones, the timing of administration can have a crucial effect on efficacy and occurrence of adverse effects[139]. Outcomes of surgical procedures can also be influenced by the time of day at which they are performed[142]. Conversely, targeting the clock could be used as a therapy itself, such as with the use of small-molecule modifiers[145]. Either way, differences between individuals should be taken into account when researching circadian rhythms in cardiovascular disease because various cardiovascular processes have been shown to differ between the sexes[156,157]. Lastly, clock disruption in our everyday lives and hospitals should be avoided and/or minimized when possible. Shift work and jetlag have been shown to have detrimental effects on cardiovascular health[83,84].

Targeting the time

Many cardiovascular risk factors vary throughout the day or have an abnormal circadian pattern. An important treatment strategy is, therefore, to decrease these risks at the time of day when they are highest or to restore circadian phase and/or amplitude to normal values. A good example is hypertension, a condition associated with many cardiovascular diseases. Normally, blood pressure varies throughout the day, and studies show that nocturnal hypertension, a condition often present in patients with renal disease, in particular leads to cardiovascular disease[110,131,132]. Conversely, adverse effects of antihypertensive drugs, including orthostatic hypotension, are mainly present during the day. Most diseases associated with hypertension have an

increased incidence in the early morning. Therefore, it is remarkable that most once-daily antihypertensive drugs are administered around 08:00 h. Drug plasma levels are highest during the day, when adverse effects are experienced most, and lowest in the early morning, when their desired effect is most needed.

Several studies addressed this issue and analysed the time of day of antihypertensive drug administration. Hermida and colleagues showed that bedtime administration of an angiotensin-converting enzyme inhibitor leads to lower nocturnal blood pressure than morning administration and confirmed that a decrease in nocturnal blood pressure improves cardiovascular outcome[133,134]. Other studies, some performed >2 decades ago, show that other antihypertensive drugs, such as calcium-channel blockers, angiotensin-receptor blockers and β-blockers, also have potential advantages when taken at a specific time of day and can prevent the morning peak in the incidence of cardiovascular disease[135,136]. Nevertheless, large, multicentre, randomized, controlled trials to compare morning and bedtime antihypertensive drug administration on outcome parameters such as cardiovascular death or all-cause mortality are currently lacking[137].

A second cardiovascular drug for which time-of-administration data are available is the classic platelet aggregation inhibitor aspirin. Studies show that thrombocytes are most active in the morning, when the risk of plaque rupture and myocardial infarction is highest[138]. In a study from 2015, Bonten and colleagues demonstrated that bedtime administration of aspirin leads to reduced morning platelet activity compared with morning administration[139]. As with antihypertensive drugs, large trials investigating major end points are not available. Of note, however, is that no studies indicate that morning administration of either aspirin or antihypertensive drugs is better than evening administration.

In addition to time-of-day administration of therapy, which entails lowering of the average blood pressure, the results of several studies suggest that restoring circadian amplitude to normal levels might be of greater importance[118,140,141]. Even in the absence of mesor hypertension, exceeding a threshold of circadian amplitude contributes to increased risk of cardiovascular disease[118]. The results of a small chronobiological trial involving 30 patients, in which the optimal circadian stage of administering losartan and hydrochlorothiazide was assessed, point to personalized treatment of blood pressure in which not only mesor but also circadian phase and amplitude should be taken into account[140]. In the future, ascertaining optimal treatment strategies is likely to include individual circadian blood pressure pattern (for example, reversed rhythm with high blood pressure during the night or

excessive amplitude with high blood pressure during the day) together with frequency and timing of the medication[141].

The importance of the time of day of therapy is not limited to cardiovascular drugs. Montaigne and colleagues, for example, compared the outcome of surgical aortic valve replacement[142]. A total of 600 patients were paired on the basis of preoperative and perioperative characteristics, the only difference being their time of operation (morning or afternoon). Morning operation was associated with a twofold higher rate of major complications in the short and long term. In a second, randomized study, the researchers confirmed that morning operations lead to higher perioperative myocardial damage. Observed outcomes are in accordance with the established tendency of major adverse cardiovascular events, such as myocardial infarction, to occur in the morning.

Time-specific therapy has also been introduced in stem-cell-based cardiac repair, a therapy that aims to cure the failing heart. In the clinic, both cardiac and non-cardiac patient-derived (multipotent) stem cells have been tested for their potential regenerative and paracrine effects[143]. In animal studies, cardiomyocytes derived from pluripotent stem cells (embryonic or genetically modified or induced) showed promising results for cardiac repair[144]. These stem cells have circadian clocks and clear 24-h rhythms in function and paracrine effects[14]. However, whether the use of stem cells at a specific time during the day improves patient outcomes remains to be investigated.

Targeting the clock

Another therapeutic option might be to change the phase of the circadian clock to a time-of-day setting or specific phase in physiological rhythms that is the most beneficial in a given situation. Myocardial infarction and aortic valve replacement, as described above, have a worse outcome when they occur in the morning because of reduced ischaemia tolerance. Manipulating the molecular and functional situation of the heart to resemble that in the afternoon or evening might, therefore, be an attractive strategy. Animal studies show that genetic clock changes influence infarct size[99,103,105]. Targeting the molecular clock in humans is more complicated, especially in the case of acute, unexpected disease when interventions can be initiated only after the index event, but some studies show promising results. Small-molecule modifiers, such as Rev-erbα inhibitors, can change the molecular circadian clock to a beneficial state preventing cardiac damage in animal models[142,145]. Many compounds that influence phase, amplitude and period of the circadian rhythm are available, hopefully allowing clock-targeting therapies in the near

future[146].

Are we all ticking in the same way?

In cardiovascular disease and circadian research, major differences are known to exist on the basis of sex, age and ethnicity. Large epidemiological studies show that African-American individuals have a shorter free-running circadian period than European-American individuals, whereas Chinese-American and Latin-American individuals reportedly sleep less[147,148]. Second, desynchronization of external and internal rhythms leads to more cognitive dysfunction in elderly individuals and in women than in young men, possibly caused by a different response of the autonomic nervous system[149,150]. Changes in circadian rhythms with age can be observed on different levels: a shift towards a morning chronotype as well as a preference for 'morningness' in terms of cognitive skill performance and alertness; worsened sleep quality and quantity; dampened capacity to accommodate light–dark schedule change; reduced amplitude and peak shift in hormones such as cortisol and melatonin; and progressive dampening of many metabolic rhythms, which can lead to diabetes, dyslipidaemia and hypertension, which are all risk factors for cardiovascular diseases[151].

These differences affect cardiovascular disease. The onset of myocardial infarction has a different time-of-day pattern in white European individuals in Spain from that in white European or Asian European individuals in the UK[152]. Second, risk factors might differ between ethnic groups. In African-American individuals, increased sleep duration is associated with obesity, whereas European-American individuals who sleep for a long time are less obese[153]. African-American individuals also experience more nocturnal hypertension (non-dippers) than European-American individuals and have a greater incidence of cardiovascular disease[154].

The incidence of cardiovascular disease is similar in men and women[155]. However, sex-specific differences in circadian rhythms and cardiovascular disease have not been studied thoroughly. Given that many cardiovascular processes such as cardiac metabolism differ between the sexes and that women are protected from heart disease in both animal and human studies, sex and cardiovascular diseases related to circadian disruption are likely to be linked[156,157]. Alibhai and colleagues did a pioneering study and indeed found that female mice with disrupted circadian clocks are protected from the development of metabolic changes and cardiomyopathy[158]. This protection is likely to be mediated by ovarian hormones.

In terms of diagnosis and treatment, taking into account individual differences is equally important, even among patients with the same sex, age or

ethnicity[159]. A one-size-fits-all approach to blood pressure reduction might be detrimental in some cases. Currently used reference values for blood pressure might not reveal all the vascular variability anomalies that are associated with an increased risk of adverse cardiovascular events, whereas time-specified, individualized reference values obtained by long-term monitoring might do so. With a proper diagnosis based on circadian amplitude and acrophase instead of only mesor, a simple adjustment in timing of the daily drug administration might be sufficient to treat and prevent further vascular variability anomalies.

Conclusions

The broad range of cardiovascular (patho)physiologies are subject to circadian oscillations. Circadian clocks have been found in all cell types in the heart and vasculature, and many important biological processes, such as heart rate, body temperature, blood pressure, metabolism and hormone levels, show daily fluctuations. In addition, the incidence, development and outcome of disease are linked to the circadian clock. Better understanding of molecular mechanisms underlying variability in cardiovascular disease might lead to new treatment strategies and improvement of existing approaches.

What happens when knowledge of circadian rhythms is used as a tool? Chronotherapy immediately emerges as an obvious application, given its usage for the timing of administration of several commonly used medications[160,161], such as calcium-channel blockers, angiotensin-converting enzyme inhibitors and aspirin. However, not only have the pharmacokinetics and pharmacodynamics of various drugs shown circadian variability but choosing different times of the day or phase of marker rhythms (such as blood pressure or a symptom) at which to perform some medical procedures might also be beneficial to patients[142]. In addition to cardiovascular conditions, diseases such as asthma, acute and chronic inflammations, allergies and cancer show daily fluctuations in symptoms and occurrence[162]. Accordingly, administration of treatments and analysis of the outcome for these conditions can be tailored to particular times of day[163,164].

In conclusion, time is a crucial consideration in the treatment of cardiovascular diseases. Translational application of circadian knowledge benefits patients, both by timing of therapy to maximize the desired effect and/or to minimize adverse effects and by avoiding clock disruption in our everyday lives and disease (such as in intensive care units). The use of circadian rhythms in research and in the clinic (**Box 3**) will hopefully improve the treatment and survival of patients with cardiovascular disease.

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All the authors researched data for the article, discussed its content, wrote the manuscript and reviewed and/or edited the manuscript before submission.

Competing interests

The authors declare no competing interests.

References

1. Bargiello, T. A., Jackson, F. R. & Young, M. W. Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*. *Nature* **312**, 752–754 (1984).
2. Hardin, P. E., Hall, J. C. & Rosbash, M. Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. *Nature* **343**, 536–540 (1990).
3. Siwicki, K. K., Eastman, C., Petersen, G., Rosbash, M. & Hall, J. C. Antibodies to the period gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* **1**, 141–150 (1988).
4. Zehring, W. A. et al. P-Element transformation with *period* locus DNA restores rhythmicity to mutant, arrhythmic *Drosophila melanogaster*. Cell 39, 369–376 (1984).
5. Brown, T. M. & Piggins, H. D. Electrophysiology of the suprachiasmatic circadian clock. *Prog. Neurobiol.* **82**, 229–255 (2007).
6. Zhang, R., Lahens, N. F., Ballance, H. I., Hughes, M. E. & Hogenesch, J. B. A circadian gene expression atlas in mammals: implications for biology and medicine. *Proc. Natl Acad. Sci. USA* **111**, 16219–16224 (2014).
7. Cajochen, C., Kräuchi, K. & Wirz-Justice, A. Role of melatonin in the regulation of human circadian rhythms and sleep. *J. Neuroendocrinol.* **15**, 432–437 (2003).
8. Stokkan, K. A., Yamazaki, S., Tei, H., Sakaki, Y. & Menaker, M. Entrainment of the circadian clock in the liver by feeding. *Science* **291**, 490–493 (2001).
9. Mistlberger, R. E. & Skene, D. J. Social influences on mammalian circadian rhythms: animal and human studies. *Biol. Rev. Camb. Philos. Soc.* **79**, 533–556 (2004).
10. Damiola, F. et al. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev.* **14**, 2950–2961 (2000).
11. Vitaterna, M. H., Takahashi, J. S. & Turek, F. W. Overview of circadian rhythms. *Alcohol Res. Health* **25**, 85–93 (2001).
12. Beesley, S., Noguchi, T. & Welsh, D. K. Cardiomyocyte circadian oscillations are cell-autonomous, amplified by β-adrenergic signaling, and synchronized in cardiac ventricle tissue. *PLOS ONE* **11**, e0159618 (2016).
13. Takeda, N. et al. Thrombomodulin is a clock-controlled gene in vascular endothelial cells. *J. Biol. Chem.* **282**, 32561–32567 (2007).
14. Du Pré, B. C. et al. SCA1+ cells from the heart possess a molecular circadian clock and display circadian oscillations in cellular functions. *Stem Cell Rep.* **9**, 762–769 (2017).
15. Lin, C. et al. The rhythmic expression of clock genes attenuated in human plaque-derived vascular smooth muscle cells. *Lipids Health Dis.* **13**, 14 (2014).
16. Balsalobre, A., Damiola, F. & Schibler, U. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93**, 929–937 (1998).
17. Kollias, G. E. et al. Diurnal variation of endothelial function and arterial stiffness in hypertension. *J. Hum. Hypertens.* **23**, 597 (2009).
18. Degaute, J. P., van de Borne, P., Linkowski, P. & Van Cauter, E. Quantitative analysis of the 24-hour blood pressure and heart rate patterns in young men. *Hypertension* **18**, 199–210 (1991).
19. Portaluppi, F. & Hermida, R. C. Circadian rhythms in cardiac arrhythmias and opportunities for their chronotherapy. *Adv. Drug Deliv. Rev.* **59**, 940–951 (2007).
20. Bulluck, H. et al. Circadian variation in acute myocardial infarct size assessed by

cardiovascular magnetic resonance in reperfused STEMI patients. *Int. J. Cardiol.* **230**, 149–154 (2017).
21. Manfredini, R. et al. Twenty-four-hour patterns in occurrence and pathophysiology of acute cardiovascular events and ischemic heart disease. *Chronobiol. Int.* **30**, 6–16 (2013).
22. Bozek, K. et al. Regulation of clock-controlled genes in mammals. *PLOS ONE* **4**, e4882 (2009).
23. Panda, S. et al. Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* **109**, 307–320 (2002).
24. Storch, K.-F. et al. Extensive and divergent circadian gene expression in liver and heart. *Nature* **417**, 78–83 (2002).
25. Shearman, L. P. et al. Interacting molecular loops in the mammalian circadian clock. *Science* **288**, 1013–1019 (2000).
26. Preitner, N. et al. The orphan nuclear receptor REV-ERBα controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110**, 251–260 (2002).
27. Solt, L. A., Kojetin, D. J. & Burris, T. P. The REV-ERBs and RORs: molecular links between circadian rhythms and lipid homeostasis. *Future Med. Chem.* **3**, 623–638 (2011).
28. Akashi, M. & Takumi, T. The orphan nuclear receptor RORalpha regulates circadian transcription of the mammalian core-clock Bmal1. *Nat. Struct. Mol. Biol.* **12**, 441–448 (2005).
29. Dierickx, P. et al. in *Stem Cells and Cardiac Regeneration* (ed. Madonna, R.) 57–78 (Springer International Publishing, 2016).
30. Mendoza-Viveros, L. et al. Molecular modulators of the circadian clock: lessons from flies and mice. *Cell. Mol. Life Sci.* **74**, 1035–1059 (2017).
31. Du Pré, B. C. et al. Circadian rhythms in cell maturation. *Physiology* **29**, 72–83 (2014).
32. Dierickx, P. et al. Circadian networks in human embryonic stem cell-derived cardiomyocytes. *EMBO Rep.* **18**, 1199–1212 (2017).
33. Kowalska, E., Moriggi, E., Bauer, C., Dibner, C. & Brown, S. A. The circadian clock starts ticking at a developmentally early stage. *J. Biol. Rhythms* **25**, 442–449 (2010).
34. Yagita, K. et al. Development of the circadian oscillator during differentiation of mouse embryonic stem cells in vitro. *Proc. Natl Acad. Sci. USA* **107**, 3846–3851 (2010).
35. Weger, M., Diotel, N., Dorsemans, A.-C., Dickmeis, T. & Weger, B. D. Stem cells and the circadian clock. *Dev. Biol.* **431**, 111–123 (2017).
36. Davidson, A. J., London, B., Block, G. D. & Menaker, M. Cardiovascular tissues contain independent circadian clocks. *Clin. Exp. Hypertens.* **27**, 307–311 (2005).
37. McNamara, P. et al. Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock. *Cell* **105**, 877–889 (2001).
38. Nonaka, H. et al. Angiotensin II induces circadian gene expression of clock genes in cultured vascular smooth muscle cells. *Circulation* **104**, 1746–1748 (2001).
39. Chalmers, J. A. et al. Vascular circadian rhythms in a mouse vascular smooth muscle cell line (Movas-1). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R1529–R1538 (2008).
40. Welsh, D. K., Yoo, S.-H., Liu, A. C., Takahashi, J. S. & Kay, S. A. Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian

rhythms of clock gene rxpression. *Curr. Biol.* **14**, 2289–2295 (2004).
41. Durgan, D. J. The intrinsic circadian clock within the cardiomyocyte. *Am. J. Physiol. Heart Circ. Physiol.* **289**, H1530–H1541 (2005).
42. Sato, F. et al. Smad3 and Bmal1 regulate p21 and S100A4 expression in myocardial stromal fibroblasts via TNF-α. *Histochem. Cell Biol.* **148**, 617–624 (2017).
43. Panza, J. A., Epstein, S. E. & Quyyumi, A. A. Circadian variation in vascular tone and its relation to α-sympathetic vasoconstrictor activity. *N. Engl. J. Med.* **325**, 986–990 (1991).
44. Millar-Craig, M. W., Bishop, C. N. & Raftery, E. B. Circadian variation of blood-pressure. *Lancet* **311**, 795–797 (1978).
45. Bastianini, S., Silvani, A., Berteotti, C., Lo Martire, V. & Zoccoli, G. Mice show circadian rhythms of blood pressure during each wake-sleep state. *Chronobiol. Int.* **29**, 82–86 (2012).
46. Sei, H. et al. Diurnal amplitudes of arterial pressure and heart rate are dampened in *Clock* mutant mice and adrenalectomized mice. *Endocrinology* **149**, 3576–3580 (2008).
47. Viswambharan, H. et al. Mutation of the circadian clock gene *Per2* alters vascular endothelial function. *Circulation* **115**, 2188–2195 (2007).
48. Curtis, A. M. et al. Circadian variation of blood pressure and the vascular response to asynchronous stress. *Proc. Natl Acad. Sci. USA* **104**, 3450–3455 (2007).
49. Martino, T. et al. Day/night rhythms in gene expression of the normal murine heart. *J. Mol. Med.* **82**, 256–264 (2004).
50. Leibetseder, V. et al. Clock genes display rhythmic expression in human hearts. *Chronobiol. Int.* **26**, 621–636 (2009).
51. Martino, T. A. & Young, M. E. Influence of the cardiomyocyte circadian clock on cardiac physiology and pathophysiology. *J. Biol. Rhythms* **30**, 183–205 (2015).
52. Young, M. E. The circadian clock within the heart: potential influence on myocardial gene expression, metabolism, and function. *Am. J. Physiol. Heart Circ. Physiol.* **290**, H1–H16 (2006).
53. Paschos, G. K. & FitzGerald, G. A. Circadian clocks and vascular function. *Circ. Res.* **106**, 833–841 (2010).
54. Bray, M. S. et al. Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism, and gene expression. *Am. J. Physiol. Heart Circ. Physiol.* **294**, H1036–H1047 (2008).
55. Durgan, D. J. & Young, M. E. The cardiomyocyte circadian clock: emerging roles in health and disease. *Circ. Res.* **106**, 647–658 (2010).
56. Shostak, A. Circadian clock, cell division, and cancer: from molecules to organism. *Int. J. Mol. Sci.* **1**8, E873 (2017).
57. Scheiermann, C., Gibbs, J., Ince, L. & Loudon, A. Clocking in to immunity. *Nat. Rev. Immunol.* **18**, 423–437 (2018).
58. Kondratova, A. A. & Kondratov, R. V. The circadian clock and pathology of the ageing brain. *Nat. Rev. Neurosci.* **13**, 325–335 (2012).
59. Penev, P. D., Kolker, D. E., Zee, P. C. & Turek, F. W. Chronic circadian desynchronization decreases the survival of animals with cardiomyopathic heart disease. *Am. J. Physiol.* **275**, H2334–H2337 (1998).
60. Martino, T. A. et al. Circadian rhythm disorganization produces profound cardiovascular and renal disease in hamsters. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R1675–R1683 (2008).

61. Martino, T. A. et al. Disturbed diurnal rhythm alters gene expression and exacerbates cardiovascular disease with rescue by resynchronization. *Hypertensio*n **49**, 1104–1113 (2007).
62. Lefta, M., Campbell, K. S., Feng, H.-Z., Jin, J.-P. & Esser, K. A. Development of dilated cardiomyopathy in Bmal1-deficient mice. *Am. J. Physiol. Heart Circ. Physiol.* **303**, H475–H485 (2012).
63. Jeyaraj, D. et al. Circadian rhythms govern cardiac repolarization and arrhythmogenesis. *Nature* **483**, 96–99 (2012).
64. Young, M. E. et al. Cardiomyocyte-specific BMAL1 plays critical roles in metabolism, signaling, and maintenance of contractile function of the heart. *J. Biol. Rhythms* **29**, 257–276 (2014).
65. Cheng, B. et al. Tissue-intrinsic dysfunction of circadian clock confers transplant arteriosclerosis. *Proc. Natl Acad. Sci. USA* **108**, 17147–17152 (2011).
66. Rüger, M. & Scheer, F. A. J. L. Effects of circadian disruption on the cardiometabolic system. *Rev. Endocr. Metab. Disord.* **10**, 245–260 (2009).
67. Thosar, S. S., Butler, M. P. & Shea, S. A. Role of the circadian system in cardiovascular disease. *J. Clin. Invest.* **128**, 2157–2167 (2018).
68. Portaluppi, F. et al. Circadian rhythms and cardiovascular health. *Sleep Med. Rev.* **16**, 151–166 (2012).
69. Reutrakul, S. & Knutson, K. L. Consequences of circadian disruption on cardiometabolic health. *Sleep Med. Clin.* **10**, 455–468 (2015).
70. Saxena, R. et al. Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. *Science* **316**, 1331–1336 (2007).
71. Zeggini, E. et al. Multiple type 2 diabetes susceptibility genes following genome-wide association scan in UK samples. *Science* **316**, 1336–1341 (2007).
72. Woon, P. Y. et al. Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) is associated with susceptibility to hypertension and type 2 diabetes. *Proc. Natl Acad. Sci. USA* **104**, 14412–14417 (2007).
73. Scott, E. M., Carter, A. M. & Grant, P. J. Association between polymorphisms in the Clock gene, obesity and the metabolic syndrome in man. *Int. J. Obes.* **32**, 658–662 (2008).
74. Fabbian, F. et al. Chronotype, gender and general health. *Chronobiol. Int.* **33**, 863–882 (2016).
75. Laugsand, L. E., Strand, L. B., Platou, C., Vatten, L. J. & Janszky, I. Insomnia and the risk of incident heart failure: a population study. *Eur. Heart J.* **35**, 1382–1393 (2014).
76. Laugsand, L. E., Vatten, L. J., Platou, C. & Janszky, I. Insomnia and the risk of acute myocardial infarction: A population study. *Circulation* **124**, 2073–2081 (2011).
77. Vyas, M. V. et al. Shift work and vascular events: systematic review and meta-analysis. *BMJ* **345**, e4800 (2012).
78. Esquirol, Y. et al. Shift work and cardiovascular risk factors: new knowledge from the past decade. *Arch. Cardiovasc. Dis.* **104**, 636–668 (2011).
79. Kawachi, I. et al. Prospective study of shift work and risk of coronary heart disease in women. *Circulation* **92**, 3178–3182 (1995).
80. Tepas, D. I. Do eating and drinking habits interact with work schedule variables? *Work Stress* **4**, 203–211 (1990).
81. Lo, S. H. et al. Working the night shift causes increased vascular stress and delayed recovery in young women. *Chronobiol. Int.* **27**, 1454–1468 (2010).

82. Pan, A., Schernhammer, E. S., Sun, Q. & Hu, F. B. Rotating night shift work and risk of type 2 diabetes: two prospective cohort studies in women. *PLOS Med.* **8**, e1001141 (2011).
83. St-Onge, M.-P. et al. Sleep duration and quality: impact on lifestyle behaviors and cardiometabolic health: a Scientific Statement from the American Heart Association. *Circulation* **134**, e367–e386 (2016).
84. Knutsson, A., Jonsson, B. G., Akerstedt, T. & Orth-Gomer, K. Increased risk of ischaemic heart disease in shift workers. *Lancet* **328**, 89–92 (1986).
85. Paul, T. & Lemmer, B. Disturbance of circadian rhythms in analgosedated intensive care unit patients with and without craniocerebral injury. *Chronobiol. Int.* **24**, 45–61 (2007).
86. Dessap, A. M. et al. Delirium and circadian rhythm of melatonin during weaning from mechanical ventilation an ancillary study of a weaning trial. *Chest* **148**, 1231–1241 (2015).
87. Buchman, T. G., Stein, P. K. & Goldstein, B. Heart rate variability in critical illness and critical care. *Curr. Opin. Crit. Care* **8**, 311–315 (2002).
88. Cornélissen, G., Halberg, F., Otsuka, K., Singh, R. B. & Chen, C.-H. Chronobiology predicts actual and proxy outcomes when dipping fails. *Hypertension* **49**, 237–239 (2007).
89. Muller, J. E. et al. Circadian variation in the frequency of sudden cardiac death. *Circulatio*n **75**, 131–138 (1987).
90. Viskin, S. et al. Circadian variation of symptomatic paroxysmal atrial fibrillation: data from almost 10000 episodes. *Eur. Heart J.* **20**, 1429–1434 (1999).
91. Matsuo, K. et al. The circadian pattern of the development of ventricular fibrillation in patients with Brugada syndrome. *Eur. Heart J.* **20**, 465–470 (1999).
92. Manfredini, R. et al. Chronobiology of rupture and dissection of aortic aneurysms. J. *Vasc. Surg.* **40**, 382–388 (2004).
93. Muller, J. E. et al. Circadian variation in the frequency of onset of acute myocardial infarction. *N. Engl. J. Med.* **313**, 1315–1322 (1985).
94. Shen, M. J. & Zipes, D. P. Role of the autonomic nervous system in modulating cardiac arrhythmias. *Circ. Res.* **114**, 1004–1021 (2014).
95. Manfredini, R., Gallerani, M., Portaluppi, F. & Fersini, C. Relationships of the circadian rhythms of thrombotic, ischemic, hemorrhagic, and arrhythmic events to blood pressure rhythms. *Ann. NY Acad. Sci.* **783**, 141–158 (1996).
96. Shea, S. A., Hilton, M. F., Hu, K. & Scheer, F. A. J. L. Existence of an endogenous circadian blood pressure rhythm in humans that peaks in the evening. *Circ. Res.* **108**, 980–984 (2011).
97. Dashti, H. S. et al. Clock genes explain a large proportion of phenotypic variance in systolic blood pressure and this control is not modified by environmental temperature. *Am. J. Hypertens.* **29**, 132–140 (2016).
98. Scheer, F. A. J. L. & Shea, S. A. Human circadian system causes a morning peak in prothrombotic plasminogen activator inhibitor-1 (PAI-1) independent of the sleep/wake cycle. *Blood* **123**, 590–593 (2014).
99. Eckle, T. et al. Adora2b-elicited Per2 stabilization promotes a HIF-dependent metabolic switch crucial for myocardial adaptation to ischemia. *Nat. Med.* **18**, 774–782 (2012).
100. Reiter, R., Swingen, C., Moore, L., Henry, T. D. & Traverse, J. H. Circadian

dependence of infarct size and left ventricular function after ST elevation myocardial infarction. *Circ. Res.* **110**, 105–110 (2012).
101. Fournier, S. et al. Circadian variations of ischemic burden among patients with myocardial infarction undergoing primary percutaneous coronary intervention. *Am. Heart J.* **163**, 208–213 (2012).
102. Ammirati, E., Maseri, A. & Cannistraci, C. V. Still need for compelling evidence to support the circadian dependence of infarct size after ST-elevation myocardial infarction. *Circ. Res.* **113**, e43–e44 (2013).
103. Durgan, D. J. et al. Short communication: Ischemia/ reperfusion tolerance is time-of-day-dependent: mediation by the cardiomyocyte circadian clock. *Circ. Res.* **106**, 546–550 (2010).
104. Bennardo, M. et al. Day-night dependence of gene expression and inflammatory responses in the remodeling murine heart post-myocardial infarction. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **311**, R1243–R1254 (2016).
105. Alibhai, F. J. et al. Short-term disruption of diurnal rhythms after murine myocardial infarction adversely affects long-term myocardial structure and function. *Circ. Res.* **114**, 1713–1722 (2014).
106. Kung, T. A. et al. Rapid attenuation of circadian clock gene oscillations in the rat heart following ischemia- reperfusion. *J. Mol. Cell. Cardiol.* **43**, 744–753 (2007).
107. Mohri, T. et al. Alterations of circadian expressions of clock genes in Dahl salt-sensitive rats fed a high-salt diet. *Hypertension* **42**, 189–194 (2003).
108. Young, M. E., Razeghi, P. & Taegtmeyer, H. Clock genes in the heart: characterization and attenuation with hypertrophy. *Circ. Res.* **88**, 1142–1150 (2001).
109. Maruo, T. et al. Circadian variation of endothelial function in idiopathic dilated cardiomyopathy. *Am. J. Cardiol.* **97**, 699–702 (2006).
110. Dhaun, N. et al. Diurnal variation in blood pressure and arterial stiffness in chronic kidney disease: the role of endothelin-1. *Hypertension* **64**, 296–304 (2014).
111. Floras, J. S. Sleep apnea and cardiovascular risk. J. Cardiol. **63**, 3–8 (2014).
112. Kaneko, Y. et al. Cardiovascular effects of continuous positive airway pressure in patients with heart failure and obstructive sleep apnea. *N. Engl. J. Med.* **348**, 1233–1241 (2003).
113. Vacas, S. et al. The feasibility and utility of continuous sleep monitoring in critically ill patients using a portable electroencephalography monitor. *Anesth. Analg.* **123**, 206–212 (2016).
114. Tsimakouridze, E. V. et al. Chronomics of pressure overloadinduced cardiac hypertrophy in mice reveals altered day night gene expression and biomarkers of heart disease. *Chronobiol. Int.* **29**, 810–821 (2012).
115. Tsimakouridze, E. V., Alibhai, F. J. & Martino, T. A. Therapeutic applications of circadian rhythms for the cardiovascular system. *Front. Pharmacol.* **6**, 77 (2015).
116. Podobed, P. et al. The day/night proteome in the murine heart. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **307**, R121–R137 (2014).
117. Dominguez-Rodriguez, A., Abreu-Gonzalez, P., Garcia-Gonzalez, M. & Reiter, R. J. Prognostic value of nocturnal melatonin levels as a novel marker in patients with ST-segment elevation myocardial infarction. *Am. J. Cardiol.* **97**, 1162–1164 (2006).
118. Cornelissen, G. et al. Chronobiologically interpreted ambulatory blood pressure monitoring: past, present, and future. *Biol. Rhythm Res.* **50**, 46–62 (2019).
119. Fournier, S. et al. Circadian rhythm of blood cardiac troponin T concentration. *Clin.*

Res. Cardiol. **106**, 1026–1032 (2017).
120. du Pre, B. C. et al. Analysis of 24-h rhythm in ventricular repolarization identifies QT diurnality as a novel clinical parameter associated with previous ventricular arrhythmias in heart failure patients. *Front. Physiol.* **8**, 590 (2017).
121. Oldham, M. A., Lee, H. B. & Desan, P. H. Circadian rhythm disruption in the critically ill: an opportunity for improving outcomes. *Crit. Care Med.* **44**, 207–217 (2016).
122. Van Rompaey, B. et al. Risk factors for delirium in intensive care patients: a prospective cohort study. *Crit. Care* **13**, R77 (2009).
123. Perras, B., Meier, M. & Dodt, C. Light and darkness fail to regulate melatonin release in critically ill humans. *Intensive Care Med.* **33**, 1954–1958 (2007).
124. Carden, S. M. Entrainment of free-running circadian rhythms by melatonin in blind people: melatonin, circadian rhythms and sleep (Editorial). *Surv. Ophthalmol.* **46**, 299–300 (2001).
125. Al-Aama, T. et al. Melatonin decreases delirium in elderly patients: a randomized, placebo-controlled trial. Int. J. Geriatr. *Psychiatry* **26**, 687–694 (2011).
126. de Jonghe, A. et al. Effect of melatonin on incidence of delirium among patients with hip fracture: a multicentre, double-blind randomized controlled trial. *Can. Med. Assoc. J.* **186**, E547–E556 (2014).
127. Purnell, M. T., Feyer, A. M. & Herbison, G. P. The impact of a nap opportunity during the night shift on the performance and alertness of 12-h shift workers. *J. Sleep Res.* **11**, 219–227 (2002).
128. Neil-Sztramko, S. E., Pahwa, M., Demers, P. A. & Gotay, C. C. Health-related interventions among night shift workers: a critical review of the literature. *Scand. J. Work Environ. Health* **40**, 543–556 (2014).
129. Sarafidis, P. et al. Prevalence and control of hypertension by 48-h ambulatory blood pressure monitoring in haemodialysis patients: a study by the European Cardiovascular and Renal Medicine (EURECA-m) working group of the ERA-EDTA. *Nephrol. Dial. Transplant.* **33**, 1872 (2018).
130. Culleton, B. et al. Effect of frequent nocturnal hemodialysis versus conventional hemodialysis. *J. Am. Med. Assoc.* **298**, 1291–1299 (2007).
131. Boggia, J. et al. Prognostic accuracy of day versus night ambulatory blood pressure: a cohort study. *Lancet* **370**, 1219–1229 (2007).
132. Svensson, P., de Faire, U., Sleight, P., Yusuf, S. & Ostergren, J. Comparative effects of ramipril on ambulatory and office blood pressures: a HOPE substudy. *Hypertension* **38**, E28–E32 (2001).
133. Hermida, R. C. & Ayala, D. E. Chronotherapy with the angiotensin-converting enzyme inhibitor ramipril in essential hypertension: improved blood pressure control with bedtime dosing. *Hypertension* **54**, 40–46 (2009).
134. Hermida, R. C., Ayala, D. E., Mojón, A. & Fernández, J. R. Decreasing sleep-time blood pressure determined by ambulatory monitoring reduces cardiovascular risk. *J. Am. Coll. Cardiol.* **58**, 1165–1173 (2011).
135. De Giorgi, A., Mallozzi Menegatti, A., Fabbian, F., Portaluppi, F. & Manfredini, R. Circadian rhythms and medical diseases: does it matter when drugs are taken? *Eur. J. Intern. Med.* **24**, 698–706 (2013).
136. Manfredini, R., Gallerani, M., Salmi, R. & Fersini, C. Circadian rhythms and the heart: Implications for chronotherapy of cardiovascular diseases. *Clin. Pharmacol. Ther.* **56**, 244–247 (1994).

137. Zhao, P., Xu, P., Wan, C. & Wang, Z. Evening versus morning dosing regimen drug therapy for hypertension. *Cochrane Database Syst. Rev.* **10**, CD004184 (2011).
138. Scheer, F. A. J. L. et al. The human endogenous circadian system causes greatest platelet activation during the biological morning independent of behaviors. *PLOS ONE* **6**, e24549 (2011).
139. Bonten, T. N. et al. Time-dependent effects of aspirin on blood pressure and morning platelet reactivity: a randomized cross-over trial. *Hypertension* **65**, 743–750 (2015).
140. Watanabe, Y., Halberg, F., Otsuka, K. & Cornelissen, G. Toward a personalized chronotherapy of high blood pressure and a circadian overswing. *Clin. Exp. Hypertens.* **35**, 257–266 (2013).
141. Shinagawa, M. et al. Impact of circadian amplitude and chronotherapy: relevance to prevention and treatment of stroke. *Biomed. Pharmacother.* **55**, 125s–132s (2001).
142. Montaigne, D. et al. Daytime variation of perioperative myocardial injury in cardiac surgery and its prevention by Rev-Erbα antagonism: a single-centre propensity-matched cohort study and a randomised study. *Lancet* **391**, 59–69 (2017).
143. Madonna, R. et al. Position Paper of the European Society of Cardiology Working Group Cellular Biology of the Heart: cell-based therapies for myocardial repair and regeneration in ischemic heart disease and heart failure. *Eur. Heart J.* **37**, 1789–1798 (2016).
144. Van Laake, L. W., Passier, R., Doevendans, P. A. & Mummery, C. L. Human embryonic stem cell-derived cardiomyocytes and cardiac repair in rodents. *Circ. Res.* **102**, 1008–1010 (2008).
145. Woldt, E. et al. Rev-erb-α modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy. *Nat. Med.* **19**, 1039–1046 (2013).
146. Chen, Z., Yoo, S. H. & Takahashi, J. S. Small molecule modifiers of circadian clocks. *Cell. Mol. Life Sci.* **70**, 2985–2998 (2013).
147. Eastman, C. I., Suh, C., Tomaka, V. A. & Crowley, S. J. Circadian rhythm phase shifts and endogenous free-running circadian period differ between African- Americans and European-Americans. *Sci. Rep.* **5**, 8381 (2015).
148. Chen, X. et al. Racial/ethnic differences in sleep disturbances: the Multi-Ethnic Study of Atherosclerosis (MESA). *Sleep* **38**, 877–888 (2015).
149. Santhi, N. et al. Sex differences in the circadian regulation of sleep and waking cognition in humans. *Proc. Natl Acad. Sci. USA* **113**, E2730–E2739 (2016).
150. Jarczok, M. N. et al. The heart's rhythm 'n' blues: sex differences in circadian variation patterns of vagal activity vary by depressive symptoms in predominantly healthy employees. *Chronobiol. Int.* **35**, 896–909 (2018).
151. Hood, S. & Amir, S. The aging clock: circadian rhythms and later life. *J. Clin. Invest.* **127**, 437–446 (2017).
152. López, F. et al. Are there ethnic differences in the circadian variation in onset of acute myocardial infarction? A comparison of 3 ethnic groups in Birmingham, UK and Alicante. Spain. *Int. J. Cardiol.* **100**, 151–154 (2005).
153. Donat, M. et al. Linking sleep duration and obesity among black and white US adults. *Clin. Pract.* https://doi.org/10.2217/cpr.13.47 (2013).
154. Yamasaki, F., Schwartz, J. E., Gerber, L. M., Warren, K. & Pickering, T. G. Impact of shift work and race/ ethnicity on the diurnal rhythm of blood pressure and catecholamines. *Hypertension* **32**, 417–423 (1998).
155. Manfredini, R. et al. Sex and circadian periodicity of cardiovascular diseases: are

women sufficiently represented in chronobiological studies? *Heart Fail. Clin.* **13**, 719–738 (2017).
156. Berry, J. D. et al. Lifetime risks of cardiovascular disease. *N. Engl. J. Med.* **366**, 321–329 (2012).
157. Forman, D. E., Cittadini, A., Azhar, G., Douglas, P. S. & Wei, J. Y. Cardiac morphology and function in senescent rats: gender-related differences. *J. Am. Coll. Cardiol.* **30**, 1872–1877 (1997).
158. Alibhai, F. J. et al. Female ClockΔ19/Δ19 mice are protected from the development of age-dependent cardiomyopathy. *Cardiovasc. Res.* **114**, 259–271 (2018).
159. Halberg, F. et al. Diagnosing vascular variability anomalies, not only MESOR-hypertension. *Am. J. Physiol. Heart Circ. Physiol.* **305**, H279–H294 (2013).
160. Smolensky, M. H., Hermida, R. C., Ayala, D. E., Tiseo, R. & Portaluppi, F. Administration-time-dependent effects of blood pressure-lowering medications: basis for the chronotherapy of hypertension. *Blood Press. Monit.* **15**, 173–180 (2010).
161. Racca, C. et al. Aspirin intake in the morning is associated with suboptimal platelet inhibition, as measured by serum thromboxane B2, during infarct-prone early-morning hours. *Platelets* **30**, 871-877 (2018).
162. Smolensky, M. H. et al. Diurnal and twenty-four hour patterning of human diseases: acute and chronic common and uncommon medical conditions. *Sleep Med. Rev.* **21**, 12–22 (2015).
163. Bruguerolle, B. & Labrecque, G. Rhythmic pattern in pain and their chronotherapy. *Adv. Drug Deliv. Rev.* **59**, 883–895 (2007).
164. Lévi, F., Altinok, A., Clairambault, J. & Goldbeter, A. Implications of circadian clocks for the rhythmic delivery of cancer therapeutics. *Phil. Trans. R. Soc. A.* **366**, 3575–3598 (2008).
165. de Mairan, J.-J. Observation botanique [French]. Hist. Acad. R. Sci. 1729, 35 (1729).
166. Konopka, R. J. & Benzer, S. Clock mutants of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* **68**, 2112–2116 (1971).
167. Sehgal, A. et al. Rhythmic expression of timeless: a basis for promoting circadian cycles in period gene autoregulation. *Science* **270**, 808–810 (1995).
168. Price, J. L. et al. *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell* **94**, 83–95 (1998).
169. Stujanna, E. N. et al. Rev-erb agonist improves adverse cardiac remodeling and survival in myocardial infarction through an anti-inflammatory mechanism. *PLOS ONE* **12**, e0189330 (2017).
170. Van Beek, M. H. C. T. et al. The prognostic effect of physical health complaints with new cardiac events and mortality in patients with a myocardial infarction. *Psychosomatics* **58**, 121–131 (2017).
171. Anea, C. B. et al. Vascular disease in mice with a dysfunctional circadian clock. *Circulation* **119**, 1510–1517 (2009).
172. Wang, Q., Maillard, M., Schibler, U., Burnier, M. & Gachon, F. Cardiac hypertrophy, low blood pressure, and low aldosterone levels in mice devoid of the three circadian PAR bZip transcription factors DBP, HLF, and TEF. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **299**, R1013–R1019 (2010).
173. Durgan, D. J. et al. Evidence suggesting that the cardiomyocyte circadian clock modulates responsiveness of the heart to hypertrophic stimuli in mice. *Chronobiol. Int.* **28**, 187–203 (2011).

174. Schroder, E. A. et al. The cardiomyocyte molecular clock, regulation of Scn5a, and arrhythmia susceptibility. *Am. J. Physiol. Cell Physiol.* **304**, C954–C965 (2013).
175. Xie, Z. et al. Smooth-muscle BMAL1 participates in blood pressure circadian rhythm regulation. *J. Clin. Invest.* **125**, 324–336 (2015).

3

CHAPTER

Minimally Invasive Ways of Determining Circadian Rhythms in Humans

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Abstract

Circadian rhythm exerts a critical role in mammalian health and disease. A malfunctioning circadian clock can be a consequence, as well as the cause of several pathophysiologies. Clinical therapies and research may also be influenced by the clock. Since the most suitable manner of revealing this rhythm in humans is not yet established, we discuss existing methods and seek to determine the most feasible ones.

Keywords: Circadian Rhythm, Peripheral Clock, Human Clock, Clock Genes, Minimally Invasive, Diurnal Rhythm

Introduction

All life is shaped by Earth's rotation around its axis, leading to a daily light/dark cycle. Defined as biological rhythms with a period of 24 hours, circadian rhythms allow organisms to prepare for the changes brought upon these cycles and adapt their physiology and behaviour accordingly. Hints towards the importance of this mechanism started unfolding already in the 18th century[1], but it was not until the 1980s that the 2017 Nobel Laureates Jeffrey C. Hall, Michael Rosbash, and Michael W. Young discovered the foundation for the understanding of the molecular machinery underpinning the biological clock[2-4]. Due to their findings, researchers are now more aware of the role and importance that circadian rhythms hold both in physiology and disease.

Spanning from body temperature and hormonal homeostasis to cardiovascular function, liver metabolism and immune system, circadian rhythms influence almost every aspect of human physiology (**Figure 1**)[5-8]. Most of these findings were collected from animal or *in vitro* studies which, although informative and necessary, are somewhat limited in depicting human physiology when it comes to circadian rhythms[9]. For example, it may be difficult to compare nocturnal animals, such as mice, with diurnal humans that have diametrically opposite rest/activity cycles. Therefore, thorough investigation in humans is warranted, potentially leading to new insights about human (patho)physiology, as well as elucidating new circadian biomarkers or finding innovative therapeutic targets. Additionally, since efficacy and side effects of clinical therapies may be time-dependent, and results in clinical research could be modified and confounded by the circadian clock, it is of importance to determine the circadian phase of the subjects. However, a reliable outcome requires sampling every few hours during a 24-hour (or, even better, 48-hour) period, while human organs of interest, such as brain or liver, are not accessible for repeated sampling. Consequently, in most research, sampling is performed at only one time-point – from surgical samples[10], deceased donors[11] or organ transplantation[12]. Samples are then pooled into clusters of similar times of origin, commonly spanning four to 12 hours, to create a signature of a time period.

Clearly, organ biopsies and samples from the deceased give us valuable organ-specific information about one time-point per subject, but are intrinsically hampered by interindividual variation, thus necessitating large groups with still suboptimal reliability when it comes to determining the diurnal phase of the subject. Skin biopsies[13], while less invasive than other organ sampling, provide high-quality samples and sufficient amounts of genetic material, but are still considered too invasive to warrant frequent

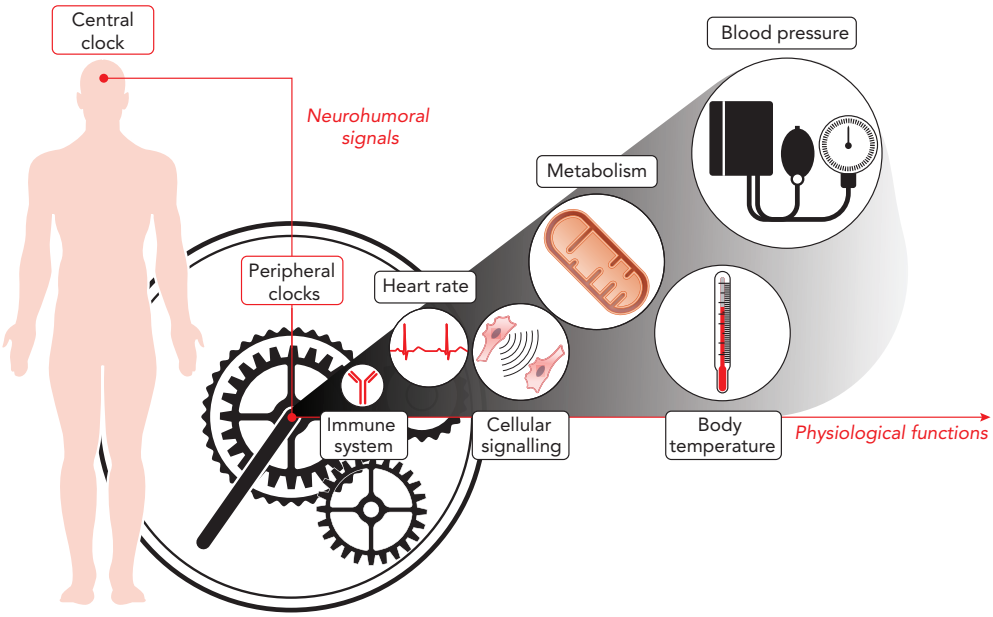

Figure 1 | **Functional aspects of human physiology influenced by circadian rhythms.** Circadian rhythms are regulated by circadian clocks. Central clock is located in the suprachiasmatic nucleus of the brain and it synchronizes peripheral clocks via various neurohumoral signals (e.g. melatonin). Heart rate, blood pressure, body temperature, cellular signaling and responsiveness, energy metabolism, and immune system, are some of the many aspects controlled by the peripheral circadian clocks residing in virtually all cell types and tissues.

sampling. Thus, less invasive alternatives are needed to investigate human peripheral clock.

In this review, we first present minimally invasive sources of human peripheral circadian rhythms, used to study clock genes: blood, oral mucosa, hair and beard follicles. These are more easily obtained and subjected to repeated individual measurements, with least damage to the tested subject. Then, we discuss the feasibility and reliability of their usage.

The molecular basis of circadian rhythms

Circadian rhythms are regulated by circadian clocks. The central circadian clock, also called "master" clock, is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and comprises around 20.000 neurons[14]. This central "pacemaker" for circadian rhythms is driven by external influences, with light being the main clock input-signal (Zeitgeber)[15]. Light cues received through the

retina are transmitted to the central clock which subsequently synchronizes peripheral clocks via neurohumoral signals (e.g. melatonin)[15,16]. Aside from light/dark cycles, peripheral clocks can directly be influenced by behavioral factors, via non-photic entrainment[17,18]. Officially the term *circadian* should only be used to describe processes that persist under constant environmental conditions, meaning that they are not synchronized by any cyclic change in the physical environment. The term *diurnal* (or *nocturnal*) rhythms, however, should only be used when referring to rhythms that are a response to 24-hour environmental changes. For practical reasons, and since almost all diurnal rhythms are also found to be circadian[19], we use circadian in this Review as a term that encompasses all 24-hour oscillatory processes.

Peripheral clocks are present in virtually every tissue, influencing various bodily functions via complex molecular feedback loops (**Figure 2**)[20,21]. Core clock genes BMAL (brain and muscle ARNT-like), circadian locomoter output cycles kaput (CLOCK), cryptochrome (CRY) and period (PER), constitute the main feedback loop[22]. BMAL and CLOCK are activators: after forming a heterodimer, they bind to enhancer box elements (E-boxes) of *PER* and *CRY*, along many other clock-controlled genes (CCG), thereby activating their transcription. In turn, PER and CRY accumulate and heterodimerize in the cytoplasm. The PER-CRY heterodimer, acting as a repressor, then translocates back to the nucleus and inhibits the transcriptional activity of the BMAL-CLOCK heterodimer, thus forming a negative feedback loop. This loop is active until PER and CRY are degraded through ubiquitin-dependent pathways and their nuclear levels decrease. Consequently, the repression on the CLOCK-BMAL heterodimer is lifted and a new transcriptional cycle with ~ 24-hour periodicity begins again. Additional genes, such as orphan nuclear receptors ROR and REV-ERB, form the secondary feedback loop which participates in fine-tuning and maintaining a robust circadian rhythm[23,24]. Activated by the BMAL-CLOCK heterodimer, ROR and REV-ERB compete for the retinoic acid-related orphan receptor response element (*RORE*), located in the *BMAL* promoter. When ROR binds to *RORE*, this leads to activation of *BMAL*, whereas binding of REV-ERB leads to the repression of its transcriptional activity. The interplay of all these transcription-translation feedback loops is crucial for the accuracy of circadian timing and determination of the optimal physiological circadian output[20].

Methods of detecting human peripheral circadian rhythms

Assessing the human peripheral circadian rhythm can be done *in vitro* or *in vivo*. For the purpose of this review, we will shortly clarify differences between these two conditions, reflected only by peripheral clock gene expression. Aside

from these, human circadian rhythms can be assessed by a plethora of methods, ranging from plasma or salivary dim light melatonin secretion onset time (DLMO) as the gold standard for estimating circadian phase, determination of body temperature and the hormonal output levels of melatonin and cortisol, to various, more subjective, chronotype (individual's preferred timing of sleep and activity during a 24-hour period) and sleep/activity questionnaires, as discussed elsewhere in more detail[25,26].

In vitro methods entail culturing human cells and collecting them at regular intervals for, most often, gene expression analysis by quantitative polymerase chain reaction (qPCR)[27,28]. Alternatively, expression of clock genes – as reflected by promotor activity – can be determined in a less cumbersome manner, by means of real-time bioluminescence recordings[29-31]. Either way, cells in culture lack the influence of SCN and therefore need to be artificially synchronized prior to the analysis, either by serum[32] or other compounds such as forskolin[33]. The advantage of this method is the reusability of obtained cells, meaning that they can be expanded, stored, and used for various long- and short-term experiments, and under different experimental conditions (e.g. testing different compounds or medication). However, although a convenient and relatively easy tool to study human cellular rhythms, *in vitro* methods do not completely reflect the *in vivo* clock physiology. As mentioned, these cells are not under the influence of the central clock and its hormonal output, and after being cultured for a longer period of time, they become sensitive to culturing conditions (e.g. medium change and seeding density) that differ from the *in vivo* situation[34,35]. Also, conflicting results are reported from different studies where the correlation of the *in vitro* human clock (e.g. cultured primary fibroblasts) with corresponding physiological period, estimated through the secretion pattern of melatonin, was assessed[36,37].

In vivo methods of exploring human peripheral clocks are different in such a way that they require frequent sampling, usually at regular intervals of one or several hours for a period of 24 to 48 hours. This renders the *in vivo* approach time-consuming and inconvenient for involved researchers and subjects. Furthermore, sleep patterns are often somewhat disturbed when sampling nightly time-points. Nevertheless, thus far this approach most reliably represents the actual physiological situation of circadian clocks in humans. *In vivo* sampling methods can be roughly divided into non-invasive and invasive approaches. Non-invasive approaches encompass, among many others, body temperature measurements and various questionnaires[26]. Fully invasive methods are surgical and tissue biopsies or post-mortem material[10,12,13].

For the sake of this review, we opted for classification of minimally invasive methods, the umbrella under which we subsume any method that does

not leave the tissue permanently scarred or damaged after sampling (e.g. biopsy), and is easily obtained. These methods – peripheral blood, oral mucosa, and hair and beard follicles – emerged as a compromise to allow molecular diagnostics as well as repeated sampling, and are further discussed in the following section.

Peripheral *in vivo* sources of human circadian rhythm

As mentioned above, several minimally invasive tissue sources are currently being used to study circadian oscillations of clock genes in humans (**Figure 2A**). In the following section, we summarize findings obtained from peripheral blood, oral mucosa, and hair and beard follicles, discussing the advantages and limitations of each approach used to study the *in vivo* human circadian system.

Peripheral blood: whole blood and peripheral blood mononuclear cells (PBMCs)

Organ specific RNA transcripts have been identified in peripheral blood [38], making it a possible surrogate tissue for investigating rhythmic clock gene expression in humans[38,39]. Clock gene expression analysis in blood is usually performed either on whole blood or in isolated peripheral blood mononuclear cells (PBMCs).

Whole blood. Transcriptomic analysis in studies on insufficient and mistimed versus normal sleep revealed that up to 9% of genes isolated from whole blood have rhythmic expression[40,41]. A number of studies utilizing whole blood as a source of peripheral circadian rhythms investigated the correlation of sleep or sleep disturbances with clock gene expression[42-46]. Differences have been found in rhythmicity of gene expression between individuals resistant or sensitive to the sleep loss caused by experimental sleep deprivation[43]. A significant reduction in amplitude of the rhythmic clock genes was associated with resistance to sleep loss. Core clock genes *PER1-3* and *BMAL1* displayed significant oscillations, whereas *CRY1-2* and *CLOCK* did not exhibit circadian rhythmicity in both groups.

Overall, whole blood was used to find important links between various diseases and circadian rhythms. *CRY1* and *PER2* expression was found to be significantly decreased in septic patients, when compared to non-septic patients, admitted to the hospital for various reasons such as cerebral hemorrhage, craniocerebral injury, liver and kidney failure, acute poisoning, and coronary heart disease[47]. Circadian clock gene expression was found to be disrupted in patients with obstructive sleep apnea syndrome (OSAS)[48,49]. A study on 133 patients with OSAS and 11 healthy controls showed that the

oscillating pattern of *BMAL1*, *CLOCK*, and *CRY2*, was abolished in patients[49]. Furthermore, various effects of external cues on changes in clock gene expression, such as blue-light exposure[50] and influence of breakfast on healthy and diabetic subjects[51], were also investigated in whole blood. Blue light caused significant phase advances in the timing of *PER3* and melatonin peak expression[50], while skipping the breakfast caused alteration in clock gene expression in healthy and individuals with type 2 diabetes[51]. Aside from observing differences between diseased and healthy condition, peripheral blood can be used to study the effect of certain compounds, such as endotoxins[52] and β_2-adrenoceptor agonists[53], on clock gene expression (80-90% reduced gene expression of *CLOCK*, *CRY1-2*, *PER3*, *CSNK1epsilon*, *RORA*, and *REV-ERB*, and increased expression of *PER1*, respectively).

Moreover, several studies observed the effect of the treatment on clock gene expression in RNA isolated from whole blood, although only by comparing two time-points: at the start and after the completion of the therapy[54,55]. Similarly, aging, glucose intolerance and irregular working hours, were found to affect the rhythmic expression of clock genes in 70 healthy women, by comparing only morning time-points[56]. By conducting a study at a single time-point, for example before and after the intervention, intra- and inter-individual differences of clock gene expression at a given time-point can be observed. This approach, however, does not provide information on circadian dynamics of clock genes.

Peripheral blood mononuclear cells (PBMCs). Aside from full blood, circadian rhythms were investigated in a subpopulation of cells, namely PBMCs, which consist mainly of lymphocytes, monocytes and dendritic cells. PBMCs can be isolated from venous blood by various centrifugation-based density methods, including the most commonly known Ficoll-Paque technique[57].

Boivin *et al.*[58] found significant *PER* oscillations in PBMCs isolated from three healthy male subjects, with peak expression during the active period (subjective day). This study was performed under constant routine conditions, with the goal of revealing the endogenous circadian rhythms without the influence of outside factors such as posture, light changes, or food intake. This was in line with previous work[59] in which significant differences between morning and evening *PER* expression were found in PBMCs isolated from 9 healthy volunteers. The same study, however, did not reveal significant differences between morning and evening expression of the *CLOCK* gene. Afterwards, Kusanagi *et al.* confirmed the existence of similar *PER1* oscillation in different peripheral blood cell type fractions, mononuclear cells and polymorphonuclear neutrophils[60].

More research followed, encompassing the influence of shifted[61] and

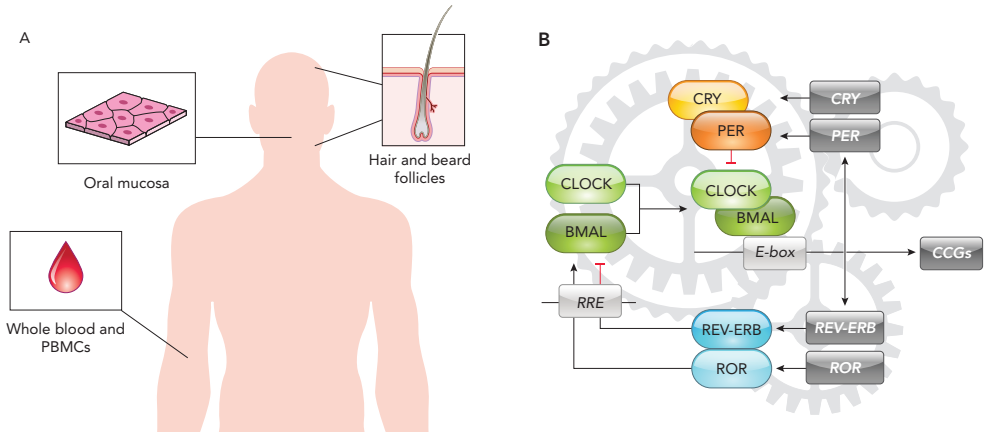

Figure 2 | **Circadian clock molecular machinery in human peripheral tissues. (A)** Minimally invasive sources of the human circadian clock include tissues such as blood (whole blood or peripheral blood mononuclear cells (PBMCs)), oral mucosa, and hair and beard follicles. **(B)** The core clock mechanism consists of interconnecting positive and negative feedback loops. BMAL (brain and muscle ARNT-like) and circadian locomoter output cycles kaput (CLOCK) heterodimerize and bind to enhancer box elements (E-boxes) of cryptochrome (*CRY*) and period (*PER*), along many other clock-controlled genes (CCG), thus initiating their transcription. In turn, PER and CRY form a heterodimer, inhibiting CLOCK-BMAL activity. This constitutes the main feedback loop. A secondary feedback loop consists of orphan nuclear receptors ROR and REV-ERB, which either activate or inhibit *BMAL* transcription, depending on which protein binds first to retinoic acid-related orphan receptor response element (*RORE*), located in the *BMAL* promoter.

normal[62] sleep/wake schedules on circadian expression patterns in PBMCs; the comparison of aberrant clock gene expression profiles in different diseases with healthy individuals[63,64]; or simply evaluation of expression of a bigger range of circadian core clock genes in PBMCs, isolated from healthy individuals under constant conditions[65]. In the latter study, and in most of the other studies that utilized PBMCs as a source of peripheral circadian clock, *PER* almost always showed the most robust circadian pattern, as opposed to other core clock genes, and it was most often reported. For example, Hida *et al.*[66] investigated differences between *PER1-3* expression in younger and older subjects, finding altered *PER3* expression profiles upon aging, with later peak time and earlier trough time relative to the sleep phase. Watanabe *et al.*[67] found *PER1* to be significantly oscillating in 9 subjects, *PER2* in three, and *PER3* in all 13 of the tested subjects, with the phases of *PER1* and *PER3* significantly correlating with those of physiological rhythms. The expression of *BMAL1* was quantified as well, and found to oscillate significantly in 8 out of 13 subjects.

Two other studies recruited a rather uncommon study population for circadian rhythms research: PBMCs were collected from 32 breast-milk jaundiced neonates hospitalized for hyperbilirubinemia[68] and 10 premature neonates in order to study the effect of phototherapy and non-nutritive sucking (NNS)[69], respectively, on changes in *BMAL1* and *CRY1* gene expression. Blue light phototherapy was found to decrease the expression of *BMAL1* and plasma melatonin levels, while increasing the expression of *CRY1*[68]. No differences were found in expression levels of *BMAL1* and *CRY1* gene in PBMCs of preterm neonates, following the NNS[69]. Here, however, only two time-points were evaluated to determine the clock gene expression status before and after the treatment, and therefore no conclusion on 24-hour circadian fluctuations can be made. More recent research made use of isolated PBMCs to study the influence of alcohol on circadian clock changes[70], and clock disturbance in sleep apnoea patients and their response to therapy[71]. Alcohol significantly increased the amplitude of peripheral clock genes, namely *CLOCK*, *BMAL*, *PER1*, and *CRY1*, in both day and night workers, while the amplitude of *CRY2* was increased in night workers and decreased in day workers. The loss of rhythmicity was observed in inflammatory cytokines such as lipopolysaccharide-binding protein, lipopolysaccharide, and interleukin 6, after alcohol consumption in both groups[70]. In sleep apnoea patients, changes observed in *CLOCK* expression were not reverted by standard continuous positive airway pressure treatment[71].

Limitations and opportunities. Compared to other minimally invasive sources discussed further in this review, blood remains the most researched source of the human peripheral clock thus far. However, some differences in clock gene expression can be observed across different studies. As mentioned before, mRNA levels of *PER* almost always showed robust oscillations, however, oscillations or phases of other clock genes sometimes varied across different studies[65,67]. Indeed, we investigated expression of core clock genes in the peripheral blood of heart failure patients and healthy controls, without observing clear circadian oscillations in gene expression for majority of included subjects (data not shown). 70 subjects were enrolled in the study: 46 patients with heart failure with reduced ejection fraction (HFrEF; left ventricular ejection fraction (LVEF) < 40% by echocardiography; 32.6% in NYHA class II and 67.4% in NYHA class III) and 24 controls which were matched for age and sex (**Table S1** and **Supplemental Methods**). The majority of participants were male (71.7% for patients and 70.8% for controls) with a median age of 60 years for patients and 56 for controls. In both groups, cosinor analysis revealed significant cosine shaped 24-hour oscillations only in

normalised *BMAL1* values (P=0.001 for heart failure patients and P=0.006 for controls, **Figure 3A**), while *CLOCK*, *PER2*, and *CRY2* seemed to fluctuate randomly throughout the 24-hour period (**Figure 3B-D**).

This variation in gene expression, coming from the same peripheral source, could be traced to differential proportion of cell subsets in blood, or to endogenous factors of tested subjects, such as age, sex, hormones, or underlying comorbidities[72]. As Teboul *et al.*[73] suggested, the variation in the abundance of a certain cell type in blood at a time-point of collection could theoretically bias the results towards observing molecular oscillations. Although it has been shown that peripheral blood cells share more than 80% of the transcriptome with several other organs[39], blood is one of the most dynamic tissues which comprises RNA from various origins (e.g. extracellular vesicles or other secreted RNA products)[74] and therefore might not entirely represent gene expression in each specific peripheral organ.

Interestingly and most relevant, different blood collection and processing methods from the same subjects may lead to different gene expression profiles[75]. First, differences between the two above-described cell sources of clock transcripts – whole blood and PBMCs – arise when it comes to the processing of blood samples. Translation-wise, the use of whole blood might be more feasible if using PAXGene blood RNA tubes, as RNA extraction can be deferred up to 72 hours without affecting the quality of transcripts (according to manufacturer's protocol). PBMCs, however, require more labor-intensive and direct processing after blood sampling[58,75], which can be challenging especially during the night. Aside from the practical translational aspect, differences in blood processing, RNA isolation methods, physical handling of the samples and time delays in processing of the cell separation might affect clock gene expression levels, consequently hampering the reproducibility of the results when using blood as a source of peripheral rhythms[75,76].

However, despite these drawbacks, and based on copious research performed on blood, this peripheral tissue seems to be a valuable tool for clock gene expression profiling in humans. For example, more frequent sampling can be done via intravenous cannulation of hospitalized subjects[77], which is especially important for nightly time-points when blood can be collected with minimal or no disruption of subjects' sleep. Researchers should, however, carefully approach and aim for standardization of all the processes including blood collection, RNA isolation, storage, and handling, in order not to affect downstream processes of gene expression profiling, which would confound internal and external validity[75].

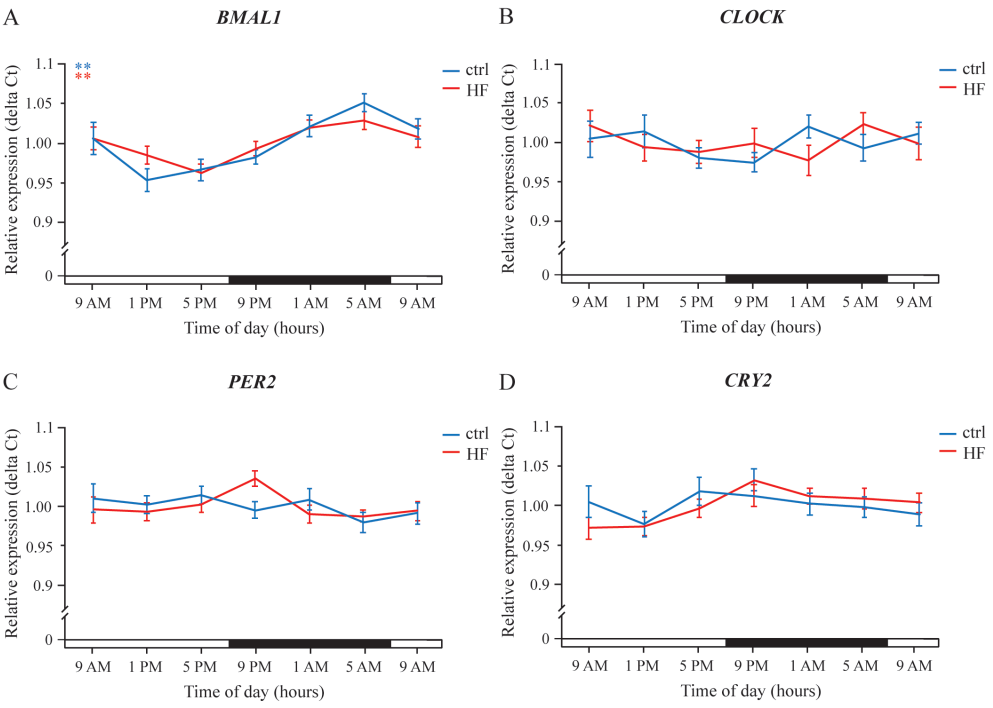

Figure 3 | **24-hour expression profiles of core clock genes in human peripheral blood.** Cosinor analysis of normalized **(A)** *BMAL1* **(B)** *CLOCK* **(C)** *PER2* and **(D)** *CRY2* per each HFrEF patient (N=37-44 per each time-point) and control (N=19-22 per each time-point), in a period of 24 hours. Per subject, each time-point was normalized against its mean delta Ct value of the entire day. Higher delta Ct values indicate lower gene expression. Values are mean ± standard error of mean (SEM). P<0.05 was used as a cut-off for significance. A significance of cosinor analysis is indicated at the top left graph corner: **P<0.01. Horizontal bar indicates day (=white) and night (=black). *BMAL1*, Brain and Muscle ARNT-Like 1; *CLOCK*, Circadian Locomotor Output Cycles Kaput; *CRY2*, Cryptochrome Circadian Regulator 2; HFrEF, heart failure with reduced ejection fraction; *PER2*, Period Circadian Regulator 2.

Oral mucosa

Another minimally invasive way to study the human clock is via oral mucosa samples. Previously, small punch biopsies were taken under a local anaesthetic[78]. However, more recently, mucosa samples are scraped off with pipette tips, rendering this approach much less invasive than before, while still providing enough material for subsequent analysis[79].

The first study showing rhythmic expression of core clock genes in human oral mucosa was done by Bjarnason *et al.*[78] in 2001. By obtaining samples from healthy male subjects, they found that *PER1*, *CRY1*, and *BMAL1*

oscillate in a circadian manner, with patterns of oscillation similar to those reported in rodents. They further suggested that there is a potential link between cell cycle and the clock, since peaks of *PER1* and *BMAL1* expression coincided with the peak expression of *p53* and *cyclin β1*, bearing importance for oncology research, and timing of chemo- and radiotherapy[78,80]. This was confirmed in the study by Zieker *et al.* in which a 24-hour comparative microarray analysis of oral mucosa samples obtained from healthy male volunteers revealed 33 differentially regulated clock genes and various CCGs, including cell cycle-related-, tumor suppressor- and oncogenes[81].

Having this easily accessible indicator of circadian rhythms available has enabled researchers to study various diseases, sleep disturbances, and changes in daily schedules that may influence the human clock. For example, in two separate studies Cajochen *et al.*[79] and Jud *et al.*[82] investigated if the effect of light exposure, as main Zeitgeber, can be observed in *PER2* expression in human mucosa. As a society, we are overwhelmingly exposed to different artificial lights, potentially leading to circadian rhythm disturbances and negatively affecting our health and well-being. The results indicated that evening and morning exposure to blue light acutely stimulates *PER2* expression in oral mucosa, and that this expression is wavelength- and age-dependent[79,82]. Another study aimed to elucidate if the disturbed sleep and melatonin secretion in children with Smith-Magenis syndrome – a rare developmental disorder – was reflected by an alteration of the molecular mechanism of the circadian clock[83]. The SCN is mainly responsible for driving the rhythm of pineal melatonin production, but inaccessible for sampling in humans. The peripheral clock from oral mucosa therefore served as a substitute and a marker of SCN function. Nováková *et al.* further showed that the circadian phases of the *PER1*, *PER2*, and *REV-ERBα* expression were advanced in the early chronotypes, as opposed to late chronotypes, and that the individual chronotype affects the phasing of both daily melatonin rhythm in saliva and *PER1-2*, and *REV-ERBα* clock gene expression profiles in oral mucosa[84]. Finally, oral mucosa samples were most recently used to determine peripheral clock gene expression during a polar expedition with compromised light/dark cycles[85]. In addition, the disruption of several clock genes, namely *PER1-3* and *REV-ERBα*, was demonstrated in police officers undergoing a week of night shifts[86]. The researchers showed that the two-week polar expedition to Svalbard had an effect on melatonin by decreasing its concentration and delaying its peak time during the night, as well as on the clock genes which displayed a higher amplitude when compared to the amplitude measured in the same subjects before the expedition[85].

Limitations and opportunities. Although convenient for determining human circadian rhythms, sampling from oral mucosa has several drawbacks. Ideally, for most precise measurement of circadian gene expression profile changes, tissue samples would be collected in one- or two-hour intervals throughout the day. However, even though minimally invasive, the method cannot be performed unnoticed (unlike blood collection from an intravenous cannula) and usually a minimum of four-hour intervals are acceptable to study subjects[83]. Furthermore, subjects are sometimes excluded from the study due to difficulties in obtaining mucosa samples, or not having enough material for subsequent analysis[79,86]. Additional hurdles may arise due to the sensitivity of material. Oral mucosa samples are highly sensitive to RNA degradation and, if not handled properly, may result in fragmentation of RNA, and thus hinder reproducibility of results[76].

Hair and beard follicles

Another source of human peripheral circadian rhythms was found by Akashi *et al.* in 2010[76], when they determined expression of clock genes in cells isolated from scalp hair follicles. Based on the influence of circadian rhythms on skin in general this does not come as a surprise: among others, temperature and blood flow of the skin, proliferation of the keratinocytes, and loss of trans-epidermal water, all vary in a circadian manner[87]. Furthermore, it has been shown that peripheral core clock genes modulate the hair follicle cycling[88,89].

Using samples of hair follicle cells, obtained from either the head or chin, enabled research into human peripheral circadian rhythms. This new method emerged as a less invasive alternative to blood sampling and oral mucosa biopsies, providing high-quality RNA, and reliable and accurate means to study human clock gene expression[76]. By means of DNA microarray analysis Akashi *et al.*[76] provided an extensive list (>200) of oscillating genes that originate from hair follicles. Furthermore, they showed that using hair follicles is feasible to test the influence of both endogenous (behavioral) and environmental factors (shift work) on the individual peripheral circadian clock. In another study, Watanabe *et al.* provided further validation of this method[67] by collecting beard hairs every two hours from 13 healthy male subjects kept under constant routine conditions, in order to assess their circadian phases when interfering factors are kept to a minimum. Finally, other studies aimed to link individual chronotypes to circadian gene expression[90,91]. Ferrante *et al.* identified differences in expression of clock genes, *PER3* and *NR1D2*, in hair follicle cells collected from 14 individuals of extreme morning or evening chronotypes[90]. Almost three-hour phase delay of clock gene expression was found in extreme evening compared to extreme morning

chronotypes, proving this method feasible of determining clock phase differences between different diurnal preferences.

Limitations and opportunities. Some of the advantages of using hair follicles to study circadian rhythms are the relatively high quantity of RNA obtained, around 100 ng from each follicle, as well as the possibility of analyzing the gene expression by commonly used qPCR[76]. Another advantage of this method includes absence of additional cell separation steps, since cells are readily available just by plucking the hair[76]. Both head and chin hair follicles can be used to isolate high-quality RNA, needing approximately 10 head hairs or five chin hairs per time-point, according to Akashi *et al.*[76].

However, the amount of genetic material attached to each hair varies between individuals in terms of hair thickness and sex, therefore the number of needed hairs will be different in each subject. Consequently, Watanabe *et al.*[67] could not obtain enough RNA for subsequent analysis from five out of 13 subjects, indicating that further optimization for this method is required. Lastly, while this method is minimally invasive for the subject, the sampling will not go unnoticed, as it would with blood collection from intravenous cannula. This could bare significance during the nightly time-points, where disturbed sleep caused by hair sampling could influence the subjects' circadian rhythms.

Perspectives and clinical utility

Circadian rhythms drive various important processes in human physiology and pathophysiology. With more than 80% of mammalian protein-coding genes showing diurnal variation in their mRNA levels[21,92-95], the circadian clock influences for example the immune system[8], cardiovascular biology and disease[5], the nervous system[96], metabolic processes[97] and cancer[98]. Understanding the biology around circadian variation could potentially lead to novel or improved therapies or strategies to prevent certain pathophysiologies. Aside from circadian focused research, determining the circadian phase of the body clock could have an impact on clinical research and therapies in general. Therapy efficacy and side effects may be time-dependent, and circadian clock can modify and confound clinical research. Thus, it is of importance to study the impact of circadian rhythms on human health and disease directly at the source – by using human tissue samples.

Aside from the SCN, as a central circadian clock, rhythms can be found in virtually every tissue, directly regulated by peripheral clocks[20]. Having suitable sources to study peripheral circadian rhythms directly in humans, as

discussed in this Review, will provide important insights into the internal rhythms of both healthy and diseased individuals. This could lead to discovering new links between disturbed regulation of circadian clock and disease in question. For example, correlating dysregulation of the clock with diseases like cancer might lead to detection of people more prone to developing the disease, or allow for monitoring of tumor progression and timing of the therapy[78,80,81]. Furthermore, as seen in shift workers, various environmental circumstances may disrupt the clock, eventually leading to serious health problems[76,86,99]. Observing changes in circadian rhythms of these workers can help explain the adverse health conditions they develop as a consequence of disrupted day/night cycling (e.g. cardiovascular diseases[100]), and allow for development of strategies that could ameliorate those negative effects (e.g. by strict food intake schedules or light-exposure adjustments).

Taking into consideration the immense impact circadian rhythms have on human health and disease, it is of utmost importance to develop convenient and reliable methods for studying these rhythms. Aside from blood, oral mucosa, and hair and beard follicles, other minimally invasive sources could potentially be used for human circadian research. For example, tape stripping could be used to sample the human epidermis[101]. Using this approach, the authors successfully collected RNA from normal and inflamed skin, and performed qPCR analysis. Obtained gene expression profile in this study was not related to clock genes, however it does open possibilities towards circadian application of tape stripping, as a less invasive alternative method to skin biopsy. Furthermore, besides blood, other bodily fluids, such as urine[102,103], saliva[104], and breastmilk[105,106], could be used to study circadian gene expression. So far, urine and breastmilk have been used to determine non-circadian gene expression profiles in physiological and pathophysiological conditions[103,105,106], while clock gene expression has been confirmed in human salivary glands[104]. Owing to the minimal invasiveness in obtaining these tissues, and the confirmed possibility of using them for gene expression analysis, they could represent novel sources for studying human peripheral rhythms.

Besides, several new methods of determining internal circadian time based on human transcriptomic data have been developed[13,107-110]. One of the assays (BodyTime) is based on monocyte gene expression profiles derived from single blood sample[110]. It utilizes only a handful of clock genes (≤12), and in a less cumbersome way estimates a person's DLMO. Traditionally, sampling to determine the time-point when endogenous melatonin is secreted needs to be performed under controlled dim-light conditions, and once or twice per hour for a period of 5 to 6 hours. With only one blood sample needed, taken

anytime during the day, BodyTime offers a clinically more translatable method of chronotyping individuals. Similarly, Wu *et al.*[13] explored human epidermis as an alternative source for determining circadian phase on a population level. By using a CYCLOPS machine learning-type algorithm[107], they identified and characterized a robust biomarker set capable of reporting circadian phase to within 3 hours from a single skin sample. These methods represent new personalized diagnostic tools for tailoring future health care according to the individual's internal clock.

Interesting, however out of scope of this Review, would be to correlate findings from peripheral tissues to other organs, and central clock in the brain. A study from Hughey *et al.*[111] hinted towards this direction by comparing the phasing of circadian gene expression in human brain, blood, skin and hair follicle, and across the species, including organs from nocturnal mouse, rat, and Siberian hamster. Their results suggest that the circadian clocks in, for example, human brain and blood are progressing similarly, but are phase-shifted, with 6 to 8-h difference in peak times for majority of tested clock genes. Generally, peripheral clocks were phase-shifted by approximately 12 hours between diurnal and nocturnal species, with an exception of SCN in brain, whose phase was different from the ones in human blood, skin and hair follicles. Further research is needed to determine alignment between circadian phases of different human organs, as well as to investigate possible misalignment in various pathophysiologies. Also, taking into account the growing body of evidence that indicates the existence of sex differences in circadian rhythms[112], it would be of interest to investigate how these influence the circadian clock in peripheral sources outlined in this Review. Although many studies did include both male and female participants[43,79,84,85,90], the majority of research is still performed in males. In the present research, no apparent differences were observed between the sexes, however they were not the main focus of the studies and therefore no concrete conclusions can be drawn.

Of note, activity and temperature monitoring devices, as other minimally invasive techniques, have been extensively studied and shown to accurately predict the human body's circadian rhythm, strongly correlating with the central clock outputs[113]. One of the key differences and advantage of the methods presented in this Review, when compared with other wearable devices, is the intention to provide phase and expression of core clock genes and other CCGs directly in the corresponding peripheral tissues. Overall, profiling of peripheral clock gene expression can be used to determine functional circadian rhythms, as well as to recognize causes, environmental or endogenous, and consequences of deviating rhythms.

Summary and conclusions

Until recently, *in vitro* cultures were one of the only sources to study human molecular circadian rhythms[26,114], along with the timewise uncontrolled biopsy samples obtained either during surgery[12] or via a deceased donor[11]. The latter two options would result in only one time-point per person, therefore lacking the possibility to study individual changes in circadian rhythms.

Due to the difficulty of obtaining human tissue biopsies at regularly occurring intervals, researchers focused on finding less invasive alternatives to investigate the human circadian clock and to determine the circadian phase of the subjects – blood, oral mucosa, and hair and beard follicles. In this review, we provided an overview of research done on those peripheral sources (key findings summarized in **Table 1**), indicating advantages and disadvantages for each source (**Figure 4**).

Several requirements have to be met when considering human tissues as a reflection of the molecular circadian clock: samples must be obtained with minimal damage and pain to human subjects, and they need to be accessible for repeated sampling while containing a sufficient amount and quality of genetic material for subsequent analysis of clock gene expression. More frequent sampling, for example every hour, would give a clearer picture of circadian expression, however this is rarely acceptable to tested subjects. So far, hair follicles as a source of circadian rhythms fulfill most requirements for human clock research: they represent the least invasive way of obtaining samples while providing RNA of sufficient quality and quantity for downstream gene expression analysis[76]. However, more research needs to be done in order to verify the reproducibility and feasibility of this relatively novel source for studying human circadian rhythms, and thereby indicate translational aspects for biomarker use, disease monitoring, and therapeutic intervention windows.

The peripheral indicators of the molecular circadian clock described in this review overcame the biggest obstacle in studying individual circadian rhythms *in vivo*: finding a method for direct regular sampling of human tissues which is acceptable both for human subjects in terms of invasiveness and discomfort, and for the quality of the subsequent research. Circadian rhythms are essential components of human physiology and disease. Having a possibility to monitor and assess the human circadian clock is crucial for understanding the impact of internal and external environmental changes on human circadian rhythms and consequently health and disease. Since circadian rhythms have been implicated in various pathologies, either as a symptom or a causative factor, it is necessary to have means for their easy and reliable

determination. Additionally, to have readily available ways to measure circadian timing in humans is of importance for the recently proposed field, termed circadian medicine[115,116]. By bridging the clinical practice with circadian rhythms, our understanding of the underlying disease mechanisms will be improved, timing of the drug administration or other therapeutic interventions optimized, and new potential (circadian) therapeutic targets for certain diseases unravelled.

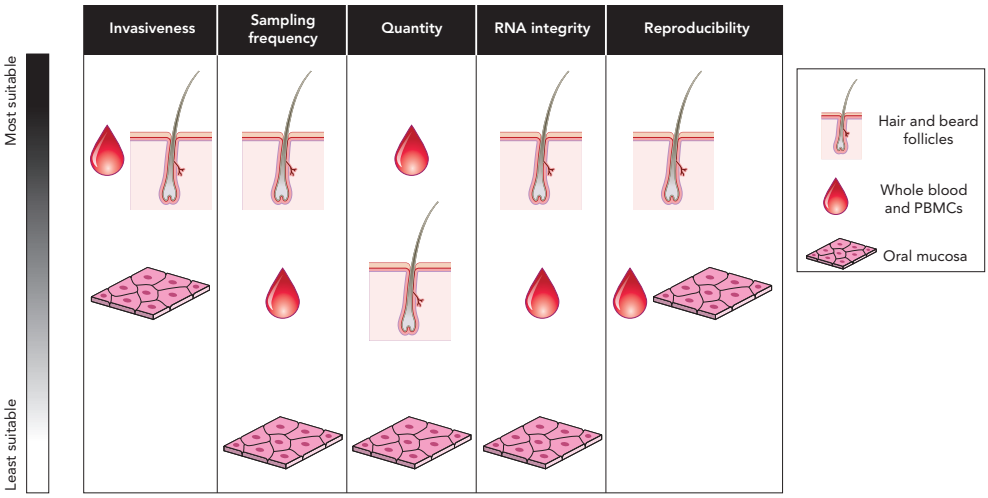

Figure 4 | **Advantages and disadvantages of human peripheral sources of circadian rhythms.** Depicted are comparisons between minimally invasive sources of the human peripheral clock, namely blood, oral mucosa, and hair and beard follicles, based on invasiveness and acceptable frequency of sampling in regards to subjects' discomfort, quantity and integrity of obtained RNA, and reported reproducibility of clock gene expression results.

Table 1 | **Summary of key findings from peripheral blood, oral mucosa, and hair and beard follicles.**

(Patho)physiology and subjects	Sampling frequency	Gene expression	Mathematical methods	References
Whole blood				
Healthy subjects and a patient with circadian rhythm sleep disorder	24-hour period; 4-hour interval	Significant daily variations of *PER1*, *PER2*, *PER3*, *BMAL1*, and *CLOCK* in healthy subjects; restored expression of clock genes in a patient after combined therapy (light, exercise, and pharmacotherapy)	ANOVA	Takimoto *et al.* 2005
Healthy young and old subjects under blue and green light exposure	Day 1 and day 3 of 3 laboratory sessions; 1-hour interval	*PER3* amplitude was reduced in older subjects; observed phase advances after blue- (but not green-) light exposure regardless of the age	Cosinor analysis	Ackermann *et al.* 2009
Healthy subjects under CR condition (sleep deprivation)	30-hour period; 3-hour interval	Expression of 711 genes changed by insufficient sleep; reduction of the number of genes with a circadian expression profile from 1.855 to 1.481 (reduced amplitude); affected genes included *PER1*, *PER2*, *PER3*, *CRY2*, *CLOCK*, *NR1D1*, *NR1D2*, *RORA*, *DEC1*, *CSNK1E*, as well as genes implicated in metabolism, immune and stress response, inflammation, and oxidative stress	Sine analysis	Möller-Levet *et al.* 2013
Healthy subjects under sleep deprivation	72-hour period; 4-hour interval	*BMAL1*, *NPAS2*, *REV-ERB*, *PER1*, *PER2*, *PER3*, *CSNK1D*, and *CSNK1E* had a circadian rhythm, while *CRY1*, *CRY2*, *RORA*, *RORB*, *RORC*, *DEC1*, *DEC2*, and *CLOCK* did not; *SREBF1* and *CPT1A* had a significantly	Cosinor analysis	Arnardottir *et al.* 2014

Table 1 | **Continued**

(Patho)physiology and subjects	Sampling frequency	Gene expression	Mathematical methods	References
		changed expression during SD		
Healthy subjects under CR and S/SD condition	33-hour period for CR and 48-hour interval for S/SD; 2-hour interval	*STAT3*, *SREBF1*, *TRIB1*, and *THRA1* were rhythmic in both conditions; *MKNK2* rhythmic only in S/SD; *PER1*, *PER3*, and *REV-ERBα* rhythmic in the CR; *BMAL1* and *HSPA1B* not rhythmic in CR	A time-domain analysis fitting sinusoidal curve	Lech *et al.* 2016
Obstructive sleep apnoea patients and healthy controls	18-hour period; 6-hour interval	*BMAL1*, *CLOCK*, and *CRY2* expression were abolished in patients	Univariate analysis of General Linear Model (GLM) with post hoc Bonferroni comparison under GLM	Yang *et al.* 2019
Peripheral blood mononuclear cells (PBMCs)				
Healthy subjects	32-hour period; 2-hour interval	*PER1*, *PER2*, *PER3*, and *DEC1* oscillate in a circadian manner; peak during activity period	A dual-harmonic regression model	Boivin *et al.* 2003
Healthy subjects	42-hour period; 2-hour interval	*PER1* oscillates in a circadian manner in mononuclear and polymorphonuclear cells; peak during activity period	Cosinor analysis, least squares method	Kusanagi *et al.* 2004
Healthy subjects	72-hour period; 2-hour interval	*PER1* and *PER2* have comparable oscillations under L-D and CR conditions, while *BMAL1*	A dual-harmonic	James *et al.* 2007

Table 1 | **Continued**

(Patho)physiology and subjects	Sampling frequency	Gene expression	Mathematical methods	References
		peak expression changes in regards to a different sleep/wake condition	regression model	
Healthy subjects	24-hour period; 2-hour interval	Out of 10 genes (*PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CLOCK*, *BMAL1*, *DEC1*, *DEC2*, *TIM*) only *PER1*, *PER2* and *PER3* were consistently rhythmic	A 12/24-h composite cosine model	Kusanagi *et al.* 2008
Chronic myeloid leukemia patients and healthy subjects	18-hour period; 6-hour interval	*PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, and *CKIε* expression was disrupted in patients; *CLOCK* and *TIM* were not rhythmic in both groups	Repeated-measures ANOVA	Yang *et al.* 2011
Type 2 diabetes patients and healthy subjects	18-hour period; 6-hour interval	*PER1*, *PER2*, *PER3*, and *BMAL1* expression dampened in patients	Friedman test	Ando *et al.* 2009
Healthy older subjects	24-hour period; 2-hour interval	Altered *PER3* expression compared to young controls (shifted peak/nadir time); no changes in daily rhythm of *PER1* and *PER2*	A 24/12-hour composite cosine model using ChronoLab	Hida *et al.* 2009
Healthy subjects	32-hour period; 2-hour interval	Significant circadian rhythmicity was found for *PER1*, *PER2*, *PER3*, *BMAL1*; *PER1* and *PER3* expression correlated with physiological rhythms	A 24/12 h composite cosine model using ChronoLab	Watanabe *et al.* 2012
Alcohol consumption influence on healthy day and night workers	24-hour period; 4-hour interval	Alcohol increased the amplitude of *CLOCK*, *BMAL*, *PER1*, and *CRY1*, in both groups; amplitude of *CRY2* was increased in night workers and decreased in day workers;	Cosinor analysis	Swanson *et al.* 2016

Table 1 | **Continued**

(Patho)physiology and subjects	Sampling frequency	Gene expression	Mathematical methods	References
		loss of rhythmicity was observed in lipopolysaccharide-binding protein, lipopolysaccharide, and interleukin 6 in both groups		
Oral mucosa				
Healthy subjects	24-hour period; 4-hour interval	*PER1*, *CRY1*, and *BMAL1* oscillate in a circadian manner, with similar pattern to rodents and SCN; *CLOCK* and *TIM* are not rhythmic; *PER1* peak coincides with the peak of *p53*, *BMAL1* with cyclin *B1*	Cosinor analysis using a non-linear, least squares method	Bjarnason *et al.* 1999 and 2001
Healthy subjects	48-hour period; 6-hour interval	Microarray analyses: differentially regulated 16 clock genes (*BMAL1*, *CRY1*, *PER2*) and 17 tumor suppressor- and oncogenes (*SMAD5*, *CCRN4L*); *CLOCK* and *TIM* were not rhythmic	Fisher's g-statistic, topoGraph.R, cosine curves correlation, Lomb-Scargle test statistic for periodicity	Zieker *et al.* 2010
Healthy subjects under blue light exposure	30-hour period; 3-hour interval	Significant *PER2* oscillations; evening and morning exposure to blue light acutely stimulates wavelength-dependent *PER2* expression	Sinusoidal non-linear least-square fitting analysis based upon the	Cajochen *et al.* 2006

Table 1 | **Continued**

(Patho)physiology and subjects	Sampling frequency	Gene expression	Mathematical methods	References
			Marquardt–Levenberg algorithm	
Extreme morning or evening chronotype	24-hour period; 4-hour interval	Advanced phases of *PER1*, *PER2*, and *REV-ERBα* expression in the early chronotypes	The least squares regression method	Nováková *et al.* 2013
Shift workers	2 separate laboratory visits: 24-h period for each visit; 4-hour interval	Disrupted rhythmicity of *PER1-3* and *REV-ERBα* after a week of night shifts	A single-harmonic regression model	Koshy *et al.* 2019
Healthy subjects on a polar expedition	2 phases: 24-h period for each phase; 4-hour interval	Decreased melatonin concentration and delayed peak time; higher amplitude of clock genes (*PER1* and *NR1D1*) after the 2-week expedition; no oscillation in *BMAL1*	Cosinor analysis	Weissová *et al.* 2019
Hair and beard follicles				
Healthy subjects and shift workers	48-hour period; 3-hour interval	DNA microarray analysis; >200 oscillating genes, including *BMAL1*, *PER3*, *NR1D1*, *NR1D2*; phase delay in shift work	Cosinor analysis using a non-linear, least squares method	Akashi *et al.* 2010
Healthy subjects	32-hour period; 2-hour	Significant circadian rhythmicity was found	A 24/12 h	Watanabe *et*

Table 1 | **Continued**

(Patho)physiology and subjects	Sampling frequency	Gene expression	Mathematical methods	References
	interval	for *PER1*, *PER2*, *PER3*, *BMAL1*	composite cosine model using ChronoLab	*al.* 2012
Extreme morning and evening chronotype	24-hour period; 3 time-points	*PER3* and *NR1D2* phase differences between morning and evening chronotypes	Cosinor analysis using a non-linear, least squares method	Ferrante *et al.* 2015; Ingram *et al.* 2016

CR, constant routine; L-D, light-dark; S/SD, sleep/sleep deprivation

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References

1. De Mairan, J.-J. d'Ortous. Observation Botanique. *Hist. Académie R. Sci.* 35 (1729).
2. Bargiello, T. A., Jackson, F. R. & Young, M. W. Restoration of circadian behavioural rhythms by gene transfer in Drosophila. *Nature* **312**, 752 (1984).
3. Hardin, P. E., Hall, J. C. & Rosbash, M. Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. *Nature* **343**, 536 (1990).
4. Siwicki, K. K., Eastman, C., Petersen, G., Rosbash, M. & Hall, J. C. Antibodies to the period gene product of Drosophila reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* **1**, 141–150 (1988).
5. Crnko, S., Du Pré, B. C., Sluijter, J. P. G. & Van Laake, L. W. Circadian rhythms and the molecular clock in cardiovascular biology and disease. *Nat. Rev. Cardiol.* **16**, 437–447 (2019).
6. Gnocchi, D. & Bruscalupi, G. Circadian Rhythms and Hormonal Homeostasis: Pathophysiological Implications. *Biology* **6**, 10 (2017).
7. Reinke, H. & Asher, G. Crosstalk between metabolism and circadian clocks. *Nat. Rev. Mol. Cell Biol.* **20**, 227 (2019).
8. Scheiermann, C., Kunisaki, Y. & Frenette, P. S. Circadian control of the immune system. *Nat. Rev. Immunol.* **13**, 190–198 (2013).
9. Xie, Y. et al. New Insights Into the Circadian Rhythm and Its Related Diseases. *Front. Physiol.* **10**, 682 (2019).
10. Lin, Y.-M. et al. Disturbance of circadian gene expression in hepatocellular carcinoma. *Mol. Carcinog.* **47**, 925–933 (2008).
11. Stamenkovic, J. A. et al. Regulation of core clock genes in human islets. *Metabolism* **61**, 978–985 (2012).
12. Leibetseder, V. et al. Clock genes display rhythmic expression in human hearts. *Chronobiol. Int.* **26**, 621–636 (2009).
13. Wu, G. et al. Population-level rhythms in human skin with implications for circadian medicine. *Proc. Natl. Acad. Sci. U. S. A.* **115**, 12313–12318 (2018).
14. Brown, T. M. & Piggins, H. D. Electrophysiology of the suprachiasmatic circadian clock. *Prog. Neurobiol.* **82**, 229–255 (2007).
15. Challet, E. Minireview: Entrainment of the suprachiasmatic clockwork in diurnal and nocturnal mammals. *Endocrinology* **148**, 5648–5655 (2007).
16. Cajochen, C., Kräuchi, K. & Wirz-Justice, A. Role of melatonin in the regulation of human circadian rhythms and sleep. *J. Neuroendocrinol.* **15**, 432–437 (2003).
17. Mistlberger, R. E. & Skene, D. J. Social influences on mammalian circadian rhythms: animal and human studies. *Biol. Rev. Camb. Philos. Soc.* **79**, 533–556 (2004).
18. Mistlberger, R. E. & Skene, D. J. Nonphotic entrainment in humans? *J. Biol. Rhythms* **20**, 339–352 (2005).
19. Vitaterna, M. H., Takahashi, J. S. & Turek, F. W. Overview of circadian rhythms. *Alcohol Res. Health J. Natl. Inst. Alcohol Abuse Alcohol.* **25**, 85–93 (2001).
20. Partch, C. L., Green, C. B. & Takahashi, J. S. Molecular Architecture of the Mammalian Circadian Clock. *Trends Cell Biol.* **24**, 90–99 (2014).
21. Zhang, R., Lahens, N. F., Ballance, H. I., Hughes, M. E. & Hogenesch, J. B. A circadian gene expression atlas in mammals: implications for biology and medicine. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 16219–16224 (2014).

22. Shearman, L. P. et al. Interacting molecular loops in the mammalian circadian clock. *Science* **288**, 1013–1019 (2000).
23. Preitner, N. et al. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110**, 251–260 (2002).
24. Sato, T. K. et al. A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron* **43**, 527–537 (2004).
25. Pandi-Perumal, S. R. et al. Dim light melatonin onset (DLMO): a tool for the analysis of circadian phase in human sleep and chronobiological disorders. Prog. Neuropsychopharmacol. *Biol. Psychiatry* **31**, 1–11 (2007).
26. Saini, C., Brown, S. A. & Dibner, C. Human Peripheral Clocks: Applications for Studying Circadian Phenotypes in Physiology and Pathophysiology. *Front. Neurol.* **6**, (2015).
27. Du Pré, B. C. et al. SCA1+ Cells from the Heart Possess a Molecular Circadian Clock and Display Circadian Oscillations in Cellular Functions. *Stem Cell Rep.* **9**, 762–769 (2017).
28. Mazzoccoli, G. et al. Circadian transcriptome analysis in human fibroblasts from Hunter syndrome and impact of iduronate-2-sulfatase treatment. *BMC Med. Genomics* **6**, 37 (2013).
29. Dierickx, P. et al. Circadian networks in human embryonic stem cell-derived cardiomyocytes. *EMBO Rep.* **18**, 1199–1212 (2017).
30. Fang, M., Kang, H.-G., Park, Y., Estrella, B. & Zarbl, H. In Vitro Bioluminescence Assay to Characterize Circadian Rhythm in Mammary Epithelial Cells. *J. Vis. Exp. JoVE* (2017) doi:10.3791/55832.
31. Yoshikawa, A. et al. Establishment of human cell lines showing circadian rhythms of bioluminescence. *Neurosci. Lett.* **446**, 40–44 (2008).
32. Balsalobre, A., Damiola, F. & Schibler, U. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93**, 929–937 (1998).
33. Yagita, K. & Okamura, H. Forskolin induces circadian gene expression of rPer1, rPer2 and dbp in mammalian rat-1 fibroblasts. *FEBS Lett.* **465**, 79–82 (2000).
34. Lee, S. K. et al. Extracellular low pH affects circadian rhythm expression in human primary fibroblasts. *Biochem. Biophys. Res. Commun.* **416**, 337–342 (2011).
35. Noguchi, T., Wang, L. L. & Welsh, D. K. Fibroblast PER2 circadian rhythmicity depends on cell density. *J. Biol. Rhythms* **28**, 183–192 (2013).
36. Hasan, S. et al. Assessment of circadian rhythms in humans: comparison of real-time fibroblast reporter imaging with plasma melatonin. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **26**, 2414–2423 (2012).
37. Pagani, L. et al. The physiological period length of the human circadian clock in vivo is directly proportional to period in human fibroblasts. *PloS One* **5**, e13376 (2010).
38. Kohane, I. S. & Valtchinov, V. I. Quantifying the white blood cell transcriptome as an accessible window to the multiorgan transcriptome. *Bioinforma. Oxf. Engl.* **28**, 538–545 (2012).
39. Liew, C.-C., Ma, J., Tang, H.-C., Zheng, R. & Dempsey, A. A. The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool. *J. Lab. Clin. Med.* **147**, 126–132 (2006).
40. Archer, S. N. et al. Mistimed sleep disrupts circadian regulation of the human transcriptome. *Proc. Natl. Acad. Sci. U. S. A.* **111**, E682-691 (2014).

41. Möller-Levet, C. S. et al. Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome. *Proc. Natl. Acad. Sci. U. S. A.* **110**, E1132-1141 (2013).
42. Archer, S. N., Viola, A. U., Kyriakopoulou, V., von Schantz, M. & Dijk, D.-J. Inter-individual differences in habitual sleep timing and entrained phase of endogenous circadian rhythms of BMAL1, PER2 and PER3 mRNA in human leukocytes. *Sleep* **31**, 608–617 (2008).
43. Arnardottir, E. S. et al. Blood-gene expression reveals reduced circadian rhythmicity in individuals resistant to sleep deprivation. *Sleep* **37**, 1589–1600 (2014).
44. Lech, K. et al. Dissecting Daily and Circadian Expression Rhythms of Clock-Controlled Genes in Human Blood. *J. Biol. Rhythms* **31**, 68–81 (2016).
45. Reszka, E. et al. Circadian gene expression in peripheral blood leukocytes of rotating night shift nurses. Scand. J. Work. Environ. *Health* **39**, 187–194 (2013).
46. Takimoto, M. et al. Daily expression of clock genes in whole blood cells in healthy subjects and a patient with circadian rhythm sleep disorder. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **289**, R1273-1279 (2005).
47. Li, C.-X. et al. Altered melatonin secretion and circadian gene expression with increased proinflammatory cytokine expression in early-stage sepsis patients. *Mol. Med. Rep.* **7**, 1117–1122 (2013).
48. Burioka, N. et al. Clock gene dysfunction in patients with obstructive sleep apnoea syndrome. *Eur. Respir. J.* **32**, 105–112 (2008).
49. Yang, M.-Y. et al. Alternations of Circadian Clock Genes Expression and Oscillation in Obstructive Sleep Apnea. *J. Clin. Med.* **8**, 1634 (2019).
50. Ackermann, K. et al. Blue-Light Phase Shifts PER3 Gene Expression in Human Leukocytes. *Chronobiol. Int.* **26**, 769–779 (2009).
51. Jakubowicz, D. et al. Influences of Breakfast on Clock Gene Expression and Postprandial Glycemia in Healthy Individuals and Individuals With Diabetes: A Randomized Clinical Trial. *Diabetes Care* **40**, 1573–1579 (2017).
52. Haimovich, B. et al. In vivo endotoxin synchronizes and suppresses clock gene expression in human peripheral blood leukocytes. *Crit. Care Med.* **38**, 751–758 (2010).
53. Burioka, N. et al. Treatment with β_2-Adrenoceptor Agonist in Vivo Induces Human Clock Gene, Per1, mRNA Expression in Peripheral Blood. *Chronobiol. Int.* **24**, 183–189 (2007).
54. Balmforth, A. J. et al. Inter-subject differences in constitutive expression levels of the clock gene in man. *Diab. Vasc. Dis. Res.* **4**, 39–43 (2007).
55. Kaneshiro, K. et al. Expressions of circadian clock genes represent disease activities of RA patients treated with biological DMARDs. *Mod. Rheumatol.* **30**, 293–300 (2020).
56. Ando, H. et al. Influence of age on clock gene expression in peripheral blood cells of healthy women. J. Gerontol. *A. Biol. Sci. Med. Sci.* **65**, 9–13 (2010).
57. Grievink, H. W., Luisman, T., Kluft, C., Moerland, M. & Malone, K. E. Comparison of Three Isolation Techniques for Human Peripheral Blood Mononuclear Cells: Cell Recovery and Viability, Population Composition, and Cell Functionality. *Biopreservation Biobanking* **14**, 410–415 (2016).
58. Boivin, D. B. et al. Circadian clock genes oscillate in human peripheral blood mononuclear cells. *Blood* **102**, 4143–4145 (2003).
59. Takata, M. et al. Daily expression of mRNAs for the mammalian Clock genes Per2 and clock in mouse suprachiasmatic nuclei and liver and human peripheral blood

mononuclear cells. *Jpn. J. Pharmacol.* **90**, 263–269 (2002).
60. Kusanagi, H. et al. Similar profiles in human period1 gene expression in peripheral mononuclear and polymorphonuclear cells. *Neurosci. Lett.* **365**, 124–127 (2004).
61. James, F. O., Cermakian, N. & Boivin, D. B. Circadian rhythms of melatonin, cortisol, and clock gene expression during simulated night shift work. *Sleep* **30**, 1427–1436 (2007).
62. James, F. O., Boivin, D. B., Charbonneau, S., Bélanger, V. & Cermakian, N. Expression of clock genes in human peripheral blood mononuclear cells throughout the sleep/wake and circadian cycles. *Chronobiol. Int.* **24**, 1009–1034 (2007).
63. Ando, H. et al. Clock gene expression in peripheral leucocytes of patients with type 2 diabetes. *Diabetologia* **52**, 329–335 (2009).
64. Yang, M.-Y. et al. Altered expression of circadian clock genes in human chronic myeloid leukemia. *J. Biol. Rhythms* **26**, 136–148 (2011).
65. Kusanagi, H. et al. Expression profiles of 10 circadian clock genes in human peripheral blood mononuclear cells. *Neurosci. Res.* **61**, 136–142 (2008).
66. Hida, A. et al. Expression profiles of PERIOD1, 2, and 3 in peripheral blood mononuclear cells from older subjects. *Life Sci.* **84**, 33–37 (2009).
67. Watanabe, M. et al. Rhythmic expression of circadian clock genes in human leukocytes and beard hair follicle cells. *Biochem. Biophys. Res. Commun.* **425**, 902–907 (2012).
68. Chen, A., Du, L., Xu, Y., Chen, L. & Wu, Y. The effect of blue light exposure on the expression of circadian genes: bmal1 and cryptochrome 1 in peripheral blood mononuclear cells of jaundiced neonates. *Pediatr. Res.* **58**, 1180–1184 (2005).
69. Chen, A, Du, J & Du, LZ. The Effect of Non-nutritive Sucking on Expression of BMAL1 and CRY1 in Peripheral Blood Mononuclear Cells of Preterm Neonates: A Preliminary Study. *HK J Paediatr (New Series)* **19**, 233–239 (2014).
70. Swanson, G. R. et al. Night workers with circadian misalignment are susceptible to alcohol-induced intestinal hyperpermeability with social drinking. *Am. J. Physiol. Gastrointest. Liver Physiol.* **311**, G192-201 (2016).
71. Moreira, S. et al. Changes in Expression of the CLOCK Gene in Obstructive Sleep Apnea Syndrome Patients Are Not Reverted by Continuous Positive Airway Pressure Treatment. *Front. Med.* **4**, 187 (2017).
72. Whitney, A. R. et al. Individuality and variation in gene expression patterns in human blood. *Proc. Natl. Acad. Sci.* **100**, 1896–1901 (2003).
73. Teboul, M. et al. Atypical patterns of circadian clock gene expression in human peripheral blood mononuclear cells. *J. Mol. Med.* **83**, 693–699 (2005).
74. Savelyeva, A. V. et al. Variety of RNAs in Peripheral Blood Cells, Plasma, and Plasma Fractions. *BioMed Res. Int.* **2017**, 7404912 (2017).
75. Gautam, A. et al. Investigating gene expression profiles of whole blood and peripheral blood mononuclear cells using multiple collection and processing methods. *PLOS ONE* **14**, e0225137 (2019).
76. Akashi, M. et al. Noninvasive method for assessing the human circadian clock using hair follicle cells. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 15643–15648 (2010).
77. Crnko, S. et al. Prognostic biomarker soluble ST2 exhibits diurnal variation in chronic heart failure patients. *ESC Heart Fail.* **7**, 1224–1233 (2020).
78. Bjarnason, G. A. et al. Circadian expression of clock genes in human oral mucosa and skin: association with specific cell-cycle phases. *Am. J. Pathol.* **158**, 1793–1801 (2001).

79. Cajochen, C. et al. Evening exposure to blue light stimulates the expression of the clock gene PER2 in humans. *Eur. J. Neurosci.* **23**, 1082–1086 (2006).
80. Bjarnason, G. A., Jordan, R. C. & Sothern, R. B. Circadian variation in the expression of cell-cycle proteins in human oral epithelium. *Am. J. Pathol.* **154**, 613–622 (1999).
81. Zieker, D. et al. Circadian expression of clock- and tumor suppressor genes in human oral mucosa. Cell. Physiol. Biochem. *Int. J. Exp. Cell. Physiol. Biochem. Pharmacol.* **26**, 155–166 (2010).
82. Jud, C. et al. Age-dependent alterations in human PER2 levels after early morning blue light exposure. *Chronobiol. Int.* **26**, 1462–1469 (2009).
83. Nováková, M., Nevsímalová, S., Príhodová, I., Sládek, M. & Sumová, A. Alteration of the circadian clock in children with Smith-Magenis syndrome. *J. Clin. Endocrinol. Metab.* **97**, E312-318 (2012).
84. Nováková, M., Sládek, M. & Sumová, A. Human chronotype is determined in bodily cells under real-life conditions. *Chronobiol. Int.* **30**, 607–617 (2013).
85. Weissová, K., Škrabalová, J., Skálová, K., Bendová, Z. & Kopřivová, J. The Effect of a Common Daily Schedule on Human Circadian Rhythms During the Polar Day in Svalbard: A Field Study. *J. Circadian Rhythms* **17**, 9 (2019).
86. Koshy, A., Cuesta, M., Boudreau, P., Cermakian, N. & Boivin, D. B. Disruption of central and peripheral circadian clocks in police officers working at night. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **33**, 6789–6800 (2019).
87. Lyons, A. B., Moy, L., Moy, R. & Tung, R. Circadian Rhythm and the Skin: A Review of the Literature. *J. Clin. Aesthetic Dermatol.* **12**, 42–45 (2019).
88. Al-Nuaimi, Y. et al. A meeting of two chronobiological systems: circadian proteins Period1 and BMAL1 modulate the human hair cycle clock. *J. Invest. Dermatol.* **134**, 610–619 (2014).
89. Lin, K. K. et al. Circadian clock genes contribute to the regulation of hair follicle cycling. *PLoS Genet.* **5**, e1000573 (2009).
90. Ferrante, A. et al. Diurnal Preference Predicts Phase Differences in Expression of Human Peripheral Circadian Clock Genes. *J. Circadian Rhythms* **13**, 4 (2015).
91. Ingram, K. K. et al. Molecular insights into chronotype and time-of-day effects on decision-making. *Sci. Rep.* **6**, 29392 (2016).
92. Li, H. et al. Endogenous circadian time genes expressions in the liver of mice under constant darkness. *BMC Genomics* **21**, 224 (2020).
93. Mure, L. S. et al. Diurnal transcriptome atlas of a primate across major neural and peripheral tissues. *Science* **359**, eaao0318 (2018).
94. Panda, S. et al. Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* **109**, 307–320 (2002).
95. Storch, K.-F. et al. Extensive and divergent circadian gene expression in liver and heart. *Nature* **417**, 78–83 (2002).
96. Kondratova, A. A. & Kondratov, R. V. The circadian clock and pathology of the ageing brain. *Nat. Rev. Neurosci.* **13**, 325–335 (2012).
97. Serin, Y. & Acar Tek, N. Effect of Circadian Rhythm on Metabolic Processes and the Regulation of Energy Balance. *Ann. Nutr. Metab.* **74**, 322–330 (2019).
98. Shostak, A. Circadian Clock, Cell Division, and Cancer: From Molecules to Organism. *Int. J. Mol. Sci.* **18**, 873 (2017).
99. Roenneberg, T. & Merrow, M. The Circadian Clock and Human Health. *Curr. Biol. CB* **26**, R432-443 (2016).

100. Vyas, M. V. et al. Shift work and vascular events: systematic review and meta-analysis. *BMJ* **345**, e4800 (2012).
101. Wong, R. et al. Use of RT-PCR and DNA microarrays to characterize RNA recovered by non-invasive tape harvesting of normal and inflamed skin. *J. Invest. Dermatol.* **123**, 159–167 (2004).
102. Noh, J.-Y. et al. Circadian rhythms in urinary functions: possible roles of circadian clocks? *Int. Neurourol. J.* **15**, 64–73 (2011).
103. Song, Y. et al. Gene Expression Profiles Identified Novel Urine Biomarkers for Diagnosis and Prognosis of High-Grade Bladder Urothelial Carcinoma. *Front. Oncol.* **10**, 394 (2020).
104. Tomita, T., Mori, T. & Onishi, Y. β-Arrestin 1 (ARRB1) serves as a molecular marker of the peripheral circadian rhythm. *Int. J. Oral Sci.* **11**, 32 (2019).
105. Sharp, J. A., Lefèvre, C., Watt, A. & Nicholas, K. R. Analysis of human breast milk cells: gene expression profiles during pregnancy, lactation, involution, and mastitic infection. *Funct. Integr. Genomics* **16**, 297–321 (2016).
106. Twigger, A.-J. et al. Gene expression in breastmilk cells is associated with maternal and infant characteristics. *Sci. Rep.* **5**, 12933 (2015).
107. Anafi, R. C., Francey, L. J., Hogenesch, J. B. & Kim, J. CYCLOPS reveals human transcriptional rhythms in health and disease. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 5312–5317 (2017).
108. Braun, R. et al. Universal method for robust detection of circadian state from gene expression. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E9247–E9256 (2018).
109. Laing, E. E. et al. Blood transcriptome based biomarkers for human circadian phase. *eLife* **6**, e20214 (2017).
110. Wittenbrink, N. et al. High-accuracy determination of internal circadian time from a single blood sample. *J. Clin. Invest.* **128**, 3826–3839 (2018).
111. Hughey, J. J. & Butte, A. J. Differential Phasing between Circadian Clocks in the Brain and Peripheral Organs in Humans. *J. Biol. Rhythms* **31**, 588–597 (2016).
112. Nicolaides, N. C. & Chrousos, G. P. Sex differences in circadian endocrine rhythms: Clinical implications. *Eur. J. Neurosci.* **52**, 2575–2585 (2020).
113. Martinez-Nicolas, A. et al. Assessing Chronotypes by Ambulatory Circadian Monitoring. *Front. Physiol.* **10**, 1396 (2019).
114. Beaulé, C., Granados-Fuentes, D., Marpegan, L. & Herzog, E. D. In vitro circadian rhythms: imaging and electrophysiology. *Essays Biochem.* **49**, 103–117 (2011).
115. Abbott, S. M., Malkani, R. G. & Zee, P. C. Circadian disruption and human health: A bidirectional relationship. *Eur. J. Neurosci.* **51**, 567–583 (2020).
116. Panda, S. The arrival of circadian medicine. *Nat. Rev. Endocrinol.* **15**, 67–69 (2019).

Supporting information

Methods

Study design and participants

The study was conducted according to the principles of the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO). It was approved by the Medical Ethics Committee of University Medical Centre Utrecht (Study Number 14/471). Upon admission to the University Medical Centre Utrecht, study participants provided written informed consent after which the blood withdrawal began.

Laboratory measurements

At seven subsequent time points (9 AM, 1 PM, 5 PM, 9 PM, 1 AM, 5 AM, 9 AM), PAXGene blood RNA tubes (PreAnalytix) were used to collect blood from an intravenous cannula. After all samples from all subjects had been collected, RNA was isolated using the PAXGene blood RNA kit (IVD, PreAnalytix) and 500 ng was used to synthesize cDNA (qScript cDNA synthesis kit, QuantaBio). High-throughput real-time qPCR (Fluidigm, BioMark™ Systems) was used according to the manufacturer's protocol to analyse gene expression profiles:

PPIA
forward TCTGGTTCCTTCTGCGTGAA
reverse CCAGGGAATACGTAACCAGACA)
ARNTL
forward GACGATGAATTGAAACACCTCA
reverse GAGTATCTTCCCTCGGTCAC
CLOCK
forward GCCAGTGATGTCTCAAGCTA
reverse CAGATGTTGCATGGCTCCTA
CRY2
forward CCAGCCGGCTTAACATTGAA
reverse GGAAGGGACAGATGCCAGTA)
PER2
forward GCCTGATGATGGCAAAATCTGAA
reverse GTGTGTGTCCACTTTCGAAGAC.

Statistical analysis

Differences between the baseline characteristics of patients and controls were

compared by using Mann–Whitney U test for non-normal distributions and independent Student's t test for normal distributions. A cosinor analysis was used to determine rhythmicity of 24-hour clock genes concentrations. P<0.05 was considered significant.

Table S1 | **Baseline characteristics of study participants**

	Characteristics	**Patients (n = 46)**	**Controls (n = 24)**	***P* value**
	Age (years)	60 (14)	56 (16)	0.207
	Male sex (%)	71.7	70.8	1.000
	BMI (kg/m²)	25.5 ± 4.6	24.9 ± 3.5	0.566
	CKD-EPI GFR (mL/ min/1.73 m²)	68.0 (39.8)	65.5 (22.0)	0.981
Comorbidities (%)	Diabetes mellitus	21.7	0.0	0.012*
	Atrial flutter/fibrillation	30.4	0.0	0.001*
Severity of heart failure (%)	NYHA class II	32.6	NA	
	NYHA class III	67.4	NA	
	Ejection fraction (%)	22.7 ± 8.0	NA	

Values are mean ± standard deviation, median (interquartile range (IQR) or percentage. Differences between the baseline characteristics of the patient and control group were compared by using the independent two-sample T-test for normal distributions and the Mann-Whitney U test for non-normal distributions (**P* value <0.05). BMI, body mass index; CKD-EPI GFR, the estimated glomerular filtration rate calculated with the Chronic Kidney Disease Epidemiology Collaboration equation; NYHA class, New York Heart Association functional classification of heart failure severity.

4

CHAPTER

The Circadian Clock Remains Intact, but with Dampened Hormonal Output in Heart Failure

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Abstract

Aim. Circadian (24-hour) rhythms are important regulators in cardiovascular physiology and disease. Various neurohormones relevant in heart failure (HF) are under influence of circadian rhythms, potentially interacting with each other. Whether HF affects the functionality of the circadian clock remains unknown. Therefore, we sought to characterize circadian rhythmicity in HF patients as reflected by the rhythmic expression of main endocrine products of the central clock: melatonin and cortisol.

Methods and Results. In this study, 46 patients with HF with reduced ejection fraction and 24 age- and sex-matched controls were included. Blood was collected at seven subsequent time-points during a 24-hour period (320 HF and 167 control samples in total), and melatonin, cortisol, and cardiac troponin T were measured from serum.

Next, we analyzed the functionality of the peripheral circadian clock in animal models of HF: nocturnal mice and diurnal zebrafish based on oscillatory expression of core clock genes *Bmal*, *Per*, *Cry* and *Clock* in heart, kidneys, and liver.

Melatonin and cortisol concentrations followed a diametrically opposite 24-hour pattern, as expected. Maximum melatonin values clustered at nighttime (modal value: 5 AM, N=70), with no difference between patients and controls (p=0.770). However, diurnal melatonin variation ([maximum]/[minimum]) was lower in HF patients than controls: median 27.5 versus 47.8 (p=0.003). Similarly, diurnal cortisol variation was lower in HF patients: median 3.9 versus 6.3 (p=0.006). The physiological nocturnal blood pressure dip was absent in 77.8% of HF patients, and physiological nocturnal heart rate dip was also absent in 77.8%.

No differences between clock gene expression profiles were found in animal HF models and control groups in all organs, pointing to preserved peripheral clock functionality in HF. Concordantly, serum cardiac troponin T concentrations in HF patients revealed significant cosine shaped 24-hour oscillations.

Conclusions. Central clock output is dampened in HF patients, corresponding with a non-dipping blood pressure and heart rate pattern, while the molecular peripheral clock remains intact. This emphasizes the importance of taking timing into account in research and therapy for HF, and sets the stage for a novel dimension of diagnostic, prognostic and therapeutic approaches.

Keywords: Human Heart Failure, Circadian Rhythms, Biological Clock, Zebrafish, Mouse, Melatonin, Cortisol

Introduction

Molecular clocks that control circadian (24-hour) rhythms are increasingly seen as important regulators in cardiovascular physiology and disease.[1] They can be divided into a central clock located in the suprachiasmatic nucleus of the brain and peripheral clocks found in almost every tissue. The central clock synchronizes peripheral clocks via various neurohumoral signals (e.g. cortisol and melatonin) while, in addition to central clock signaling, peripheral clocks respond to tissue-specific synchronizers such as food intake and exercise. Being present in each of the cardiovascular cell types, the molecular clock influences many myocardial processes, such as heart rate, metabolism, cellular responsiveness to stimuli, signaling, contractility, and heart growth and regeneration.[2,3] Disruption of the molecular clock has been linked with the development and incidence of cardiovascular diseases. For example, acute myocardial infarction[4] and arrhythmias[5] peak in the morning, and a greater risk of cardiac damage and major cardiac events has been described after morning heart surgery, in comparison to afternoon surgery.[6]

Several observations suggest a possible connection between the circadian clock and heart failure (HF): relevant neurohormones such as glucocorticoids, catecholamines, atrial natriuretic factor, angiotensin II, aldosterone, and renin, display day/night rhythms at least in healthy subjects.[7] Furthermore, patients with HF often suffer from insomnia and, vice versa, insomnia has been found to increase the risk of incident HF.[8] Using murine models, Duong *et al.* showed that the circadian mechanism plays an important role in neurobiology of the healthy cognitive system.[9] HF changes neuron morphology and function, potentially causing the neurocognitive impairments observed in HF patients, and the loss of the circadian mechanism alters neurobiological gene adaptations to HF. Whether HF affects the functionality of the circadian clock remains unknown, but would be plausible given the massive neurohormonal activation in HF.[10] On the other hand, the circadian clock is a very strong evolutionarily conserved mechanism that may receive priority in remaining intact during the course of a disease.

Circadian rhythms have also proven relevant in timing of the administration of therapies.[11] The risk-to-benefit ratio of angiotensin-converting enzyme (ACE)-inhibition varies in a diurnal fashion at least in hypertensive patients.[12] Diurnal oscillations of pharmacokinetics/dynamics, receptor expression on target organs, and dynamic intrinsic cellular function, may all affect the efficacy and side effects of drugs.[13] In addition, non-pharmacological HF interventions such as exercise, diet and pacemaker/implantable cardioverter-defibrillator (ICD) therapy may vary in their

effectiveness and optimal settings according to the time of day. Finally, it should be noted that HF therapy does not only affect signaling pathways in the heart, but also influences other organs such as the kidneys, which are regulated by the circadian clock as well.[14]

Although it is increasingly recognized that circadian rhythms are important in cardiovascular health and disease, their involvement in HF genesis, progression and treatment is yet unknown. Given the potential implications for tailoring standard therapy and opportunities to develop new therapies targeting components of the circadian systems, it is important to establish whether the circadian clock is impaired following HF.

Here, we take a translational approach to characterize circadian rhythmicity in human HF patients, and provide insight into molecular peripheral clock function of multiple HF-related organs in two independent animal models of HF.

Methods

The clinical study complies with the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and the Medical Research Involving Human Subjects Act (WMO). It was approved by the medical ethical committee of University Medical Center (UMC) Utrecht (study number 14/471). All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, with prior approval by the local Animal Ethical Experimentation Committee (Utrecht University: mice; Luxembourg Institute of Health: zebrafish). Upon reasonable request, the data of this study are available to other researchers.

Detailed description of the methods, including inclusion and exclusion criteria, animal models primer sequences (**Table S1**) and statistical analysis can be found in **Supplemental Methods**.

Study overview

This study characterized circadian rhythmicity in HF patients as reflected by the rhythmic expression of main endocrine products of the central clock: melatonin and cortisol. Since the human heart is not accessible for repeated sampling needed to reliably determine the circadian phase of the subject, we utilized 24-hour cardiac troponin T (cTnT) serum concentration as a cardiac-specific surrogate clock marker.[15,16] Additionally, we analyzed the functionality of the peripheral circadian clock directly in the hearts of nocturnal mice and diurnal zebrafish with HF, to further confirm our findings in humans. Finally, we corroborated the conservation of circadian rhythmicity in other target

organs in HF: kidneys and liver (**Figure S1**).

Study design: human heart failure

Participant selection. In this observational case-control study, two subject groups were included: HF with reduced ejection fraction (HFrEF) patients (NYHA II/III) and age- and sex-matched healthy individuals (UMC Utrecht, 2015-2019). All subjects signed informed consent.

Laboratory measurements. At seven predefined time-points (9 AM, 1 PM, 5 PM, 9 PM, 1 AM, 5 AM, 9 AM), blood was collected from an intravenous cannula, totaling 320 samples for HF patients and 167 for controls. Serum melatonin and cortisol concentrations were measured using liquid chromatography in combination with isotope dilution tandem mass spectrometry (LC-MS/MS),[17] and serum cTnT concentration with Troponin T-high sensitive immunoassay (Roche Diagnostics, Indianapolis; 99th percentile of 14 ng/L).

Questionnaires. The subjects completed the VOA-questionnaire (Vragenlijst Ochtend/Avond typering, translation: questionnaire morning/evening chronotype), used to determine their chronotype.[18] The Epworth Sleepiness Scale (ESS, provided by courtesy of Russcher *et al.* (slightly adjusted)[19]) was used for determining the level of daytime sleepiness and subjective sleep quality.

Activity measurements. Following hospitalization, subjects were instructed to wear a wrist actometer (Philips Respironics Actiwatch 2) for five days in total, while maintaining the usual daily sleep and activity patterns. Peak activity, mean daily activity, total sleep time, sleep latency, wake after sleep onset, sleep efficiency were used to quantify sleep and activity patterns.

24-h ambulatory blood pressure and heart rate measurements. In the HFrEF patient group, 24-hour blood pressure (BP) measurements were obtained, as a complementary approach to unveil circadian clock functionality. BP (mmHg) and heart rate (HR) measurements in beats per minute (BPM), were obtained every 30 minutes for 24 hours, to calculate mean systolic BP (SBP), diastolic BP (DBP), diurnal SBP, diurnal DBP, nocturnal SBP, nocturnal DBP, and nocturnal fall in SBP and DBP, diurnal mean arterial pressure (MAP), nocturnal MAP, nocturnal fall in MAP. The BP dip was calculated by dividing the nocturnal fall in SBP by the diurnal SBP and categorized as follows: ≥10%=dipper pattern, 0-10%=non-dipper pattern, <0%=riser pattern.[20]

Mean diurnal and nocturnal HR, and nocturnal percentual fall in HR, were calculated and categorized as follows: <10%=absent dip, ≥10%=present dip.

Study design: mouse heart failure

Mouse HF model and experimental setup. Male C57BL/6 mice (Jackson), aged 10 – 12 weeks were housed under controlled conditions in a 12-h light/12-h dark cycle (lights on at zeitgeber 0 (ZT0), lights off at ZT12). Water and food were provided *ad libitum*. Myocardial infarction (MI) was induced by ligation of the left coronary artery, as described previously.[21]

On day 28, on each time-point during a period of 24 hours (7 AM, 10 AM, 1:30 PM, 5 PM, 8:30 PM, 12 AM, 3:30 AM), randomly chosen HF and sham mice were sacrificed for tissue collection. The first time point of tissue sampling was carried out at 7 AM and represents ZT1 (one hour after lights were turned on). After termination, heart, kidneys, and liver were snap frozen for later analysis by quantitative real-time polymerase chain reaction (qPCR).

Study design: zebrafish heart failure

Zebrafish HF model and experimental setup. Wild-type AB zebrafish male adults aged 8 – 10 months were maintained in a strict 14/10-hour light/dark cycle environment (ZT0=lights on, ZT14=lights off) at 28°C on a daily basis. Animals were fed to satiety twice daily with dry food. After one week of acclimatization, treatment with phenylhydrazine hydrochloride (PHZ, Sigma-Aldrich) was started to generate HF phenotype during five weeks, as previously described.[22]

The hearts of PHZ-treated and control zebrafish were harvested every four hours over a 24-hour period (8:30 AM, 12:30 PM, 4:30 PM, 8:30 PM, 12:30 AM, 4:30 AM) at the end of the five-week phase. The first time point of tissue sampling was carried out at 8:30 AM (ZT0; time-point when lights were turned on). At each time-point, heart and kidneys were snap frozen and analyzed by qPCR reaction.

Statistical analysis

For the clinical case-control study, all outcome parameters were continuous. In both groups, diurnal melatonin fluctuations were assessed using the Friedman test. Cosinor analysis was used to determine rhythmicity of 24-hour cortisol concentrations.[23]

Additional quantitative analyses were used to assess 24-hour melatonin and cortisol variation: maximum, minimum, diurnal variation (maximum/minimum; **Figure 1A**). Melatonin values for single measurements below the

detection limit of 8 pmol/L were assigned the value of 4 pmol/L. Subjects with no detectable melatonin in all samples (n=3) were excluded for the diurnal variation analysis.[24]

Normality was checked using QQ plots and the Kolmogorov-Smirnov test. Baseline characteristics and circadian parameters were compared between patients and controls using the independent samples T-test and the Mann-Whitney U test, as appropriate. Univariable linear and logistic regression were performed to assess an association between melatonin and cortisol levels and the presence of HF. Correction for predefined confounders was performed using multivariable linear regression analysis, correcting for sex, age, body mass index (BMI), kidney function, smoking history and alcohol consumption.

An independent samples T-test for normal distributions and the Mann-Whitney U test for non-normal distributions were used to test differences between delta Ct values of each ZT of control and HF animal group. A cosinor analysis was performed to determine a significant rhythmicity of normalized cTnT data and delta Ct values.[23]

Results

Human heart failure: Hormonal clock output is conserved in HF, with dampened oscillatory pattern

Study population characteristics. A total of 70 subjects were enrolled in the study: 46 HFrEF patients, with a left ventricular ejection fraction (LVEF) of <40% by echocardiography, and 24 controls which were matched for age and sex (**Table 1** and **Supplemental Methods**).

71.7% of patients and 70.8% of controls were male, with a median age of 60 and 56 years, respectively. Renal function was equal between groups and all patients were in New York Heart Association (NYHA) class II (32.6%) or III (67.4%).

24-hour melatonin rhythmicity remains intact, but is dampened, in heart failure. The rhythmic release of melatonin is under direct regulation of the central clock, thus reflecting any changes in its functionality. Both patients and controls showed diurnal variability of melatonin concentration (Friedman test $p<0.001$ for both groups; **Figure 1B**). Almost all subjects (n=67) showed intact serum melatonin rhythmicity, according to the established pattern of low values during the day and an increase at nighttime. In 23.9% of subjects, peak melatonin values were measured at 1 AM, and in 76.1% of subjects at 5 AM. No significant differences in peak time were found between the groups ($p=0.770$). Subjects (two HF patients, one control) with all melatonin values

Table 1 | **Baseline characteristics**

	Characteristics	Patients (n = 46)	Controls (n = 24)	*P* value
	Male sex (%)	71.7	70.8	1.000
	Age (years)	60 (14)	56 (16)	0.207
	BMI (kg/m^2)	25.5 ± 4.6	24.9 ± 3.5	0.566
	CKD-EPI GFR (mL/min/1.73 m^2)	68.0 (39.8)	65.5 (22.0)	0.981
Medical history	Smoking (PY)	2 (12.5)	0 (4.5)	0.041*
	Alcohol (IU/week)	0 (4)	7 (10)	0.056
	Diabetes mellitus (%)	21.7	0.0	0.012*
	Atrial flutter/fibrillation (%)	30.4	0.0	0.001*
Medication (%)	ACEi	58.7	16.7	0.001*
	ARB	26.1	8.3	0.116
	Beta-blocker	45.7	4.2	<0.001*
	MRA	80.4	0.0	<0.001*
	Amiodarone	26.1	0.0	0.006*
	#Benzodiazepine	8.7	0.0	0.291
	PPI	19.6	12.5	0.526
Heart failure characterization (%)	NYHA II	32.6	NA	NA
	NYHA III	67.4	NA	NA
	LVEF	22.7 ± 8.0	NA	NA
	iCMP	43.5	NA	NA

Values are mean ± standard deviation, median (interquartile range (IQR)) or percentage of total. Differences between the baseline characteristics of the patient and control group were compared by using the independent two-sample T-test for normal distributions and the Mann-Whitney U test for non-normal distributions (*P value<0.05). ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BMI, body mass index in kg/m^2; eGFR, estimated glomerular filtration rate in mL/minute/1.73m^2 based on the CKD-EPI equation[49]; iCMP, ischemic cardiomyopathy; IU, international units; LVEF, left ventricular ejection fraction; MRA, mineralocorticoid receptor antagonist; NA, not applicable; NYHA, New York Heart Association functional classification[50]; PPI, proton pump inhibitor; PY, packyears (one year of smoking 1 pack of cigarettes a day). #Benzodiazepine medication was usually used by 8.7% of the patients, however not on the day of, and one day prior to, study interventions.

under the detection limit were excluded from statistical analysis of the diurnal variation over the day.[24] Maximum melatonin values were not significantly different (187.1 (IQR=219.1) pmol/L in controls vs. 126.4 (IQR=261.5) pmol/L in patients, (p=0.207)). However, minimum values were significantly lower in controls than in patients (p=0.022), and only 80.4% of patients reached a minimum value of <8pmol/L while all controls did. Strikingly, diurnal melatonin variation was significantly reduced in HF: 27.5 (IQR=40.5) for patients and 47.8 (IQR=48.6) for controls (p=0.003), indicating the dampened output of the central circadian clock in the HF patients (**Table 2**).

After correction for confounders, diurnal melatonin variation remained lower in patients than in controls (**Table S2**). Furthermore, in these multivariable analyses higher BMI was associated with lower maximum levels (p=0.005) and diurnal melatonin variation (p=0.004) (**Figure S2**). Univariable logistic regression and multivariable linear regression analyses showed no significant effect of HF on minimum melatonin level and maximum melatonin level. After addition of the possible interaction terms (sex, age, BMI) to the linear regression models, no effect modifier was identified for the relationship between HF and melatonin values ($0.064 \leq p \leq 0.711$). Thus, the dampening in melatonin oscillation in HF was present regardless of sex, age and BMI.

24-hour cortisol rhythmicity remains intact, but is dampened, in heart failure. Cortisol represents another hormone which directly reflects the central clock output. All subjects showed intact serum cortisol rhythmicity, following a pattern of decreasing values in the afternoon and night, and increasing values in the morning (p=0.005 for patients and p=0.011 for controls, **Figure 1C**). Minimum cortisol values were predominantly seen at 1 AM (64.3% of subjects), followed by 9 PM (31.4%) and 5 AM (4.3%). Distribution of trough times was similar in patients and controls. Minimum cortisol values were higher in patients than in controls, with median values of 132.4 (IQR=130.4) nmol/L vs. 77.1 (IQR=66.8) nmol/L (p=0.004), respectively. However, maximum values were similar in both groups with 538.5 (IQR=152.5) nmol/L for patients and 497.0 (IQR=153.9) nmol/L for controls (p=0.251). Similar to melatonin, diurnal cortisol variation was reduced in HF patients' median compared to controls (diurnal variation 3.9 (IQR=2.9) vs. 6.3 (IQR=3.9), respectively (p=0.006)), further confirming the dampened output of the central circadian clock in the HF patients (**Table 2**).

After correction for confounders, diurnal cortisol variation remained lower in patients than in controls. Lower eGFR was associated with higher minimum levels (p=0.048), and lower diurnal cortisol variation (p=0.023). Higher alcohol consumption was associated with higher maximum cortisol

levels (p=0.048) (**Table S3**). Addition of the interaction terms sex, age and BMI, resulted in the identification of BMI as a possible effect modifier for the relationship between HF and maximum cortisol values (B coefficient=19.07, p=0.028; **Figure S3**). For both sexes and for subjects of different age, the relationship between HF and cortisol did not vary ($0.071 \leq p \leq 0.919$).

24-h blood pressure and heart rate measurements. Both HR and BP are known to portray diurnally varying patterns, which are controlled by the circadian clock.[25] A 24-hour BP profile was acquired for 36/46 patients at median 25 days (IQR=33) after blood sampling. 10/46 patients did not participate due to patient decision (n=4), LVAD implantation (n=4) and death (n=2). The mean overall BP was 102/62 mmHg. The mean diurnal BP was 104/65 mmHg, and the mean nocturnal BP was 98/59 mmHg. Only eight patients (22.2%) had a dipper pattern, while 21 (58.3%) had a non-dipper pattern and seven (19.4%) had a riser pattern.

The mean diurnal HR of patients was 75 BPM and the mean nocturnal HR was 71 BPM. The median percentual nocturnal HR fall was 4.5% (IQR=10.4%). A nocturnal HR dip was found in eight (22.2%) patients, whereas 28 (77.8%) showed a non-dipping pattern.

Actigraphy. Since sleep-wake cycles and physical activity are interrelated with circadian rhythms, we sought to determine if changes in central clock output reflect on this aspect as well. Both peak and mean activity values were lower in

Table 2 | **Melatonin and cortisol**

		Patients		Controls		
		Median	**IQR**	**Median**	**IQR**	***P* value**
Melatonin	Minimum	4.0	0.0	4.0	0.0	0.022*
pmol/L	Maximum	126.4	261.5	187.1	219.1	0.207
	Diurnal variation	27.5	40.5	47.8	48.6	0.003*
Cortisol	Minimum	132.4	130.4	77.1	66.8	0.004*
nmol/L	Maximum	538.5	152.2	497	153.9	0.251
	Diurnal variation	3.9	2.9	6.3	3.9	0.006*

Median diurnal melatonin and cortisol levels in HF patients and controls. Minimum and maximum are the lowest and highest values determined during the 24-hour blood sampling. Diurnal variation is calculated by dividing the maximum by the minimum. Differences between patient and control groups were compared by using the independent two-sample T-test for normal distributions and the Mann-Whitney U test for non-normal distributions (*P value<0.05). For all values n=46 for patients and n=24 for controls, except diurnal variation of melatonin, for which n=44 for patients and n=23 for controls. Medians of minimum melatonin are equal in both groups; however, controls had a significantly lower mean rank. IQR, interquartile range.

patients than in controls (**Table 3**). Additionally, patients had a significantly longer total sleeping time than controls (mean difference of 49 minutes, p=0.005), but sleep interruption, latency and efficiency were not significantly different (**Table 3**).

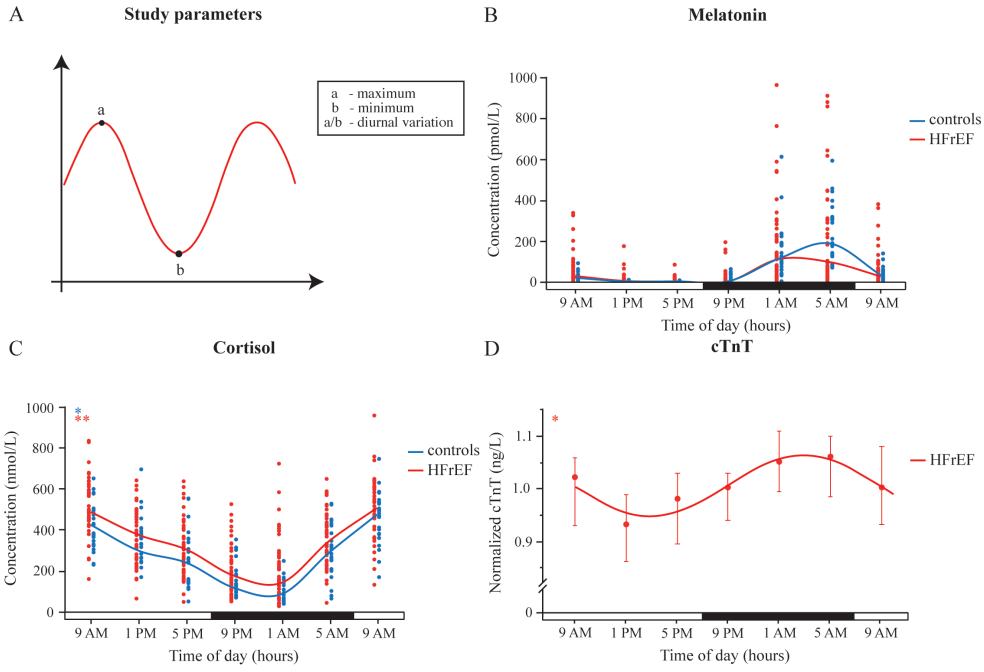


Figure 1 | **24-hour hormonal output is intact in heart failure patients. (A)** Parameters used for quantitative analysis of the 24-hour variation of melatonin and cortisol: maximum concentration (a), minimum concentration (b), diurnal variation (a/b). 24-hour **(B)** melatonin (pmol/L) and **(C)** cortisol (nmol/L) concentration. Each dot (red=patients, blue=controls) represents individual concentration at given time-point. Lines indicate median values of all patients (red; n=46) and controls (blue; n=24). **(D)** Cosinor analysis of normalized cTnT (ng/L) in HFrEF patients (n=43-45 per each time-point), in a period of 24 hours. Per subject, each time-point was normalized against its mean cTnT value of the entire day. Red line represents a fitted cosine curve. Values are median ± interquartile range (IQR). $P<0.05$ was used as a cut-off for significance. Significance of cosinor analysis for cTnT is indicated at the top left graph corner: *$P<0.05$. Horizontal bar indicates day (=white) and night (=black). cTnT, cardiac troponin T; HFrEF, heart failure with reduced ejection fraction.

Self-reported sleep quality declines in heart failure. The ESS questionnaire was used for the assessment of daytime sleepiness. A total of 11.4% of patients experienced excessive daytime sleepiness, compared to none of the control

subjects (p=0.153). According to the VOA questionnaire, 46.5% of the patients classified as morning chronotype, while 51.2% were neither evening nor morning chronotype. This was similar to the controls, with 45.8% morning and 45.8% neutral chronotypes. The evening person chronotype was scarcely represented in both groups (<10%).

Interestingly, patients reported a strongly reduced subjective sleep quality compared to controls. Patients more often experienced difficulty falling asleep (36.4% vs. 8.3%, p=0.020), felt that they woke up more frequently during the night (61.4% vs. 29.2%, p=0.021), and more often reported not to feel rested in the mornings (43.2% vs. none of the controls, p<0.001) (**Table 3**). These discrepancies remained present in a sensitivity analysis of only the 54 subjects for whom both questionnaires and and actigraphy were available, although the difference in waking up during the night became less pronounced (p=0.057; data not shown). These findings point to a need to address subjective sleep experience rather than actual sleep duration or interruption in patients suffering from HF.

Cardiac troponin T exhibits a diurnal rhythm in HF patients. To obtain information on heart-specific circadian rhythmicity, 24-hour *c*TnT concentrations were analyzed. Normalized values were calculated to visualize the overall diurnal pattern of subjects with different absolute values. In HF patients, cosinor analysis of serum *c*TnT concentrations revealed significant cosine shaped 24-hour oscillations in normalized *c*TnT values (p=0.018, n=46; **Figure 1D**). In line with previous studies reporting diurnal oscillations of *c*TnT concentration in healthy subjects[15], we observed a similar oscillatory pattern in our healthy controls although this was not statistically significant due to the insufficient sensitivity of the assay when measuring very low analyte concentrations (p=0.055, n=24; **Figure S4**). In both groups, minimum *c*TnT concentrations were between 1 PM and 5 PM, reaching peak concentration at 5 AM.

*c*TnT concentrations exhibited a normal diurnal rhythm in HF patients, suggesting preservation of the molecular clock in the heart. However, since no unequivocal cellular rhythm can be detected directly in human cardiac tissue without repeated tissue sampling, we next used animal models of HF to further confirm the *in-situ* preservation of circadian rhythms in the failing heart.

The peripheral circadian clock is conserved in heart failure in mice

Chronic HF was confirmed by gene expression analysis of natriuretic peptide type A (*Anp*) and brain natriuretic peptide (*Bnp*) in murine heart tissue

(**Figure S5**).

Core clock genes, namely brain and muscle ARNT-like 1 (*Bmal1*) and period circadian regulator 1 (*Per1*), displayed circadian cosinor-shaped oscillations in both sham control (p=0.007 and p=0.033, respectively) and HF mouse hearts (p=0.004 and p=0.011, respectively; **Figure 2A** and **2C**). Cosinor expression of circadian locomotor output cycles kaput (*Clock*) gene was observed only in the HF group (p=0.015), and not in sham control group (p=0.875; **Figure 2B**), while cryptochrome circadian regulator 2 (*Cry2*) was not significantly rhythmic in either group (p=0.425 for sham control and p=0.093 for HF group; **Figure 2D**). No differences in expression levels between sham control and HF groups per each individual ZT were found.

Similar patterns were observed in two other target organs in HF: kidneys (**Figure 2E** and **2F**) and liver (**Figure 2G** and **2H**), with *Bmal1* oscillations being most robust followed by *Per1*.

Taken together, both groups had the same expected pattern of clock gene oscillation in all three organs: *Bmal1* and *Clock* oscillated in the same phase, while *Per1* and *Cry2* had the exactly opposite peak and trough time ('anti-phase') when compared to *Bmal1* and *Clock*. *Bmal1* and *Clock* expression was highest during the beginning of the rest phase (=day), gradually declining towards the night, while *Per1* and *Cry2* expression peaked during the end of day and beginning of the active phase (=night).

Although the mouse model of HF is well-established and used extensively to study this disease, mouse is a nocturnal animal with rest/activity cycles diametrically opposite from diurnal humans. Therefore, we also used diurnal zebrafish to confirm our findings.

The peripheral circadian clock is conserved in heart failure in zebrafish

Chronic HF was confirmed by gene expression analysis of natriuretic peptide type A (*anp*) and brain natriuretic peptide (*bnp*) in zebrafish heart tissue (**Figure S6**).

All of the core clock genes, *arntl1b* (corresponding to *Bmal1* in mice), *clocka*, *per3*, and *cry1ba*, displayed statistically significant oscillations in both control (p=0.008, p<0.001, p=0.005, and p=0.020, respectively) and HF zebrafish hearts (p=0.002, p=0.001, p<0.001, and p=0.005, respectively; **Figure 3A-D**). No differences in expression levels between control and HF groups per each individual ZT were found except for ZT20 of *arntl1b* (p=0.038; **Figure 3A**) and ZT12 and ZT20 for *per3* (p=0.024 and p=0.002, respectively; **Figure 3C**).

Similar oscillations were found in kidneys for *arntl1b* (p=0.005 for control and p=0.003 for HF) and *per3* (p=0.009 for control and p=0.001 for

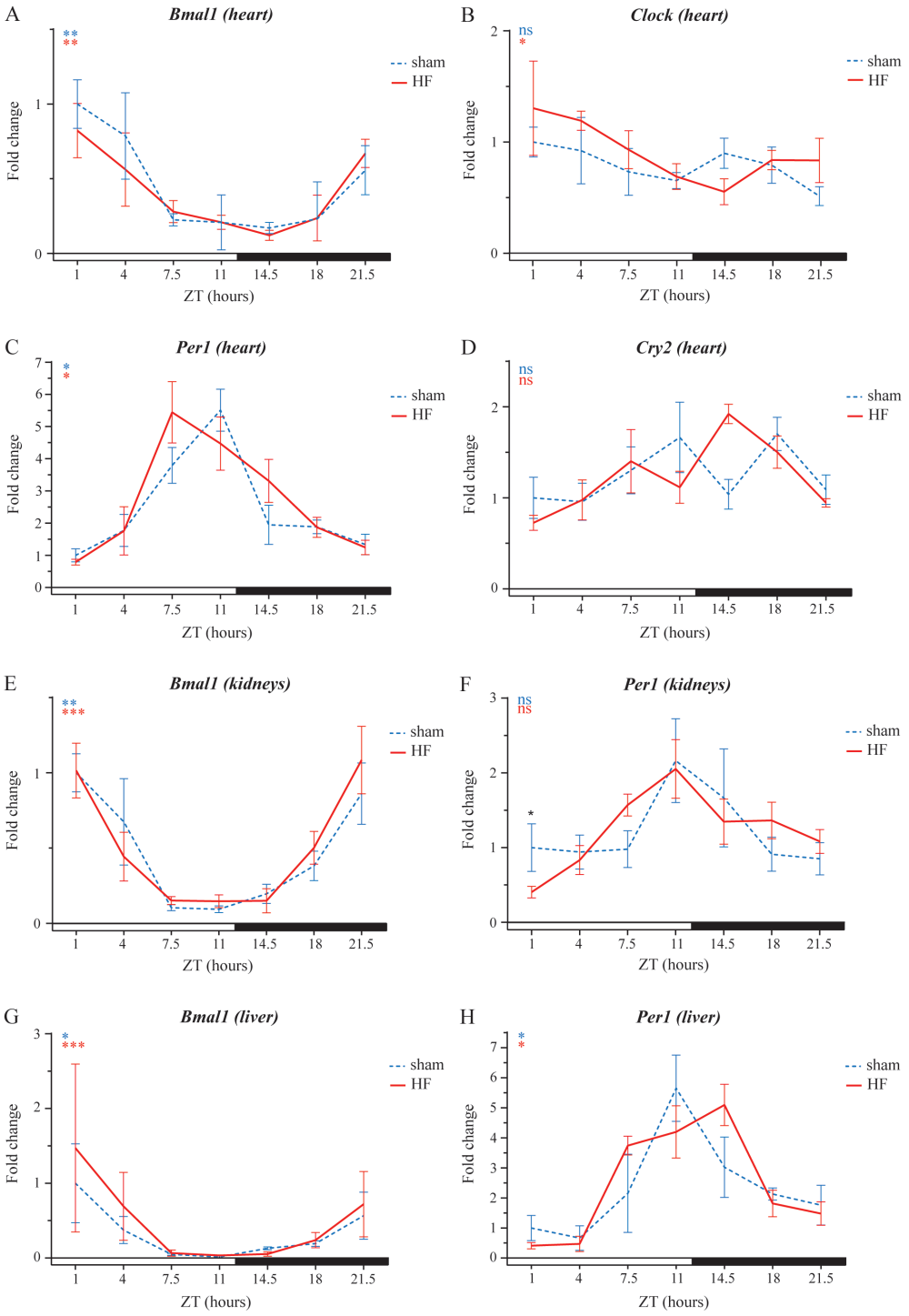


Figure 2 | **24-hour expression profiles of core clock genes in mouse heart, kidneys,**

and liver. Comparison of 24-hour **(A)** *Bmal1*, **(B)** *Clock*, **(C)** *Per1*, and **(D)** *Cry2* expression levels in mouse heart, and **(E, G)** *Bmal1* and **(F, H)** *Per1* expression levels in mouse **(E, F)** kidneys and **(G, H)** liver between sham control and heart failure group, as determined by quantitative real-time PCR reaction (n=3-5 mice for each ZT). Values are mean ± standard error of mean (SEM). Data are normalized against ZT1 of the sham control group. To test differences between sham control and treated animals at the same zeitgeber time, independent two-sample T-test or Mann-Whitney U test were used, as appropriate. $P<0.05$ was used as a cut-off for significance. Significance of independent two-sample T-test or Mann-Whitney U test is indicated above corresponding time-point, and significance of cosinor analysis is indicated at the top left graph corner: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Horizontal bar indicates lights-on (=white; ZT0) and lights-off (=black; ZT12) period. *Bmal1*, Brain and Muscle ARNT-Like 1; *Clock*, Circadian Locomotor Output Cycles Kaput; *Cry2*, Cryptochrome Circadian Regulator 2; HF, heart failure; *Per1*, Period Circadian Regulator 1; ZT, zeitgeber time.

HF group; **Figure 3E** and **3F**).

As observed in mice, both zebrafish groups had the same expected pattern of clock gene oscillation in heart and kidney: *arntl1b* and *clocka* oscillated in the same phase, while *per3* had the exactly opposite peak and trough time ('anti-phase') when compared to *arntl1b* and *clocka*. *Cry1ba*, which represents one of many variants of zebrafish *cry* gene, oscillated in the same phase as *arntl1b* and *clocka*, as previously shown.[26-28] *Arntl1b*, *clocka*, and *cry1ba* expression gradually increased during the day and peaked at the end of the active phase (=day) and beginning of the rest phase (=night), while *per3* expression was highest during the active phase (=day), with lowest values at nighttime. Furthermore, being diurnal animals, oscillations in zebrafish were expectedly in opposite phases to those of nocturnal mice.

Discussion

Central clock output is dampened in heart failure

Here, we show that the circadian clock remains functional in patients with HF, although with less robust central hormonal output as reflected by dampening of the typical oscillatory patterns of both melatonin and cortisol. HF patients had relatively lower nighttime melatonin peaks than age- and sex-matched healthy controls, while their cortisol levels remained chronically elevated throughout the day and night. Congruently, the normal nighttime dip in BP and HR was present in only a small minority of HF patients. HF patients were less physically active and reported reduced subjective sleep quality, although objective sleep parameters were similar and HF patients even slept longer than controls.

Table 3 | **Activity and sleep characteristics of HFrEF patients and controls**

	Patients (n=46)	Controls (n=24)	*P* value
Actigraphy			
	n=36	***n=23***	
Peak activity (counts per minute)	2027.0 (1106.5)	2413.0 (1043.0)	0.017*
Mean activity (counts per minute)	129.8 ± 45.8	220.9 ± 57.5	<0.001*
	n=34	***n=22***	
Total sleep time (minutes)	443.7 ± 61.9	394.9 ± 58.4	0.005*
Sleep latency (minutes)	9.5 (17.5)	7.0 (14.3)	0.562
WASO (minutes)	58.0 (27.0)	46.0 (26.5)	0.098
Sleep efficiency (%)	84.0 (7.3)	83.5 (9.3)	0.573
ESS (%)			
	n=44	***n=24***	
Normal daytime sleepiness	88.6	100.0	0.153
Excessive daytime sleepiness	11.4	0.0	
Difficulty falling asleep (%)			
Never to sometimes	63.6	91.7	0.020*
Often to very often	36.4	8.3	
Waking up during the night (%)			
Never to sometimes	38.6	70.8	0.021*
Often to very often	61.4	29.2	

Table 3 | **Continued**

	Patients (n=46)	Controls (n=24)	*P* value
Not rested in the mornings (%)			
Never to sometimes	56.8	100.0	<0.001*
Often to very often	43.2	0.0	
VOA questionnaire (%)			
	n=43	***n=24***	
Morning chronotype	46.5	45.8	0.584
Evening chronotype	2.3	8.3	
None of both	51.2	45.8	

Values are mean ± standard deviation, median (interquartile range (IQR)) or percentage of total. Differences between the baseline characteristics of the patient and control group were compared by using the independent two-sample T-test for normal distributions and the Mann-Whitney U test for non-normal distributions (*P value<0.05). Fisher's exact test was utilized to determine the existence of non-random associations between the dichotomous values of patients compared with controls. ESS, Epworth Sleepiness Scale; HFrEF, heart failure with reduced ejection fraction; VOA-questionnaire, Vragenlijst Ochtend/Avond typering (translation: questionnaire morning/evening chronotype); WASO, wake after sleep onset.

While these parameters suggest a functional, though dampened central circadian clock in HF patients, they do not provide information on the peripheral circadian clocks in the heart itself. Therefore, we investigated two animal models of HF; mice and zebrafish, at the molecular level and found intact circadian clock gene oscillation in the heart as well as in other relevant organs. We then confirmed that in human HF patients the cardiomyocyte-specific biomarker cTnT oscillated with a cosinor pattern over a period of 24 hours, a typical rhythm for proteins driven by the circadian clock.

Although this is the first multi-species mapping of circadian rhythmicity in HF, a previous study in end stage renal disease demonstrated dampening in the 24-hour concentrations of clock-related hormones.[19] Furthermore, dampened circadian rhythms and suprachiasmatic nucleus dysfunction have been linked with ageing.[29]

The high prevalence of non-dipping BP and HR patterns in our HF population is in line with previous studies.[20,30,31] In hypertensive non-dippers this has been correlated to impaired nocturnal melatonin production[32,33] and lower diurnal cortisol variation.[34] Potential mechanisms through which melatonin influences blood pressure and heart rate include its sympatholytic activity, nephroprotective action and peripheral vasodilating properties.[35] For cortisol, increased levels lead to an activation of the mineralocorticoid receptor, which in turn leads to sodium and water retention, and consequently, a rise in blood pressure besides sympathetic activation.[36] Future studies may be directed to investigate whether modulation of 24-hour rhythmicity in HF patients improves prognosis. While cortisol is –indirectly– targeted in current HF guideline recommended therapy (e.g. beta-blockers, MRA), the potential of timed suppression to restore its 24-hour diurnal variation has not been investigated. Melatonin is not used in the current therapeutic setting, although supplementation has been shown to lower nocturnal blood pressure.[37-40]

Interestingly, in our linear regression, more alcohol consumption was associated with a higher maximum cortisol value. This has also been described in previous studies, which have found that alcohol consumption is linked to circadian rhythm disruption and specifically, can cause plasma cortisol levels to increase with acute consumption, dependence, and withdrawal.[41] However, other studies were not able to confirm this association.[41] For melatonin, we found that a higher BMI was associated with lower maximum values and lower diurnal variation. This finding is in line with previous research pointing towards anti-obesogenic effects of melatonin, with possible mechanisms being energy store regulation, sleep-wake cycle control and influence on composition of gut microbes.[42]

Poor sleep quality and lower physical activity in HF patients open

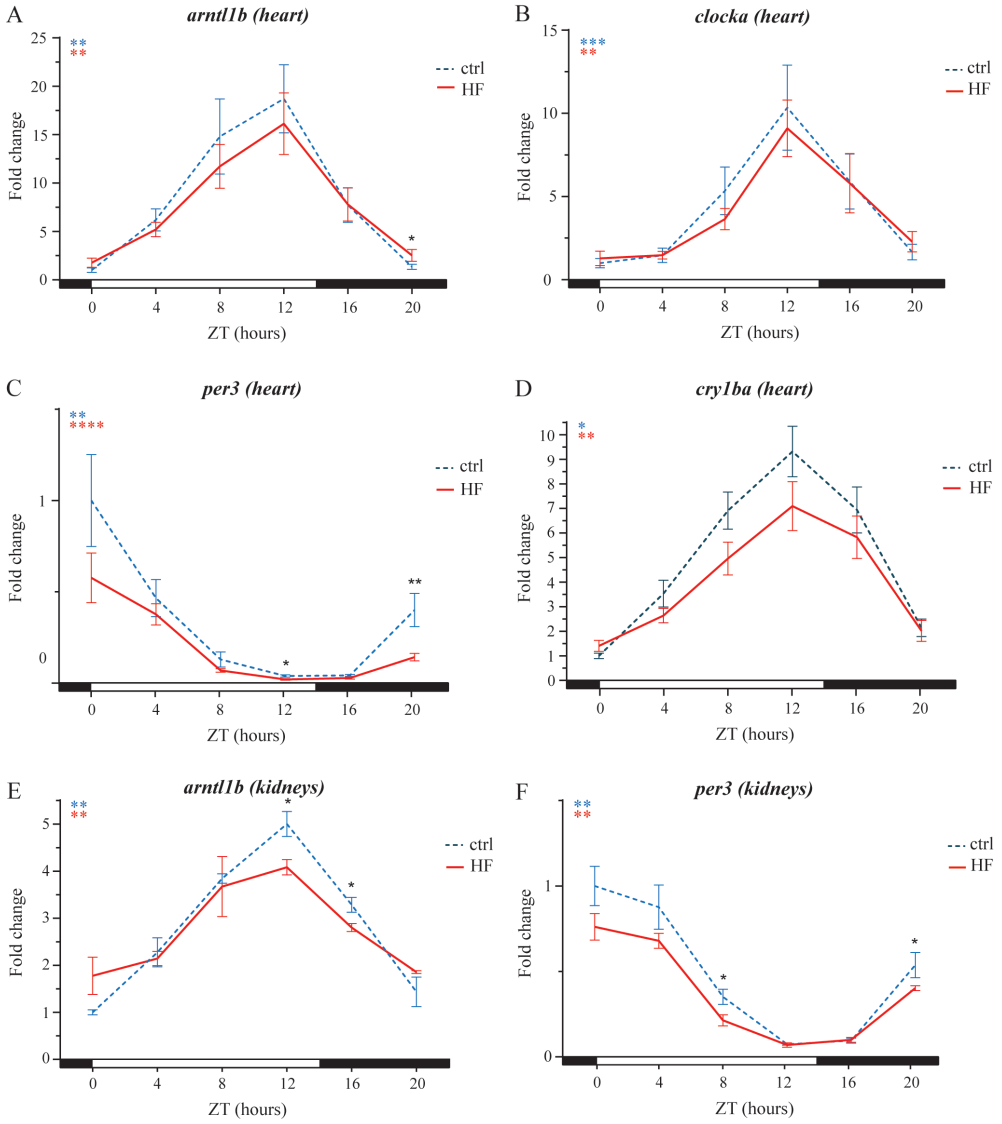


Figure 3 | **24-hour expression profiles of core clock genes in zebrafish heart and kidneys.** Comparison of 24-hour **(A)** *arntl1b*, **(B)** *clocka*, **(C)** *per3*, and **(D)** *cry1ba* expression levels in zebrafish heart, and **(E)** *arntl1b* and **(F)** *per3* expression levels in zebrafish kidneys between control and heart failure group, as determined by quantitative real-time PCR reaction (**(A-D)** n=9 zebrafish for each ZT; 3 biological replicates each including 3 pooled ventricles, **(E)** n=3 and **(F)** n=6 zebrafish for each ZT; 1 and 2 biological replicates each including 3 pooled ventricles, respectively). Values are mean ± standard error of mean (SEM). Data are normalized against ZT0 of the control group. To test differences between control and treated animals at the same zeitgeber time, independent two-sample T-test or Mann-Whitney U test were used, as appropriate. $P<0.05$ was used as a cut-off for significance.

Significance of independent two-sample T-test or Mann-Whitney U test is indicated above corresponding time-point, and significance of cosinor analysis is indicated at the top left graph corner: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. Horizontal bar indicates lights-on (=white; ZT0) and lights-off (=black; ZT14) period. *arntl1b*, Aryl Hydrocarbon Receptor Nuclear Translocator Like 1b; *clocka*, Circadian Locomotor Output Cycles Kaput a; *cry1ba*, Cryptochrome Circadian Regulator 1ba; HF, heart failure; *Per3*, Period Circadian Regulator 3; ZT, zeitgeber time.

another possibility to improve function of the central clock through behavioral interventions. As observed in treating depression with light therapy and exercise[43,44], utilizing this type of intervention could lead to better quality of life or even improved objective clinical outcomes for HF patients as well. HF patients tend to have more sleeping problems than the general population, with an insomnia prevalence of approximately 33%.[45] Although our actometer data did not show decreased sleep efficiency or increased WASO, HF patients did report lower subjective sleep quality. Poor sleep experience in HF patients might lead to the increase of sympathetic nervous system activity and stress levels, negatively affecting disease progression. Insomnia and non-restorative sleep have been correlated with the increased risk of incident HF.[8]

Lastly, we found no differences between the chronotype distribution in our HF patient and control group. To our knowledge, no previous research has been performed investigating this relationship. These findings, together with our findings on intact serum melatonin and cortisol diurnal patterns, point in the direction of no severe phase shifting in the circadian rhythmicity of HF patients.

Cardiac molecular clock is preserved in heart failure

In humans, central circadian clock output can be studied via 24-hour fluctuations of melatonin and cortisol concentrations in serum. However, a peripheral clock, that is specific for each individual tissue in the body (e.g. heart), is not always easily accessible for repeated sampling. Therefore, research of human organs is usually performed by sampling the tissue at only one time-point per subject, either during a surgical procedure and transplantation, or from deceased donors, as we recently discussed.[46] This results in clustering of samples from different origin and time period, that mimic a 24-hour pattern of organ-specific clock genes expression. Although informative, it does not provide important information on intraindividual circadian fluctuation.

For this reason, we sought to substitute cardiac tissue sample biopsies with a representative biomarker that can be measured from serum. cTnT was

the biomarker of choice, since it is excreted specifically by cardiomyocytes. Previous studies have shown that cTnT exhibits diurnal variation in both healthy and diabetic subjects.[15,16,47] Similarly, in our study, we observed a significant 24-hour oscillations of cTnT concentrations in HF patients, pointing towards a preserved circadian rhythm in the failing heart as well.

Intact peripheral clocks are also confirmed in our animal models of HF. In order to circumvent the unavailability of human organs of interest, we decided to further corroborate our findings directly in the tissues of mice and zebrafish. In both animal types, no differences between HF and control groups were found, which is consistent with our previous observation on intact cTnT oscillation in the human patients. This is in line with a previously published study conducted on orthotopic heart transplantation samples, where no difference in core clock gene expression was found between healthy controls without history of any cardiovascular disease (non-cardiac death samples), patients with coronary heart disease and patients with cardiomyopathy.[48] Taken together, our results demonstrate the overall preservation of the molecular circadian clock in the heart, and in two other organs affected by HF (liver and kidney).

Limitations

In this study, we did not use standardized regimes commonly utilized in clinical circadian experiments regarding physical activity, food intake, light exposure and sleep duration of study subjects. This is usually done to minimize external influences on circadian clock and to achieve a more comparable circadian rhythm between subjects.[16] However, we deemed it necessary to allow for a clinically more translatable setup, since patients outside the study framework will not follow these externally imposed regimes and strictly controlled environmental settings. Consequently, all consistent differences found in central circadian clock output between our study subjects are more robust and applicable to real-life situations.

Other limitations include the low sample size compared to large clinical trials. However, this study offers unique 24-hour measurements during seven time-points per each patient and control, which has not been reported previously in HF. This, in our opinion, is a valuable addition to the HF research, especially since most clinical studies disregarded circadian rhythms altogether or were performed by pooling only one time-point of each study participant with a categorical division into two or four broad time windows.[48] BP and HR measurements were not obtained from the control group because normal patterns are known from many previous studies.[1,25]

Furthermore, a possible selection bias was introduced in our patient

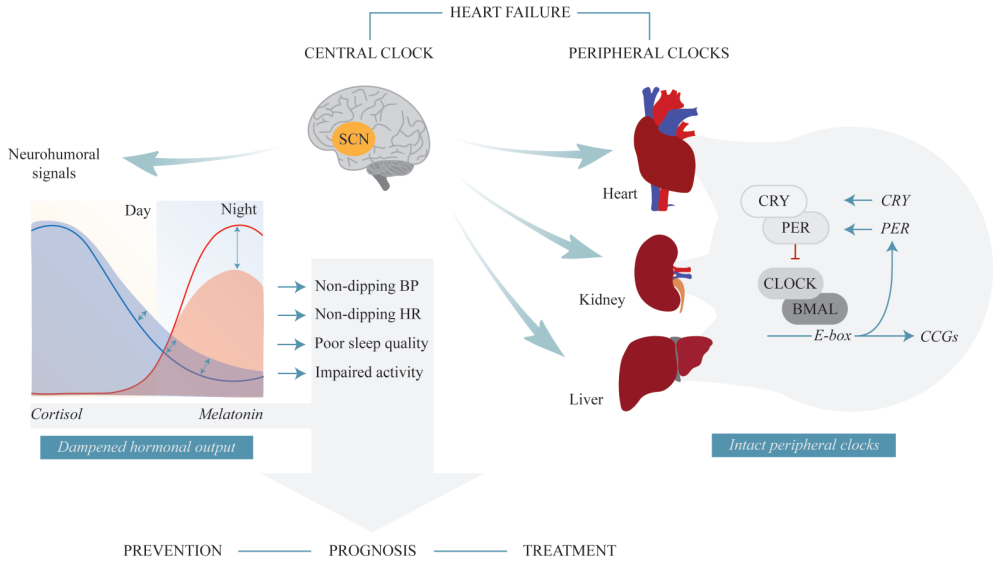
population due to exclusion of patients who received anesthesia or sedatives just prior to the study. However, this exclusion was necessary in order to obtain true circadian parameters, undisturbed by sleeping medication, and was similarly applied to patients and controls.

Conclusions

HF is a leading cause of death worldwide, with limited treatment options and poor prognosis. Considering the important role of circadian rhythms in the overall cardiovascular system, we aimed to elucidate the relation between circadian rhythms and HF. We showed that the peripheral clock machinery of heart, kidney and liver is preserved in HF, with expression comparable to that of healthy controls. However, differences were observed in the central circadian clock output, resulting in a dampened oscillatory pattern of melatonin and cortisol in HF patients. Furthermore, a high prevalence of non-dipping BP and HR patterns was observed in the HF population, possibly associated with lower nocturnal melatonin levels and higher nocturnal cortisol levels.

The fact that a significant amount of clock-governed variation remains intact in severely ill patients, here with HF, underlines the physiological importance of this regulatory system. It also implies that the timing of interventions and diagnostic measurements should be taken into account in preclinical and clinical research.

Finally, discovery of the aberrant function of specific clock components opens an exciting window of opportunity to develop new interventions targeting circadian clock components, investigate prognosis/ prevention strategies, as well as behavioral interventions for restoring patients' subjective sleep quality and physical activity, to improve the long-term quality of life and survival of patients suffering from HF (see **Take home figure**).



Take home figure | Circadian rhythms can be divided into two parts: the central clock, located in the SCN in the brain, and peripheral clocks, present in virtually every tissue in the body, including heart, kidney, and liver. The central clock synchronizes peripheral clocks via various neurohumoral signals, however peripheral clocks also respond to additional tissue-specific synchronizers. Each cell type has a molecular circadian clock. The main positive feedback loop consists of the BMAL-CLOCK heterodimer which, by binding to *E-boxes* of *PER* and *CRY*, initiates their transcription. In turn, PER and CRY form a heterodimer which inhibits transcription of *BMAL* and *CRY*, forming a negative feedback loop. This interplay results in a rhythmic expression of various clock controlled genes (*CCGs*). In HF, molecular clocks of heart, kidney and liver are preserved, with expression comparable to that of healthy controls (see animal gene expression data in the main text). Conversely, HF negatively affects the central clock output: 24-hour concentrations of melatonin and cortisol are dampened in HF, when compared to healthy controls. Furthermore, a high prevalence of non-dipping BP and HR patterns in HF population was found, correlating with lower nocturnal melatonin levels and higher nocturnal cortisol levels. Also, HF patients suffer from poor subjective sleep quality and impaired activity. Altogether, these findings open various opportunities for developing new therapeutic (clock) interventions in order to improve the long-term quality of life and survival of patients suffering from HF, and warrant further investigations (e.g. melatonin supplementation, prognosis/prevention strategies based on non-dipping BP and HR, behavioral interventions such as light and exercise therapy, chronopharmacology). BMAL, Brain and Muscle ARNT-Like; BP; blood pressure; CLOCK, Circadian Locomotor Output Cycles Kaput; CRY, Cryptochrome Circadian Regulator; HF, heart failure; HR, heart rate; PER, Period Circadian Regulator; SCN, suprachiasmatic nucleus.

What is new?

- The central circadian clock, as reflected by 24-hour melatonin and cortisol levels, is intact but dampened in patients with heart failure.
- Cardiac troponin T concentrations exhibit a diurnal rhythm in heart failure patients, further confirming the preservation of the cardiac circadian clock.
- Peripheral clocks are intact in organs of nocturnal (mouse) and diurnal (zebrafish) animal models of heart failure: heart, kidneys and liver in mice, and heart and kidneys in zebrafish.

What are the clinical implications?

- Since the circadian clock remains functional in heart failure, timing of interventions and measurements should be taken into account in clinical studies to minimize variation and maximize effect.
- The high prevalence of heart rate and blood pressure non-dippers in heart failure, combined with lower melatonin and cortisol diurnal variation provides new vantage points for future treatment options (e.g. timed melatonin supplementation) for heart failure.
- The quality of life for heart failure patients could be improved through central clock therapy by implementing behavioral interventions (e.g. light and activity therapy) focusing on central clock output and subjective sleep quality.

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Conflicts of Interest

S. Crnko, M. Printezi, L. Leiteris, A.I Lumley, L. Zhang, I. Ernens, T. Jansen, L. Homsma, D. Feyen, M. van Faassen, B. du Pré, C. Gaillard, H. Kemperman, P.P. Zwetsloot, M. Oerlemans, P. Doevendans, J. Sluijter, Y. Devaux: None. L. van Laake: Outside the current work: Consultancy fees to UMCU from Abbott, Medtronic, Vifor, Novartis. Investigator-initiated study in collaboration with Roche (cTnT kits).

References

1. Crnko, S., Du Pré, B. C., Sluijter, J. P. G. & Van Laake, L. W. Circadian rhythms and the molecular clock in cardiovascular biology and disease. *Nat. Rev. Cardiol.* **16**, 437–447 (2019).
2. Durgan, D. J. & Young, M. E. The cardiomyocyte circadian clock: emerging roles in health and disease. *Circ. Res.* **106**, 647–658 (2010).
3. Zhang, J., Chatham, J. C. & Young, M. E. Circadian Regulation of Cardiac Physiology: Rhythms That Keep the Heart Beating. *Annu. Rev. Physiol.* **82**, 79–101 (2020).
4. Muller, J. E. *et al.* Circadian variation in the frequency of onset of acute myocardial infarction. *N. Engl. J. Med.* **313**, 1315–1322 (1985).
5. Portaluppi, F. & Hermida, R. C. Circadian rhythms in cardiac arrhythmias and opportunities for their chronotherapy. *Adv. Drug Deliv. Rev.* **59**, 940–951 (2007).
6. Montaigne, D. *et al.* Daytime variation of perioperative myocardial injury in cardiac surgery and its prevention by Rev-Erbα antagonism: a single-centre propensity-matched cohort study and a randomised study. *Lancet* **391**, 59–69 (2018).
7. Sole, M. J. & Martino, T. A. Diurnal physiology: core principles with application to the pathogenesis, diagnosis, prevention, and treatment of myocardial hypertrophy and failure. *J. Appl. Physiol.* **107**, 1318–1327 (2009).
8. Laugsand, L. E., Strand, L. B., Platou, C., Vatten, L. J. & Janszky, I. Insomnia and the risk of incident heart failure: a population study. *Eur. Heart J.* **35**, 1382–1393 (2014).
9. Duong, A. T. H. *et al.* The Clock Mechanism Influences Neurobiology and Adaptations to Heart Failure in ClockΔ19/Δ19 Mice With Implications for Circadian Medicine. *Scientific Reports* **9**, 4994 (2019).
10. Hartupee, J. & Mann, D. L. Neurohormonal activation in heart failure with reduced ejection fraction. *Nat. Rev. Cardiol.* **14**, 30–38 (2017).
11. Racca, C. *et al.* Aspirin intake in the morning is associated with suboptimal platelet inhibition, as measured by serum Thromboxane B2, during infarct-prone early-morning hours. *Platelets* **30**, 871–877 (2019).
12. Hermida, R. C., Ayala, D. E., Mojón, A. & Fernández, J. R. Bedtime dosing of antihypertensive medications reduces cardiovascular risk in CKD. *J. Am. Soc. Nephrol.* **22**, 2313–2321 (2011).
13. Dallmann, R., Brown, S. A. & Gachon, F. Chronopharmacology: New Insights and Therapeutic Implications. *Annu. Rev. Pharmacol. Toxicol.* **54**, (2014).
14. Johnston, J. G. & Pollock, D. M. Circadian regulation of renal function. *Free Radic. Biol. Med.* **119**, 93–107 (2018).
15. Fournier, S. *et al.* Circadian rhythm of blood cardiac troponin T concentration. *Clin Res. Cardiol.* **106**, 1026–1032 (2017).
16. Klinkenberg, L. J. J. *et al.* Circulating Cardiac Troponin T Exhibits a Diurnal Rhythm. *Journal of the American College of Cardiology* **63**, 1788–1795 (2014).
17. van Faassen, M., Bischoff, R. & Kema, I. P. Relationship between plasma and salivary melatonin and cortisol investigated by LC-MS/MS. *Clin. Chem. Lab. Med.* **55**, 1340–1348 (2017).
18. Kerkhof, G.A. Een Nederlandse vragenlijst voor de selectie van ochtend- en avondmensen. *Ned. T.v.d. Psychologie* **39**, 281–294 (1984).
19. Russcher, M. *et al.* An observational study on disturbed peripheral circadian rhythms

in hemodialysis patients. *Chronobiology International* **32**, 848–857 (2015).
20. Ueda, T. *et al.* Differences in blood pressure riser pattern in patients with acute heart failure with reduced mid-range and preserved ejection fraction. *ESC Heart Failure* **6**, 1057–1067 (2019).
21. van Laake, L. W. *et al.* Monitoring of cell therapy and assessment of cardiac function using magnetic resonance imaging in a mouse model of myocardial infarction. *Nat. Protoc.* **2**, 2551–2567 (2007).
22. Ernens, I., Lumley, A. I. & Devaux, Y. Restoration of cardiac function after anaemia-induced heart failure in zebrafish. *J. Mol. Cell. Cardiol.* **121**, 223–232 (2018).
23. Molcan, L. Time distributed data analysis by Cosinor.Online application. *bioRxiv 805960* (2019) doi:10.1101/805960.
24. Zeitzer, J. M., Ayas, N. T., Shea, S. A., Brown, R. & Czeisler, C. A. Absence of detectable melatonin and preservation of cortisol and thyrotropin rhythms in tetraplegia. *J. Clin. Endocrinol. Metab.* **85**, 2189–2196 (2000).
25. Douma, L. G. & Gumz, M. L. Circadian Clock-Mediated Regulation of Blood Pressure. *Free Radic. Biol. Med.* **119**, 108–114 (2018).
26. Huang, D. *et al.* Zebrafish Lacking Circadian Gene per2 Exhibit Visual Function Deficiency. *Front. Behav. Neurosci.* **12**, 53 (2018).
27. Wang, M., Zhong, Z., Zhong, Y., Zhang, W. & Wang, H. The Zebrafish Period2 Protein Positively Regulates the Circadian Clock through Mediation of Retinoic Acid Receptor (RAR)-related Orphan Receptor α (Rorα). *J. Biol. Chem.* **290**, 4367–4382 (2015).
28. Liu, C. *et al.* Molecular evolution and functional divergence of zebrafish (Danio rerio) cryptochrome genes. *Scientific Reports* **5**, 8113 (2015).
29. Nakamura, T. J. *et al.* Age-Related Decline in Circadian Output. *J. Neurosci.* **31**, 10201–10205 (2011).
30. Caruana, M. P., Lahiri, A., Cashman, P. M., Altman, D. G. & Raftery, E. B. Effects of chronic congestive heart failure secondary to coronary artery disease on the circadian rhythm of blood pressure and heart rate. *Am. J. Cardiol.* **62**, 755–759 (1988).
31. Kastrup, J., Wroblewski, H., Sindrup, J., Rolighed Christensen, H. & Wiinberg, N. Diurnal blood pressure profile in patients with severe congestive heart failure: dippers and non-dippers. *Scand. J. Clin. Lab. Invest.* **53**, 577–583 (1993).
32. Jonas, M., Garfinkel, D., Zisapel, N., Laudon, M. & Grossman, E. Impaired nocturnal melatonin secretion in non-dipper hypertensive patients. *Blood Press.* **12**, 19–24 (2003).
33. Friedman, O. & Logan, A. G. Nocturnal blood pressure profiles among normotensive, controlled hypertensive and refractory hypertensive subjects. *Can. J. Cardiol.* **25**, e312–e316 (2009).
34. Holt-Lunstad, J. & Steffen, P. R. Diurnal cortisol variation is associated with nocturnal blood pressure dipping. *Psychosom. Med.* **69**, 339–343 (2007).
35. Simko, F., Reiter, R. J. & Paulis, L. Melatonin as a rational alternative in the conservative treatment of resistant hypertension. *Hypertens. Res.* **42**, 1828–1831 (2019).
36. Frey, F. J., Odermatt, A. & Frey, B. M. Glucocorticoid-mediated mineralocorticoid receptor activation and hypertension. *Curr. Opin. Nephrol. Hypertens.* **13**, 451–458 (2004).
37. Grossman, E. *et al.* Melatonin reduces night blood pressure in patients with nocturnal

hypertension. *Am. J. Med.* **119**, 898–902 (2006).
38. Arangino, S. *et al.* Effects of melatonin on vascular reactivity, catecholamine levels, and blood pressure in healthy men. *Am. J. Cardiol.* **83**, 1417–1419 (1999).
39. Burgess, H. J., Sletten, T., Savic, N., Gilbert, S. S. & Dawson, D. Effects of bright light and melatonin on sleep propensity, temperature, and cardiac activity at night. *J. Appl. Physiol.* **91**, 1214–1222 (2001).
40. Cagnacci, A. *et al.* Prolonged melatonin administration decreases nocturnal blood pressure in women. *Am. J. Hypertens.* **18**, 1614–1618 (2005).
41. Meyrel, M., Rolland, B. & Geoffroy, P. A. Alterations in circadian rhythms following alcohol use: A systematic review. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **99**, 109831 (2020).
42. Imenshahidi, M., Karimi, G. & Hosseinzadeh, H. Effects of melatonin on cardiovascular risk factors and metabolic syndrome: a comprehensive review. *Naunyn Schmiedebergs Arch Pharmacol* **393**, 521–536 (2020).
43. Pail, G. *et al.* Bright-light therapy in the treatment of mood disorders. *Neuropsychobiology* **64**, 152–162 (2011).
44. Martiny, K. *et al.* A 9-week randomized trial comparing a chronotherapeutic intervention (wake and light therapy) to exercise in major depressive disorder patients treated with duloxetine. *J. Clin. Psychiatry* **73**, 1234–1242 (2012).
45. Hayes, D., Anstead, M. I., Ho, J. & Phillips, B. A. Insomnia and chronic heart failure. *Heart Fail. Rev.* **14**, 171–182 (2009).
46. Crnko, S., Schutte, H., Doevendans, P. A., Sluijter, J. P. G. & van Laake, L. W. Minimally Invasive Ways of Determining Circadian Rhythms in Humans. *Physiology* **36**, 7–20 (2020).
47. Klinkenberg, L. J. J. *et al.* Diurnal Rhythm of Cardiac Troponin: Consequences for the Diagnosis of Acute Myocardial Infarction. *Clin. Chem.* **62**, 1602–1611 (2016).
48. Leibetseder, V. *et al.* Clock genes display rhythmic expression in human hearts. *Chronobiol. Int.* **26**, 621–636 (2009).
49. Levey, A. S. *et al.* A new equation to estimate glomerular filtration rate. *Ann. Intern. Med.* **150**, 604–612 (2009).
50. Ponikowski, P. *et al.* 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC)Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur. Heart J.* **37**, 2129–2200 (2016).

Supporting information

Study design: human heart failure
Inclusion and exclusion criteria.
Inclusion criteria:
1. Admitted to the UMC Utrecht
2. Age 18 - 85 years
3. Chronic systolic heart failure NYHA II-III (patients only), clinically euvolemic
4. Signed informed consent

Exclusion criteria:
1. Blindness
2. End stage renal failure (GFR <15mL/min)
3. Fever >38°C
4. CRP >20mg/L
5. Use of hypnotics or other medication that disturbs the normal sleep pattern during study period
6. Anesthesia within 48 hours prior to start study
7. Serious comorbidity that could influence circadian rhythms and prevents participation according to the investigators (e.g. severe neurological, psychiatric, or oncologic diseases)
8. I.v. amiodarone
9. I.v. or oral use of corticosteroids
10. I.v. diuretics or inotropes

Laboratory measurements. At seven predefined time-points (9 AM, 1 PM, 5 PM, 9 PM, 1 AM, 5 AM, 9 AM), serum-separating tubes (BD) were used to collect blood (7.5 mL per time-point) from an intravenous cannula blood was collected from an intravenous cannula, totaling 320 samples for HF patients and 167 for controls. Serum melatonin and cortisol concentrations were measured on liquid chromatography-tandem mass spectrometer (LC-MS/MS).[17] Serum cTnT concentration was measured with Troponin T-high sensitive immunoassay (Roche Diagnostics, Indianapolis), with 99th percentile of 14 ng/L. In one control subject the first time-point (9 AM) is missing due to problems with blood withdrawal, and in two patients the last time-point (9 AM) is missing due to technical problems with serum separation. Additionally, for cTnT measurements, time-points at 1 PM and 9 PM are missing for one control and two patients, respectively, due to the insufficient amount of serum necessary for the analysis.

Activity measurements. Following hospitalization, subjects were instructed to wear a wrist actometer (Philips Respironics Actiwatch 2) for five days in total, and to maintain the same daily sleep and activity patterns as usual. After data collection, the actogram was obtained and, if necessary, adjusted according to the users' manual. In order to quantify sleep and activity patterns, the following parameters were extracted: peak activity (PA) in counts per minute (CPM), mean daily activity (MA) in CPM, total sleep time (TST) in minutes, sleep latency (SL) in minutes, wake after sleep onset (WASO) in minutes, sleep efficiency (SE) in minutes. The PA was obtained by selecting the maximum activity over the entire period of data collection. The MA was calculated over a round number of complete 24-hour cycles, starting from the moment the measurement initiated. Subjects could be excluded from the actigraphy measurements for the following reasons: patient decision and important shift in clinical condition compared to the blood-sampling day.

Study design: mouse heart failure

Mouse HF model and experimental setup. Male C57BL/6 mice (Jackson), aged 10 – 12 weeks were housed under controlled conditions in a 12-h light/12-h dark cycle (lights on at ZT0, lights off at ZT12). Water and food were provided *ad libitum*. Myocardial infarction (MI) was induced by ligation of the left coronary artery, as described previously.[21] MI or sham operations were performed randomly over the day. Prior to the surgery, mice (n=4-5 per group) were anesthetized (i.p.; fentanyl 0.05 mg/kg; midazolam 5 mg/kg; medetomidine 0.5 mg/kg) and, before any incision was made, the adequacy of anesthesia was monitored by testing rear foot reflexes. Respiratory pattern, rectal temperature, and responsiveness to manipulations were continually monitored throughout the procedure.

Seven days prior to termination (day 21 after the surgery), heart function was assessed with echocardiography in order to validate the presence and effect of MI in HF mice. Heart rate, respiration and body temperature were constantly monitored, with body temperature kept between 36.0 and 38.0 °C using heating lamps. 2D images were recorded on the short axis of the heart on multiple levels in both end systole and end diastole, as well as with respiratory triggering. Subsequently, obtained images were used for complete 3D reconstruction of the heart. Image acquisition and analyses were performed using the dedicated Vevo® 2100 System and Software (Fujifilm VisualSonics Inc., Toronto, Canada).

On day 28, on each time-point during a period of 24 hours (7 AM, 10 AM, 1:30 PM, 5 PM, 8:30 PM, 12 AM, 3:30 AM), randomly chosen mice were sacrificed for tissue collection. The first time point of tissue sampling was

carried out at 7 AM and represents zeitgeber time one (ZT1; one hour after lights were turned on). After termination, heart, kidneys, and liver were snap frozen for later gene expression analysis by qPCR.

Gene expression analysis. Total RNA and protein were isolated from snap frozen organ sections using 1 mL TripureTM Isolation Reagent (Roche) according to the manufacturer's protocol. Following DNAse I (Qiagen) treatment, 500 ng total RNA was used for cDNA synthesis (iScriptTM cDNA synthesis kit, Bio-Rad). qPCR reaction was performed using 10 μL iQTM SYBR Green supermix and 10 μL cDNA. Ribosomal Protein Lateral Stalk Subunit P0 (*Rplp0*) was selected as the housekeeping gene and was used for calculation of normalized gene expression levels (ΔCt). The sequences of all primers are presented in **Table S1**.

Study design: zebrafish heart failure

Zebrafish HF model and experimental setup. Wild-type AB zebrafish male adults aged 8 – 10 months were used in this study. Before proceeding with an experiment, fish from the same breeding stock were weighed and randomized into experimental tanks in a homogenous manner so that each tank contained the same number of fish with the same range of weight. Experimental tanks were maintained in a strict 14/10-hour light/dark cycle environment (lights on at ZT0, lights off at ZT14) at 28°C on a daily basis. Animals were fed to satiety twice daily with dry food. After one week of acclimatization, treatment with phenylhydrazine hydrochloride (PHZ, Sigma-Aldrich) was started to generate HF phenotype during five weeks, as previously described.[22] In brief, PHZ was added to the fish water of the treated group for 30 min every 3 days for five weeks. PHZ was used at a concentration of 1.25 μg/mL during the first week of treatment followed by a dose increment to 2.5 μg/mL for the rest of the protocol. Each treatment phase of 30 min incubation was followed by 30 min rinsing and washing in fish water without PHZ. The control group was subjected to the same process, excluding the addition of PHZ to the fish water. At the end of the five-week period, PHZ-treated and control fish were sacrificed. Heart and kidney samples were harvested from PHZ-treated and control fish during the following 24 hours.

The hearts of PHZ-treated and control zebrafish were harvested every four hours over a 24-hour period (8:30 AM, 12:30 PM, 4:30 PM, 8:30 PM, 12:30 AM, 4:30 AM) at the end of the five-week phase. The first time point of tissue sampling was carried out at 8:30 AM (ZT0; time-point when lights were turned on). At each time-point, heart and kidneys were snap frozen for later gene expression analysis by qPCR reaction.

Gene expression analysis. Harvested intact hearts and kidneys were separately disrupted and homogenized by the Polytron Dispersing Aggregates (Kinematica) in the presence of QIAzol Lysis Reagent (Qiagen). Total RNA was isolated using the miRNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. 500 and 1000 ng of DNase I treated (Qiagen) total RNA was reverse-transcribed using the Superscript II Reverse Transcriptase for heart and kidney samples, respectively (Invitrogen). qPCR reaction was carried out using the QuantiTect SYBR Green PCR Master Mix (Qiagen) and 10-fold dilutions of cDNA. Elongation factor 1-alpha 1 (*eef1a1*) was selected as the housekeeping gene and was used for calculation of normalized gene expression levels (ΔCt). The sequences of all primers are presented in **Table S1**.

Study design: human heart failure

Univariable linear regression was performed to assess an association between melatonin and cortisol levels and the presence of HF. For non-normally distributed outcomes a natural logarithm transformation was performed before carrying out regression analyses. The resulting B-coefficients underwent retransformation. Correction for confounding variables was carried out using multivariable linear regression analysis for the following predefined variables: sex, age, BMI, kidney function, smoking history, and current alcohol use. We also tested for univariable interactions with the grouping variable (control/HF) for sex, age and BMI to detect any potential effect modification. When a univariable interaction was significant, we dichotomized the variable and ran additional multiple linear regression with mentioned confounders.

For minimum melatonin levels, data was not normally distributed. Due to this, we post-hoc dichotomized these values into detectable and undetectable values (0 or 1). This resulted in nine detectable values and 61 undetectable values, for which we ran logistic regression analysis with Firth's correction to adjust for separation and to avoid small sample bias. Taking this dichotomization in consideration, we only corrected for possible confounders univariably (alongside the grouping variable).

We are aware of the fact that these analyses included multiple independent variables with a small number of outcomes, however, we deemed correcting for confounders more important than performing an univariable analysis for this hypothesis generating endeavor.

Throughout the manuscript, the following indications for significance were used: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. All analyses were performed in IBM SPSS Statistics (v. 25).

Table S1 | **Primer sequences used for the quantitative real-time PCR reaction**

Animal	Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Mouse	*Rplp0*	*GGACCCGAGAAGACCTCCTT*	*GCACATCACTCAGAATTTCAATGG*
	Bmal1	*TGACCCTCATGGAAGGTTAGAA*	*GGACATTGCATTGCATGTTGG*
	Cry2	*CCTCGTCTGTGGGCATCAA*	*GCTTTCTTAAGCTTGTGTCCAGATC*
	Clock	*AAAGACGGCGAGAACTTGG*	*GGAGGCAGAAGGAGTTGGG*
	Per1	*TCGAAACCAGGACACCTTCTCT*	*GGGCACCCCGAAACACA*
	Anp	*CCTGTGTACAGTGCGGTGTC*	*CCTAGAAGCACTGCCGTCTC*
	Bnp	*GTTCTTTTGTGAGGCCTTGG*	*CTGAAGGTGCTGTCCCAGAT*
Zebrafish	*eef1a1*	*CTTCTCAGGCTGACTGTGC*	*CCGCTAGCATTACCCTCC*
	arntl1b	*TTCTTTTGAGGACAGTATGGAC*	*TGTTCATCTTGTCTCTGCGTCT*
	cry2a	*GCATGACATGGCAGGACACTT*	*TGCGATTTGCCGTTGTAACT*
	clocka	*GACCACCAACCTCAACCAGCA*	*TCGGCTGAGAGATCATCATGGTA*
	per1a	*TGGTAAAGACCAGCGGACAG*	*TGCCTTTGCCAGTGCTTCTA*
	anp	*GATGTACAAGCGCACACGTT*	*TCTGATGCCTCTTCTGTTGC*
	bnp	*CAGAATCGGTTCAATGTC*	*TTGTGAGGTTAAATCAAGTAG*

Anp, Natriuretic Peptide Type A; *arntl* (*bmal*), Aryl Hydrocarbon Receptor Nuclear Translocator Like; *Bmal*, Brain and Muscle ARNT-Like; *Bnp*, Brain Natriuretic Peptide; *Clock*, Circadian Locomotor Output Cycles Kaput; *Cry*, Cryptochrome Circadian Regulator; *eef1a1*, Elongation factor 1-alpha 1; *Per*, Period Circadian Regulator; *Rplp0*, Ribosomal Protein Lateral Stalk Subunit P0.

Table S2 | **Multivariable linear regression analysis for melatonin**

	Maximum melatonin**		Diurnal melatonin variation**	
	B	***P* value**	**B**	***P* value**
Group (0=control, 1=HF)	0.742	0.383	0.535	0.009*
Sex (0=male, 1=female)	0.876	0.710	0.895	0.645
Age, years	0.979	0.137	0.993	0.449
BMI, kg/m²	0.897	0.005*	0.927	0.004*
eGFR, mL/min/1.73m²	0.993	0.477	1.000	0.967
Smoking, PY	1.004	0.712	0.996	0.594
Alcohol, IU/week	0.984	0.521	0.988	0.474

**P* value <0.05, ** Prior to regression analysis these melatonin values underwent natural logarithm transformation, after which the B-coefficient was retransformed. B, B-coefficient; BMI, body mass index; eGFR, estimated glomerular filtration rate[49]; IU, international units; ln, natural logarithm; PY, packyears (one year of smoking 1 pack of cigarettes a day).

Table S3 | **Multivariable linear regression analysis for cortisol**

	Minimum cortisol**		Maximum cortisol		Diurnal cortisol variation**	
	B	***P* value**	**B**	***P* value**	**B**	***P* value**
Group (0=control, 1=HF)	1.472	0.019*	42.471	0.199	0.731	0.025*
Sex (0=male, 1=female)	0.910	0.573	-38.869	0.257	1.025	0.860
Age, years	1.011	0.106	0.590	0.655	0.992	0.131
BMI, kg/m²	0.984	0.363	-4.266	0.233	1.007	0.635
eGFR, mL/min/1.73m²	0.991	0.048*	-0.290	0.754	1.009	0.023*
Smoking, PY	1.000	0.942	0.439	0.703	1.001	0.868
Alcohol, IU/week	1.000	0.978	4.774	0.048*	1.009	0.360

**P* value <0.05, ** Prior to regression analysis these melatonin values underwent natural logarithm transformation, after which the B-coefficient was retransformed. B, B-coefficient; BMI, body mass index; eGFR, estimated glomerular filtration rate[49]; IU, international units; ln, natural logarithm; PY, packyears (one year of smoking 1 pack of cigarettes a day).

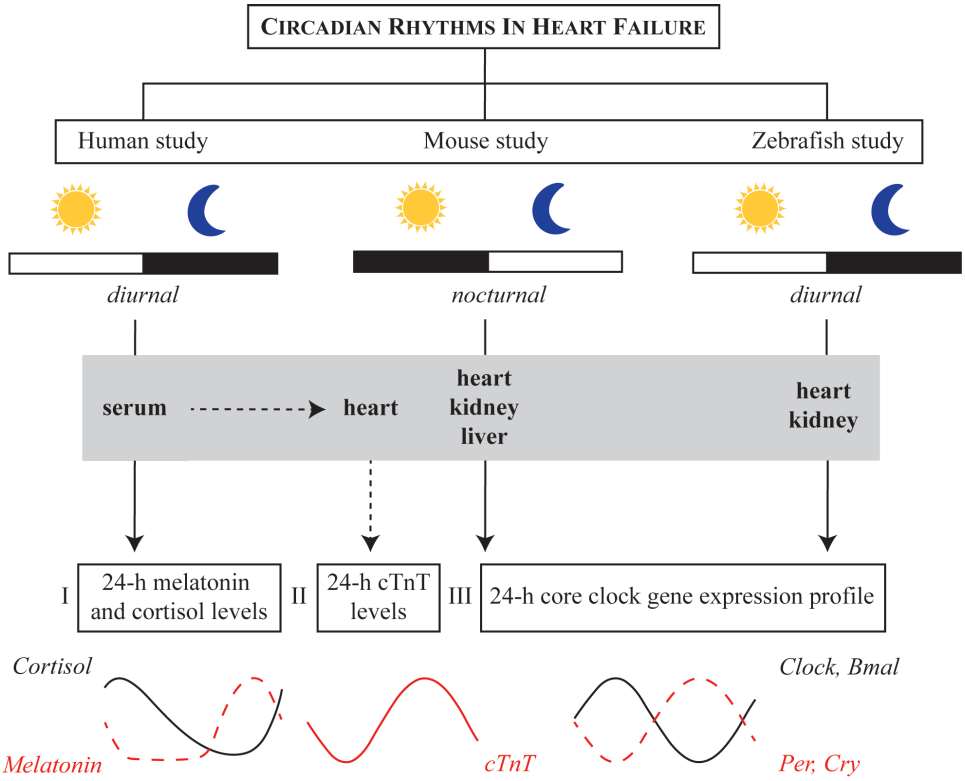

Figure S1 | **Study design overview.** In order to characterize circadian rhythmicity in heart failure patients, three separate studies were performed: human, mouse, and zebrafish study. 24-hour serum samples were collected from heart failure patients and healthy controls to assess the rhythmic expression of main endocrine products of the central clock: melatonin and cortisol. Furthermore, since human hearts are not accessible for repeated sampling, serum cardiac troponin T was used as a surrogate clock marker reflecting cardiac-specific rhythms. The functionality of the peripheral circadian clock was further analyzed in the hearts of nocturnal mice (opposite rest/activity cycles to humans) and diurnal zebrafish (the same rest/activity cycles as humans) with heart failure, as well as in their respective controls without heart failure. Finally, circadian rhythmicity was assessed in other target organs in heart failure: kidneys and liver. Of note, throughout the manuscript the term diurnal rhythms is used when referring to rhythms that exist as a response to 24-hour environmental changes. However, the reader should not be confused with the mention of diurnal and nocturnal animals, which depicts the activity/rest periods during the 24 hours: diurnal animals=active phase during the day and rest phase during the night, nocturnal animals=active phase during the night and rest phase during the day. *Bmal*, Brain and Muscle ARNT-Like; *Clock*, Circadian Locomotor Output Cycles Kaput; *Cry*, Cryptochrome Circadian Regulator; cTnT, cardiac troponin T; *Per*, Period Circadian Regulator.

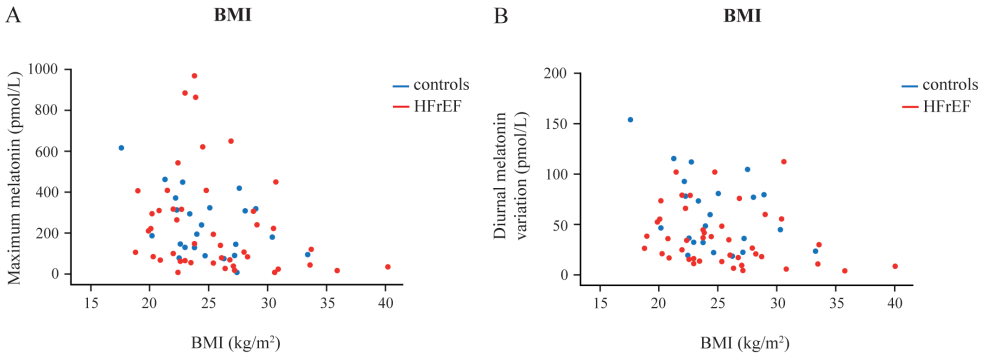

Figure S2 | **Association of BMI with (A) maximum melatonin values and (B) diurnal melatonin variation.** BMI, body mass index (kg/m²); HFrEF, heart failure with reduced ejection fraction.

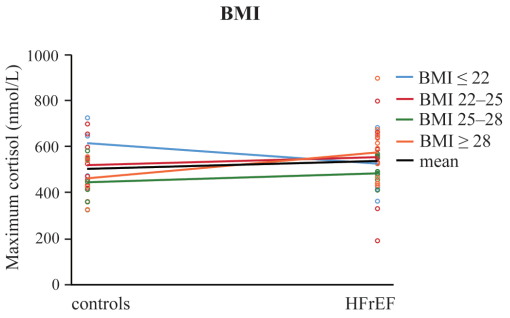

Figure S3 | **Interaction plot: effect modification of BMI on the relationship between heart failure and maximum cortisol.** BMI was identified as a possible effect modifier on the relationship between heart failure and maximum cortisol. Different relationships for different BMI subgroups are portrayed in the graph. Subgroups were chosen based on quartiles. BMI, body mass index (kg/m²); HFrEF, heart failure with reduced ejection fraction.

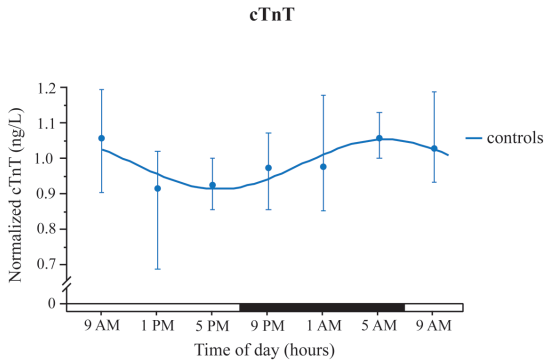

Figure S4 | **24-h analysis of cTnT values in control subjects without heart failure.** Cosinor analysis of normalized cTnT (ng/L) in controls (n=23-24 per each time-point), in a period of 24 hours. Per subject, each time-point was normalized against its mean cTnT value of the entire day. Blue line represents a fitted cosine curve. Values are median ± interquartile range (IQR). *P*<0.05 was used as a cut-off for significance. Horizontal bar indicates day (=white) and night (=black). cTnT, cardiac troponin T.

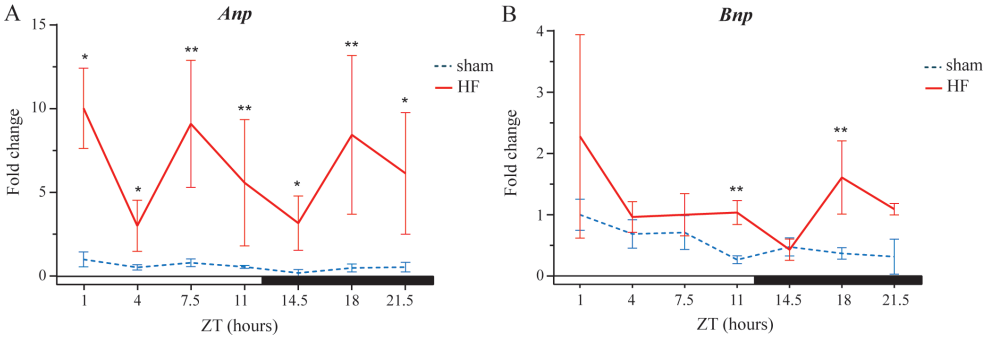

Figure S5 | **Confirmation of heart failure in mouse hearts.** Comparison of 24-hour **(A)** *Anp* and **(B)** *Bnp* expression levels in mouse heart between sham control and heart failure group, as determined by quantitative real-time PCR reaction (n=3-5 mice for each ZT). Values are mean ± standard error of mean (SEM). Data are normalized against ZT1 of the sham control group. To test differences between sham control and treated animals at the same zeitgeber time, independent two-sample T-test or Mann-Whitney U test were used, as appropriate. *P*<0.05 was used as a cut-off for significance. Significance of independent two-sample T-test or Mann-Whitney U test is indicated above corresponding time-point: **P*<0.05, ***P*<0.01, ****P*<0.001. Horizontal bar indicates lights-on (=white; ZT0) and lights-off (=black; ZT12) period. *Anp*, Natriuretic Peptide Type A; *Bnp*, Brain Natriuretic Peptide; HF, heart failure; ZT, zeitgeber time.

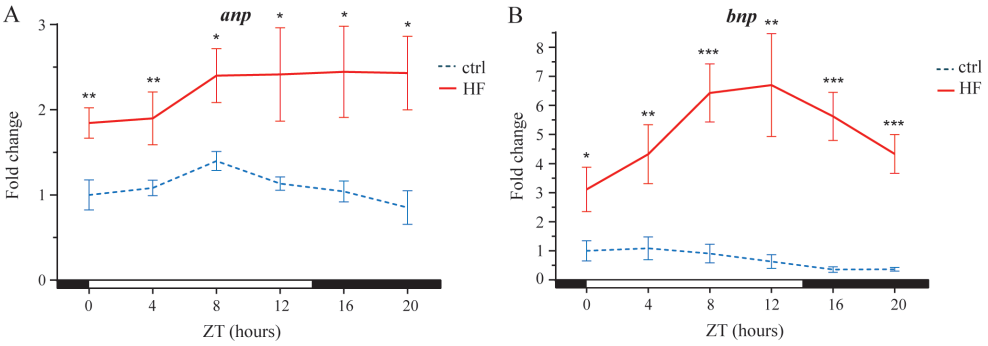

Figure S6 | **Confirmation of heart failure in zebrafish hearts.** Comparison of 24-hour **(A)** *anp* and **(B)** *bnp* expression levels in zebrafish heart between control and heart failure group, as determined by quantitative real-time PCR reaction (**(A)** n=6 and **(B)** n=9 zebrafish for each ZT; 2 and 3 biological replicates each including 3 pooled ventricles, respectively). Values are mean ± standard error of mean (SEM). Data are normalized against ZT0 of the control group. To test differences between control and treated animals at the same zeitgeber time, independent two-sample T-test or Mann-Whitney U test were used, as appropriate. $P<0.05$ was used as a cut-off for significance. Significance of independent two-sample T-test or Mann-Whitney U test is indicated above corresponding time-point: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Horizontal bar indicates lights-on (=white; ZT0) and lights-off (=black; ZT14) period. *anp*, Natriuretic Peptide Type A; *bnp*, Brain Natriuretic Peptide; HF, heart failure; ZT, zeitgeber time.

5

CHAPTER

Prognostic Biomarker Soluble ST2 Exhibits Diurnal Variation in Chronic Heart Failure Patients

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Abstract

Aim. Soluble suppression of tumorigenicity-2 (sST2) is a strong prognostic biomarker in heart failure. The emerging understanding of circadian biology in cardiovascular disease may lead to novel applications in prognosis and diagnosis and may provide insight into mechanistic aspects of the disease–biomarker interaction. So far, it is unknown whether sST2 exhibits a diurnal rhythm. Repeated measurements of sST2 may aid in clinical decision making. The goal of this study was to investigate whether sST2 exhibits diurnal variation in patients with heart failure with reduced ejection fraction (HFrEF) and in control subjects, thereby enhancing its diagnostic and prognostic values.
Methods and Results. The study comprised 32 subjects: 16 HFrEF patients and 16 controls. Blood was collected at seven subsequent time points during a 24 h time period. sST2, N-terminal pro-B-type natriuretic peptide (NT-proBNP), melatonin, and cortisol were measured from serum. Peak values of sST2 clustered at daytime (modal value: 5 PM) in 87.6% of all subjects (81.3% of patients, p=0.021; 93.8% of controls, p=0.001), and minimum concentrations at night-time (modal value: 5 AM) in 84.4% (87.5% of patients, p=0.004 81.3% of controls, p=0.021). A cosinor analysis of mean normalized sST2 values revealed significant cosine shaped 24 h oscillations of patients (p=0.026) and controls (p=0.037). NT-proBNP in contrast did not show a diurnal rhythm, while melatonin and cortisol patterns were intact in all subjects.
Conclusions. sST2 exhibits a diurnal rhythm with lower values in the morning than in the late afternoon. This new insight could lead to refinement of its diagnostic and prognostic values through specified and consistent sampling times with repeated measurements. For example, by measuring sST2 during the afternoon, when levels are at their highest, false negatives on prognosis prediction could be avoided.

Keywords: Circadian Rhythm, Diurnal Rhythm, Heart Failure, Biomarker, sST2, NT-proBNP

Introduction

Biomarkers are crucial components of clinical decision making as well as monitoring disease state and progression. Natriuretic peptides [B-type natriuretic peptide (BNP) and N-terminal pro-BNP (NT-proBNP)] are an important part of the guidelines for diagnosing heart failure (HF). Soluble suppression of tumorigenicity-2 (sST2) recently emerged as a promising prognostic tool for patients already diagnosed with HF, as well as an aid for risk stratification in identifying those at risk of mortality and prehospitalization[1]. Owing to its prognostic value, sST2 has been listed in the 2013 American College of Cardiology Foundation/American Heart Association guidelines as an important biomarker for the monitoring of HF patients[2]. However, the use of biomarkers comes with different challenges including analytical and biological variability, setting the appropriate cut-off values, and determining clinically meaningful changes in biomarker concentrations[3].

With a cut-off value set at 35 ng/mL, sST2 utilization has been shown to predict outcomes as well as to monitor and optimize therapy owing to its rapid concentration change with changing disease state[1]. An enzyme-linked immunosorbent assay has been developed by Critical Diagnostics (Presage ST2) for quantitative measurement of sST2[4], which, with coefficient of variation (CV) < 5%, has a high technical precision even when it comes to scarce analyte concentrations. Furthermore, age, elevated body mass index, or impaired renal function do not significantly affect sST2 values, while remaining common confounding situations for natriuretic peptide measurements.

Biological variation has been determined for sST2 values by means of serial measurements within the same subject over prolonged time periods only (e.g. days and months). However, while accumulating evidence has emphasized the importance of the circadian (24 h) clock in human physiology and pathophysiology[5,6], sST2 variation within a 24 h period is currently unknown. The role of circadian rhythms has also been confirmed in each type of cardiovascular tissues, influencing its normal function (e.g. heart rate and blood pressure) as well as the onset and severity of disease (e.g. myocardial infarction)[7]. Therefore, sST2 as a marker and possibly mediator of cardiac pathology might also be governed by the circadian clock.

Knowledge of the 24 h pattern in sST2 levels could have important consequences for its diagnostic and prognostic usage in clinic, by pinpointing one or several time points in a 24 h period necessary for valid assessment of disease and treatment. In addition, the existence of such a 24 h pattern may elucidate potential mechanistic aspects of the disease–biomarker interaction.

In this study, to our knowledge for the first time, within-day variations of sST2 levels are assessed in patients with HF with reduced ejection fraction (HFrEF) and healthy controls. 24 h variations of NT-proBNP were previously investigated[8], thereby serving as a control biomarker for our cohorts. Melatonin and cortisol were used as a control for circadian rhythms within our groups[9].

Methods

Study design and participants

The study was conducted according to the principles of the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO). It was approved by the Medical Ethics Committee of University Medical Centre Utrecht (Study Number 14/471).

Study participants were admitted to the University Medical Centre Utrecht. All provided written informed consent after which the blood withdrawal and biomarker analysis commenced (**Figure S1**). Furthermore, for each individual patient, health status was established based on electronic health records within a 12-month period after the blood withdrawal. For 1/16 control subject, the first time point (9 AM) is missing owing to problems with blood withdrawal.

Laboratory measurements

At seven subsequent time points (9 AM, 1 PM, 5 PM, 9 PM, 1 AM, 5 AM, and 9 AM), SST tubes (BD) were used to collect blood from an intravenous cannula (5 mL per time point). After collection, serum was separated by centrifugation and stored at 80°C until analysis. After all samples from all subjects had been collected, sST2 was quantitatively measured in triplicates by using the Presage® ST2 sandwich monoclonal immunoassay (Critical Diagnostics, San Diego, CA), according to the manufacturer's protocol. NT-proBNP was measured on a Cobas e411 analyzer using the Elecsys® NT-proBNP immunoassay (Roche Diagnostics, Indianapolis). Analysis of serum melatonin and cortisol concentrations was performed on liquid chromatography–tandem mass spectrometer[10].

Statistical analysis

Differences between the baseline characteristics of the two groups were compared by using Mann–Whitney U test, independent Student's t test, or Fisher's exact test, as appropriate. One-sample non-parametric binomial test

was used to test the chance of non-randomness of sST2 and NT-proBNP concentrations peaking at daytime or night-time. The range spread (%) was used to describe within-person difference between maximum and minimum values, calculated by dividing the range (difference between maximum and minimum) by the minimum value. Circadian parameters were calculated with cosinor analysis-based script in statistics software program R. In this program, a cosinor curve with a period of ~24 h that best fits the provided data is obtained by linear regression[11]. Throughout the manuscript, the following indications for significance were used: $^{*}P<0.05$ and $^{**}P<0.01$. All the analyses were performed in IBM SPSS Statistics (v. 23), and graphs were made in Adobe Illustrator CC (19.2.0).

Results

Patient characteristics

Thirty-two subjects were enrolled in the study: 16 patients with HFrEF (left ventricular ejection fraction < 40% by echo-cardiography), and 16 control subjects (**Table S1**). Baseline characteristics of all study participants are summarized in **Table 1**. The majority of participants were male (81.3% for patients and 75.0% for controls) with a mean age of 59 ± 13 years for patients and 54 ± 16 for controls. Among the enrolled patients, 75.0% were in New York Heart Association (NYHA) class III and 25.0% in NYHA class II. Outcomes after 1 year are shown in **Table S2**.

Circulating soluble suppression of tumorigenicity-2 exhibits distinct day and night variations

Peak sST2 values grouped clearly based on sampling time in the majority of subjects (**Figure 1**): 81.3% (p=0.021) of patients and 93.8% (p=0.001) of controls had their maximum sST2 concentrations during the day, while minimum concentrations were usually observed during night-time for both patients (87.5%; p=0.004) and controls (81.3%; p=0.021) (**Figure 1A**, **B**). Per subject, range spread of sST2 concentration spanned from a minimum of 7.8% to a maximum of 34.2% for patients (19.4% on average, n=16) (**Table 2**), and 9.6% to 60.9% for controls (23.5% on average, n=16) (data not shown). Taken together, both groups had their highest concentrations during daytime (modal value: 5 PM for 13/32 subjects; 1 PM for 9/32), declining towards their lowest values during the night (modal value: 5 AM, for 18/32 subjects). Among patients, of the three (18.8%) that deviated from the normal 24 h distribution of sST2 concentration (i.e. had their peak at night-time instead of daytime), one was in palliative setting of end-stage HF, the second recently had

an implantable cardioverter–defibrillator (ICD)-induced shock, and the third was on high-dose prednisone, which is known to affect the circadian clock[9]. These patients were included in all baseline tables and primary data analyses (n=16), but cosinor analysis for both sST2 and NT-proBNP was also performed without these three outliers to exclude confounding effects of severe clock disruption (indicated by n=13).

In order to visualize the overall diurnal patterns of patients with different absolute values, we also analysed normalized sST2 concentrations. Again, we observed a striking gradual decline from daytime values towards the night: a cosinor analysis of serum sST2 levels revealed cosine shaped 24 h oscillations in mean normalized values of HF patients (p=0.026 for n=13; p=0.072 for n=16) and controls (p=0.037, n=16) (**Figure 1C**, **D**, and **Figure S2**).

Circulating N-terminal pro-B-type natriuretic peptide fluctuates randomly within a 24 h period in heart failure with reduced ejection fraction patients and controls

Differences between minimum and maximum values of NT-proBNP ranged up to 76.5% in HFrEF patients within 24 h (**Table S3**). NT-proBNP concentration did not follow a diurnal pattern in either of the two groups, fluctuating randomly within a day (**Figure 2**). In HFrEF patients, distributions of maximum and minimum values on average were almost equally divided over day and night. During the day, NT-proBNP concentration reached its peak values in 56.3% and nadir in 43.8% of patients, while a similar peak/nadir prevalence was observed during the night (peak in 43.8% of patients, p=0.804; and nadir in 56.3%, p=0.804) (**Figure 2A**). In controls, peaks were reached in 75.0% during the day and 25.0% during the night (p=0.077), while nadir values appeared during the day in 31.3% and during the night 68.8% (p=0.210) (**Figure 2B**). Random distribution of maximum and minimum values throughout the 24 h period was also noted on mean normalized NT-proBNP concentrations (pg/mL) where cosinor rhythmicity was assessed using a fitted cosinor model in HFrEF patients and controls and showed no significance (p=0.091 for n=13; p=0.096 for n=16 patients; p=0.093 for n=16 controls) (**Figure 2C**, **2D**, and **Figure S3**). The non-circadian, non-repeating pattern is especially apparent from the very different 9 AM values that should be roughly the same in datasets with circadian distribution. Obtained data were in line with previously published studies in which no circadian or diurnal rhythm was found in circulating NT-proBNP[8].

Table 1 | **Baseline characteristics of study participants**

	Characteristics	All subjects (n=32)	Patients (n=16)	Controls (n=16)	*P* value
	Age (years)	57 ± 15	59 ± 13	54 ± 16	*0.310
	Male sex (%)	78.1	81.3	75.0	1.000
	BMI (kg/m²)	24.6 ± 3.9	24.8 ± 4.5	24.5 ± 3.3	†0.847
	Smoking (%)	53.1	75.0	31.1	0.032
	Alcohol (IU/week)	50.0	43.8	56.3	0.724
	CKD-EPI GFR (mL/min/1.73 m²)	66 ± 14	64 ± 22	69 ± 13	†0.501
Comorbidities (%)	Diabetes mellitus	15.6	31.3	0.0	0.018
	Myocardial infarction	15.6	25.0	6.3	0.600
	Atrial flutter/fibrillation	18.8	37.5	0.0	0.018
Medication (%)	ACE inhibitor	31.3	50.0	12.5	0.054
	Angiotensin receptor antagonist	18.8	25.0	12.5	0.654
	Antimineralocorticoid	34.4	68.8	0.0	<0.001
	Beta-blocker	25.0	43.8	6.3	0.037
Cause of cardiomyopathy (%)	Ischaemic		44.0		
	Non-ischaemic (valve, genetics, e.c.i.)		56.0		
Severity of heart failure (%)	NYHA class II		25.0		
	NYHA class III		75.0		
	Ejection fraction (%)		23.0 ± 7		

Values are mean ± standard deviation or percentage. Continuous variables were tested for normal distribution using skewness and kurtosis. Differences between groups were studied by the *Mann–Whitney U test, or †independent Student's t test or Fisher's exact test, as appropriate, with $P<0.05$ as a cut-off for significance. ACE inhibitor, angiotensin-converting-enzyme inhibitor; BMI, body mass index; CKD-EPI GFR, the estimated glomerular filtration rate calculated with the Chronic Kidney Disease Epidemiology Collaboration equation[21]; IU, international unit; NYHA class, New York Heart Association functional classification of heart failure severity[22]; pack-year, 1 year of smoking 20 cigarettes per day.

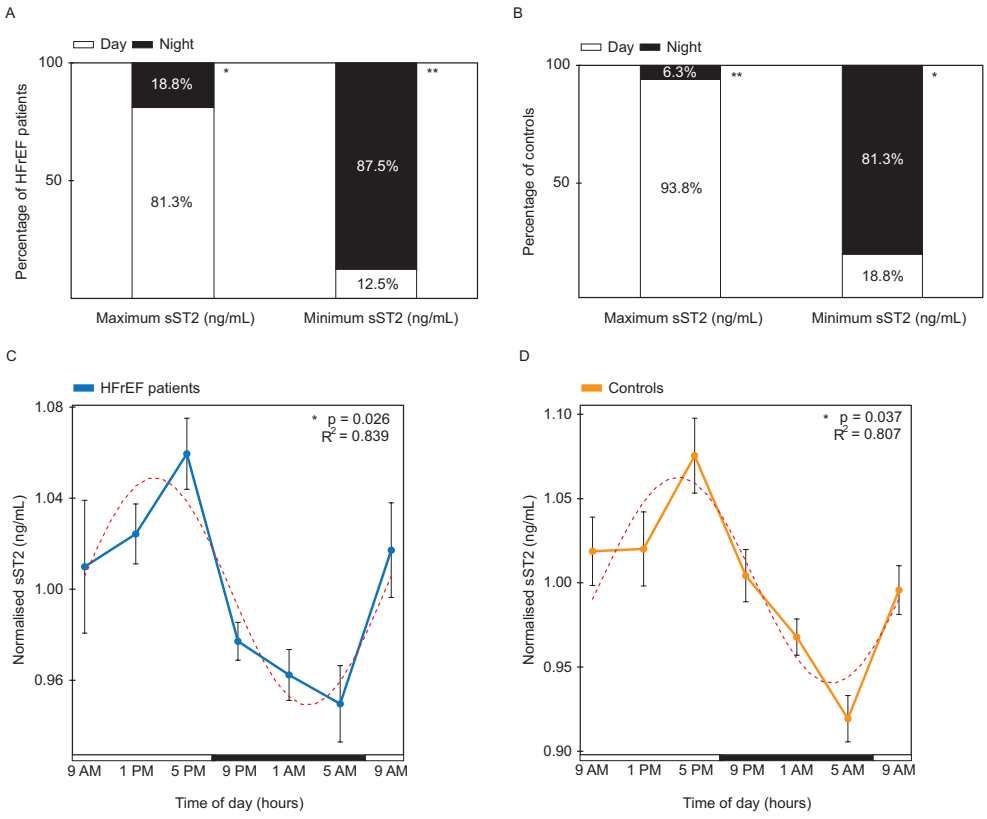

Figure 1 | **sST2 biomarker exhibits a diurnal rhythm in heart failure patients and controls. (A)** Percentage of HFrEF patients (n=16) who have their maximum and minimum sST2 values (ng/mL) during either the day (9 AM, 1 PM, and 5 PM) or night (9 PM, 1 AM, and 5 AM). **(B)** Percentage of controls (n=16) who have their maximum and minimum sST2 values (ng/mL) during either the day (9 AM, 1 PM, and 5 PM) or night (9 PM, 1 AM, and 5 AM). **(C)** Cosinor analysis of normalized sST2 (ng/mL) per each HFrEF patient (mean ± SEM), in a period of 24 h. Per subject, each time point was normalized against its mean sST2 value of the entire day (n=13). **(D)** Cosinor analysis of normalized sST2 (ng/mL) per each control (mean ± SEM), in a period of 24 h. Per subject, each time point was normalized against its mean sST2 value of the entire day (n=16). Horizontal bars indicate day (white) and night (black). One-sample non-parametric binomial test was used to test the chance of non-randomness of sST2 concentrations peaking at daytime or night-time. $P<0.05$ was used as a cut-off for significance. $^{*}P<0.05$ and $^{**}P<0.01$. R^2 indicates the proportion of the variance explained by the 24 h variation. Dashed red line represents a fitted cosine curve. HFrEF, heart failure with reduced ejection fraction; sST2, soluble suppression of tumorigenicity-2.

Table 2 | **Twenty-four-hour range of soluble suppression of tumorigenicity-2 concentration (ng/mL) in individual heart failure with reduced ejection fraction patients**

Patient number	Maximum (ng/mL)	Minimum (ng/mL)	Range (ng/mL)	Range spread (%)
1	101.9	94.5	7.4	7.8
2	**35.9**	**32.5**	**3.4**	**10.3**
3	**37.1**	**33.2**	**3.9**	**11.7**
4	19.2	16.9	2.3	13.9
5	25.6	22.5	3.1	13.9
6	47.8	41.9	5.9	14.1
7	62.7	53.9	8.9	16.5
8	101.9	87.4	14.5	16.6
9	65.9	55.6	10.2	18.4
10	54.6	45.5	9.1	19.9
11	21.6	17.7	3.9	22.0
12	64.2	51.5	12.8	24.8
13	65.4	51.1	14.3	27.9
14	33.2	25.8	7.4	28.6
15	71.4	55.3	16.1	29.2
16	33.1	24.7	8.4	34.2

Maximum = highest measured sST2 concentration (ng/mL) in 24 h period. Minimum = lowest measured sST2 concentration (ng/mL) in 24 h period. Range = maximum–minimum. Range spread = range/minimum. Patients in which maximum and minimum sST2 values fluctuate above and below 35 ng/mL cut-off value are indicated in bold. HFrEF, heart failure with reduced ejection fraction; sST2, soluble suppression of tumorigenicity-2.

Robust circadian markers remain normal in heart failure patients and controls

Serum melatonin and cortisol patterns within the 24 h period were as expected in all of 36 subjects, regardless of the presence or absence of HF (**Figure 3**). Melatonin was consistently low during daytime in both groups, with the highest concentrations during the night (modal value: 5 AM), while cortisol had diametrically opposite values and peaked during the early morning (modal value: 9 AM). Distribution of peak times for both hormones is listed in **Table S4**.

Discussion

In this study, we provide new insights in so far uninvestigated circadian properties of circulating sST2 levels in HF patients. In the vast majority of

subjects, sST2 concentration reached its peak in the afternoon, with the lowest levels during the night-time. Knowledge of this diurnal variation will lead to improved usage of sST2 as a prognostic biomarker.

Circadian rhythms in cardiovascular (patho)physiology

The importance of circadian rhythms in cardiovascular health and disease has been recognized and emphasized in many studies to date. We recently provided an in-depth explanation of circadian biology and its interplay with cardiovascular function and dysfunction[7]. Briefly, functional circadian rhythms have been found in every cell type within the cardiovascular system, influencing its function in many ways; from vascular tone, heart rate, blood pressure, signalling, and cardiac metabolism to thrombus formation, onset of myocardial infarction, and arrhythmias. Therefore, it does not come as a surprise that certain cardiovascular-related biomarkers are also under direct or indirect influence of the clock and exhibit day and night fluctuations.

The goal of the study was to investigate whether sST2 exhibits circadian variation in patients with HFrEF and controls without cardiovascular disease. While the existence of a circadian rhythm in sST2 serum concentrations was unknown, it has been previously investigated for other biomarkers. For example, cardiac troponin T was shown to exhibit non-random diurnal variation[12], while predictable daily fluctuations were absent in NT-proBNP[8] and cardiac troponin I[13] levels. Serving as a negative control in our study, NT-proBNP fluctuated randomly throughout the day and indeed did not exhibit any rhythmicity in HF patients or controls. Concentrations of melatonin and cortisol, robust circadian markers, were measured to serve as positive controls of internal circadian rhythms, with cortisol levels peaking during the day and melatonin levels during the night. Observed data were in line with expected rhythmic behaviour of these main endocrine products of the central clock[9], pointing to preserved neurohumoral circadian output in both subject groups.

Diurnal oscillation of soluble suppression of tumorigenicity-2 biomarker

To the best of our knowledge, this is the first record of the diurnality of circulating sST2 concentrations, resulting in important implications for clinical usage in terms of prognosis and therapy guidance (**Figure 4**). Circulating sST2, reflecting stress brought upon myocardial injury and adverse remodelling, recently emerged as a strong predictor of HF outcome. Indeed, within our HFrEF patients, the majority of patients with mean sST2 values higher than 35 ng/mL either died or suffered from another major adverse outcome. For detailed description of sST2 biology and its role in mediating myocardial strain, as well as in the development of vascular disease, the reader is referred

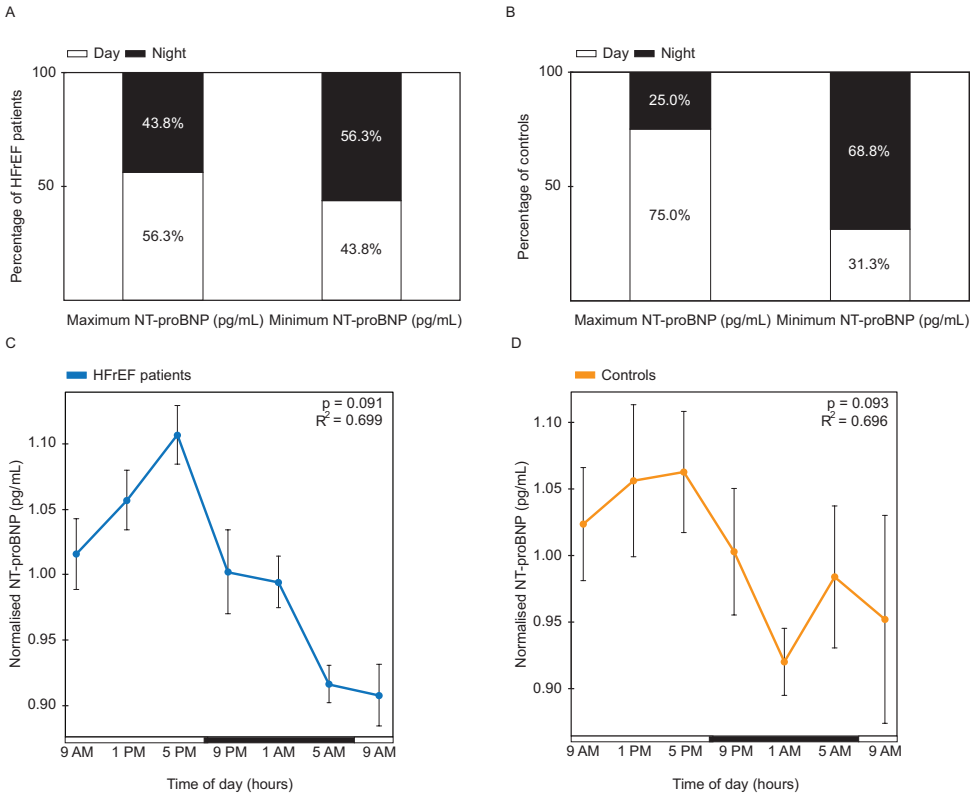

Figure 2 | **NT-proBNP biomarker fluctuates randomly within a day in heart failure patients and controls. (A)** Percentage of HFrEF patients (n=16) who have their maximum and minimum NT-proBNP values (pg/mL) during either the day (9 AM, 1 PM, and 5 PM) or night (9 PM, 1 AM, and 5 AM). **(B)** Percentage of controls (n=16) who have their maximum and minimum NT-proBNP values (pg/mL) during either the day (9 AM, 1 PM, and 5 PM) or night (9 PM, 1 AM, and 5 AM). **(C)** Cosinor analysis of normalized NT-proBNP (pg/mL) per each HFrEF patient (mean ± SEM), in a period of 24 h. Per subject, each time point was normalized against its mean NT-proBNP value of the entire day (n=13). **(D)** Cosinor analysis of normalized NT-proBNP (pg/mL) per each control (mean ± SEM), in a period of 24 h. Per subject, each time point was normalized against its mean NT-proBNP value of the entire day (n=16). Horizontal bars indicate day (white) and night (black). One-sample non-parametric binomial test was used to test the chance of non-randomness of NT-proBNP concentrations peaking at daytime or night-time. $P<0.05$ was used as a cut-off for significance. R^2 indicates the proportion of the variance explained by the 24 h variation. HFrEF, heart failure with reduced ejection fraction; NT-proBNP, N-terminal pro-B-type natriuretic peptide.

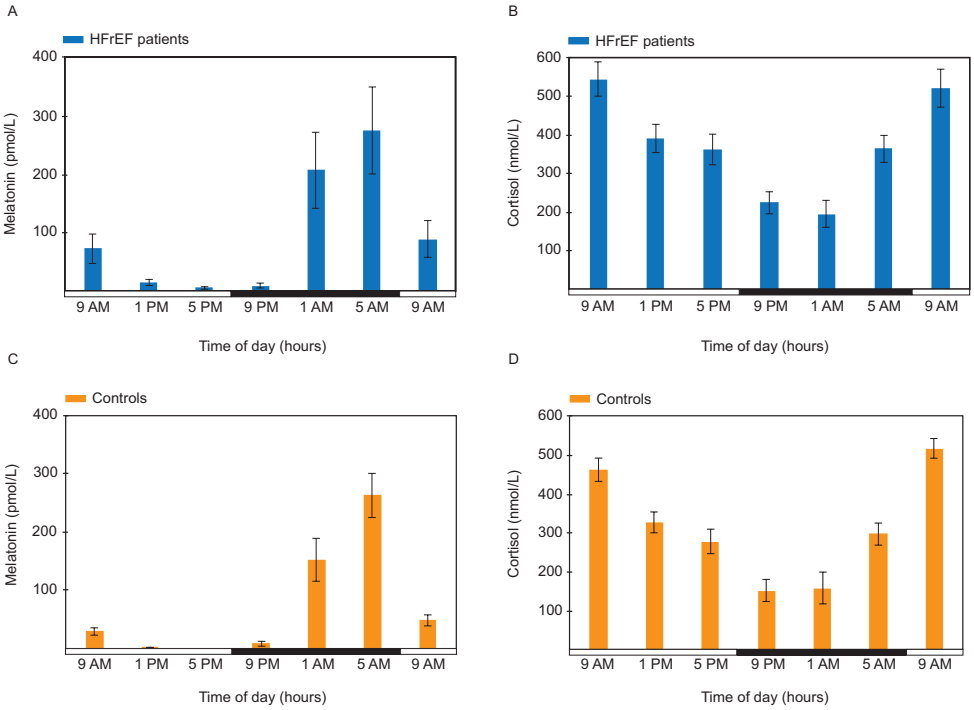

Figure 3 | **Study subjects exhibit normal melatonin and cortisol patterns.** Within-day distribution of **(A)** melatonin (pmol/L) and **(B)** cortisol concentration (nmol/L) in heart failure patients (mean ± SEM). Within-day distribution of **(C)** melatonin (pmol/L) and **(D)** cortisol concentration (nmol/L) in control subjects (mean ± SEM). Horizontal bar indicates day (white) and night (black). HFrEF, heart failure with reduced ejection fraction.

to recently published review articles[14,15]. sST2, acting as a decoy receptor for IL-33 and thereby inhibiting its beneficial cardioprotective effects, is implicated in cardiac and vascular remodelling. As favourable effects of IL-33 are blocked with the abnormal quantities of sST2 in circulation, measurement of sST2 concentration has proven useful in providing prognostic information for various cardiovascular disorders.

Aside from its prognostic value, sST2 could be used to monitor treatment effect and subsequently guide therapy; it was previously shown that patients with reduction of sST2 concentration in response to drug treatment had better outcomes than those with continued sST2 values > 35 ng/mL during the entire study period[16]. Notably, biomarkers are increasingly used in clinical HF trials as an inclusion criterion, surrogate endpoint, and target for

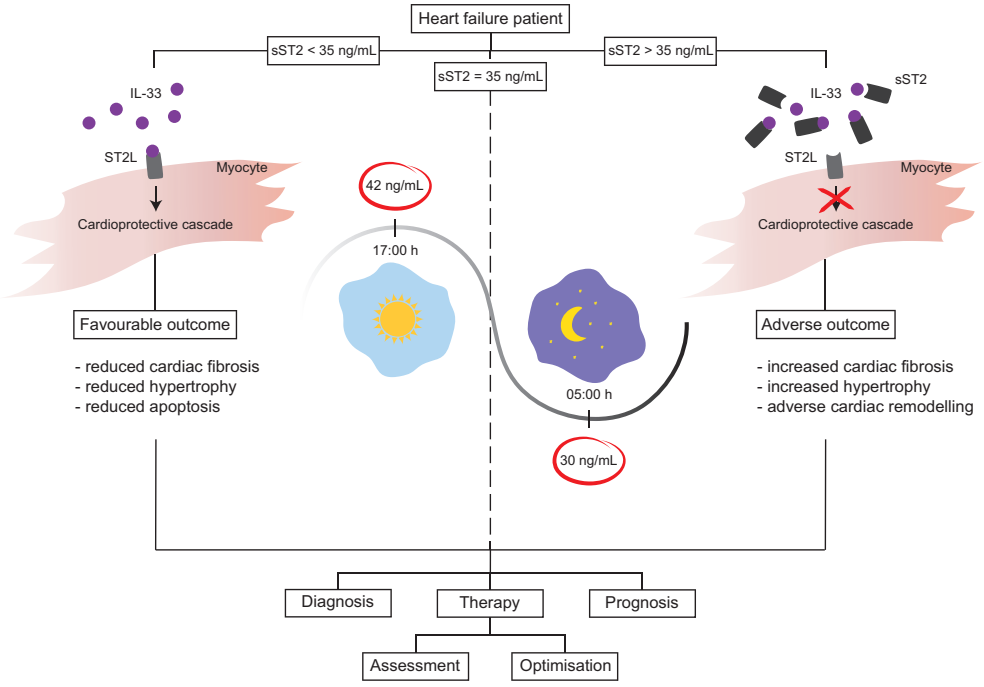

Figure 4 | **Influence of diurnal rhythms on clinical interpretation of sST2 biomarker.** Knowledge about the diurnal variation of sST2 concentration is needed for proper timing of measurements and their interpretation. Prognostic and diagnostic value of sST2 levels, as well as therapy optimization, should be refined by applying specified sampling times and repeated measurements. IL-33, interleukin-33; sST2, soluble suppression of tumorigenicity-2; ST2L, suppression of tumorigenicity-2 ligand.

therapy[17]. Thus, a rise or fall in sST2 concentration caused by physiological diurnal variation may lead to misinterpretation of treatment effect, overestimation or underestimation of prognostic warning signs, and confounding of clinical trial outcomes. It is therefore of importance to take into account at what time the blood sample was taken.

Interestingly, while the diurnal pattern of sST2 concentration was remarkably consistent across sex, age, and other baseline characteristics (**Table 1**), our data indicate that end-stage HF in palliative setting, recent ICD-induced shock, and high-dose prednisone can lead to changes in peak time of sST2 levels. Thus, while the existence of physiological diurnal variation of sST2 levels can have an important impact on clinical decision making, a deviating 24 h pattern may also have predictive value.

Clinical outlook

Although sST2 is yet to be implemented in the clinic as a standard of care, it has already proven to be a robust prognostic biomarker for both chronic and acute HFrEF[14]. As previously mentioned, the currently applied cut-off value of sST2 in chronic HF is 35 ng/mL; however, reached consensus did not take into consideration possible biological 24 h variation of its concentration[18]. Our study provides the first evidence for circadian rhythmicity of sST2, implying that time of day may be an important variable when determining sST2 levels. Therefore, clinicians are advised to be cautious while interpreting sST2 values, because a result just under 35 ng/mL in the morning may exceed this threshold in the afternoon. In **Table 2**, we show examples of this phenomenon in two patients.

Because of the diurnal rhythm of sST2, it would be recommended to measure sST2 during the afternoon, when sST2 levels are commonly at their highest, if the aim is to provide maximal negative predictive value and assure patient safety. Future studies re-evaluating the cut-off value of sST2 taking sample timing into account may further improve sST2 as a prognostic tool.

Finally, the average range spread of sST2 concentrations found in our patient group was 19.4%, with a maximum range spread of 34.2% in 24 h. Therefore, when using serial sST2 levels to assess the efficacy of initiated HF therapy and refine prognostic value[16], samples should ideally be taken around the same hour or corrected for time of day as much as possible. For example, a first measurement in the afternoon and a follow-up measurement in the morning (yielding a lower sST2 concentration) may suggest an improvement in the patient's condition and a good therapeutic response, while in fact it demonstrates circadian fluctuation.

Study limitations

This study did not make use of standardized conditions for physical activity, meals, light exposure, and sleep duration, as is commonly done for clinical circadian experiments in order to achieve a more comparable circadian rhythm between subjects[12]. Nevertheless, our set-up makes the study clinically more translatable, as patients usually do not follow an externally imposed food/activity regimen. This way, all consistent differences found within daily levels of investigated biomarkers prove to be highly robust, if evident without a controlled environmental setting.

Another limitation of this study lies in the groups assembly, as the majority of the participants were of the male gender (78.1% of total participants). Coglianese *et al.* found a significant difference in sST2 levels between men and women[19], with higher concentrations noted in men. In our

data, we observed similar diurnal patterns in men and women, but the relatively low number of women precludes definite conclusions on comparisons between both sexes. Furthermore, smoking has been shown to increase IL-33 concentrations in the airway epithelium as well as to alter the ST2 expression pattern in the lung[20]. A positive smoking status was reported in 53.1% of our subjects, however, with no obvious effect on the diurnality of sST2. Despite these limitations, the goal of our study, which was to observe diurnal differences within daily sST2 levels, was not affected and even strengthened.

Finally, sample size of the study was relatively low (n=16 for each group), and three patients were excluded from the cosinor analysis owing to the severe clock disruption. However, this was sufficient to provide the first evidence for a diurnal pattern of sST2 expression. The uniqueness of this study lies in the repeated sST2 measurements during a 24 h period within each study participant. Even though the final number of included patients and controls is modest, sST2 measurements during seven time points per each patient/control provide a resolution that has not been reported before in ST2 and HF research.

Conclusions

Biomarker sST2 concentrations exhibit significant diurnal variation with predictable peak and trough times, a novel finding that has consequences for the clinical interpretation of sST2 levels in HF patients. Besides its impact on clinical management and clinical trials, the within-day fluctuation of sST2 also gives hints for further investigation into its mechanism of action. Presumably, sST2-driven inhibition of the cardioprotective IL-33–ST2L pathway also has a time-dependent character. While clinical significance is covered within the content of this study, biological importance is yet to be determined, potentially leading towards novel therapeutic strategies.

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Conflicts of Interest

S.C., M.P., T.J., L.L., M.M., H.S., M.F., B.P., N.J., C.G., H.K., P.D., and J.S. have no conflict of interest. F.A. was supported by UCL Hospitals NIHR Biomedical Research Centre. L.L. received (related to the current work) NT-proBNP assays from Roche and ST2 assays from Sopachem BV, in a form of an investigator-initiated study, and (outside) consultancy fees from Abbott, Vifor, Novartis, and Medtronic, to the UMCU.

References

1. Boisot, S. et al. Serial sampling of ST2 predicts 90-day mortality following destabilized heart failure. *J. Card. Fail.* **14**, 732–738 (2008).
2. Yancy, C. W. et al. American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. *Circulation* **128**, e240–e327 (2013).
3. Miller, W. L. & Jaffe, A. S. Biomarkers in heart failure: the importance of inconvenient details. *ESC Heart. Fail.* **3**, 3–10 (2015).
4. Dieplinger, B. et al. Analytical and clinical evaluation of a novel high-sensitivity assay for measurement of soluble ST2 in human plasma—the Presage ST2 assay. *Clin. Chim. Acta.* **409**, 33–40 (2009).
5. Mohawk, J. A., Green, C. B. & Takahashi, J. S. Central and peripheral circadian clocks in mammals. *Annu. Rev. Neurosci.* **35**, 445–462 (2012).
6. Kelly, R. M., Healy, U., Sreenan, S., McDermott, J. H. & Coogan, A. N. Clocks in the clinic: circadian rhythms in health and disease. *Postgrad. Med. J.* **94**, 653–658 (2018).
7. Crnko, S., Du Pré, B. C., Sluijter, J. P. G. & Van Laake, L. W. Circadian rhythms and the molecular clock in cardiovascular biology and disease. *Nat. Rev. Cardiol.* **16**, 437–447 (2019).
8. Goetze, J. P., Jørgensen, H. L., Sennels, H. P. & Fahrenkrug, J. Diurnal plasma concentrations of natriuretic propeptides in healthy young males. *Clin. Chem.* **58**, 789–792 (2012).
9. Oster, H. et al. The functional and clinical significance of the 24-hour rhythm of circulating glucocorticoids. *Endocr. Rev.* **38**, 3–45 (2017).
10. van Faassen, M., Bischoff, R. & Kema, I. P. Relationship between plasma and salivary melatonin and cortisol investigated by LC-MS/MS. *Clin. Chem. Lab. Med.* **55**, 1340–1348 (2017).
11. Refinetti, R., Lissen, G. C. & Halberg, F. Procedures for numerical analysis of circadian rhythms. *Biol. Rhythm Res.* **38**, 275–325 (2007).
12. Klinkenberg, L. J. J. et al. Circulating cardiac troponin T exhibits a diurnal rhythm. *J. Am. Coll. Cardiol.* **63**, 1788–1795 (2014).
13. van der Linden, N. et al. Strong diurnal rhythm of troponin T, but not troponin I, in a patient with renal dysfunction. *Int. J. Cardiol.* **221**, 287–288 (2016).
14. McCarthy, C. P. & Januzzi, J. L. Soluble ST2 in heart failure. *Heart. Fail. Clin.* **14**, 41–48 (2018).
15. Vianello, E., Dozio, E., Tacchini, L., Frati, L. & Corsi Romanelli, M. M. ST2/IL-33 signaling in cardiac fibrosis. *Int. J. Biochem. Cell. Biol.* **116**, 105619 (2019).
16. van Vark, L. C. et al. Prognostic value of serial ST2 measurements in patients with acute heart failure. *J. Am. Coll. Cardiol.* **70**, 2378–2388 (2017).
17. Ibrahim, N. E., Gaggin, H. K., Konstam, M. A. & Januzzi, J. L. Jr. Established and emerging roles of biomarkers in heart failure clinical trials. *Circ. Heart. Fail.* **9**, e002528 (2016).
18. Januzzi, J. L., Mebazaa, A. & Di Somma, S. ST2 and prognosis in acutely decompensated heart failure: the International ST2 Consensus Panel. *Am. J. Cardiol.*

115, 26B–31B (2015).
19. Coglianese, E. E. et al. Distribution and clinical correlates of the interleukin receptor family member soluble ST2 in the Framingham Heart Study. *Clin. Chem.* **58**, 1673–1681 (2012).
20. Kearley, J. et al. Cigarette smoke silences innate lymphoid cell function and facilitates an exacerbated type I interleukin-33-dependent response to infection. *Immunity* **42**, 566–579 (2015).
21. Levey, A. S. et al. A new equation to estimate glomerular filtration rate. *Ann. Intern. Med.* **150**, 604–612 (2009).
22. Ponikowski, P. et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC) Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur. Heart. J.* **37**, 2129–2200 (2016).

Supporting information

Table S1 | **Inclusion and exclusion criteria for participants.**

Inclusion criteria
Admission to the UMC Utrecht
>18 years of age
Signed informed consent
Chronic systolic heart failure NYHA class II-III (*patients*)
No signs or symptoms of heart failure (*controls*)
Exclusion criteria
Blindness
End stage renal failure (CKD-EPI GFR < 15 ml/min/1.73m^2)
Fever > 38°C
CRP > 20 mg/L
Use of hypnotics or other medication that disturbs the normal sleep pattern during the study period
Anesthesia within 48 hours prior to start study
Serious comorbidity that could influence circadian rhythms (e.g. severe neurological, psychiatric or oncologic diseases)

CKD-EPI GFR, the estimated glomerular filtration rate calculated with the Chronic Kidney Disease Epidemiology Collaboration equation[1]; CRP, C-reactive protein; NYHA class, New York Heart Association functional classification of heart failure severity[2].

Table S2 | **12-month outcome of HFrEF patients in relation to average sST2 concentrations (ng/mL).**

Average ST2 (ng/mL)	Average NT-proBNP (pg/mL)	12-month outcome
18.0	2382	Re-hospitalization
20.2	2080	No events
23.9	160	No events
29.0	431	No events
30.0	3382	No events
34.8	6255	LVAD
35.9	778	Deceased
45.6	10769	No events
50.7	1874	No events
56.0	2437	LVAD
58.0	5235	LVAD
59.0	1502	No events

Table S2 | **Continued**

Average ST2 (ng/mL)	Average NT-proBNP (pg/mL)	12-month outcome
60.1	5399	Deceased
62.5	11141	LVAD
95.3	3162	No events
98.4	1168	Deceased

The sST2 cut-off value of 35 ng/mL is indicated by the dotted line. HFrEF, heart failure with reduced ejection fraction; LVAD, Left Ventricular Assist Device; NT-proBNP, N-terminal pro–B-type natriuretic peptide; sST2, soluble Suppression of Tumorigenicity-2.

Table S3 | **24-hour range of NT-proBNP (pg/mL) in individual HFrEF patients**

Patient number	Range (pg/mL)	Range spread
1	298	29.6%
2	693	11.6%
3	114	16.1%
4	845	32.6%
5	74	59.2%
6	1826	18.4%
7	320	22.9%
8	1072	40.8%
9	1242	26.8%
10	335	19.6%
11	330	17.6%
12	780	36.7%
13	902	18.0%
14	1828	76.5%
15	3653	37.9%
16	166	39.5%

Range = difference between maximum and minimum concentration of NT-proBNP (pg/mL) in 24-hour period. Range spread = range / minimum concentration of NT-proBNP (pg/mL) in 24-hour period. HFrEF, heart failure with reduced ejection fraction; NT-proBNP, N-terminal pro–B-type natriuretic peptide.

Table S4 | **Distribution of peak times for melatonin and cortisol in HFrEF patients and controls**

	Melatonin peak time		Cortisol peak time			
	1 AM	**5 AM**	**9 AM**	**1 PM**	**5 PM**	**1 AM**
HFrEF patients (n)	2	14	13	2	1	
Control subjects (n)	2	14	14		1	1

n, total number of subjects; HFrEF, heart failure with reduced ejection fraction.

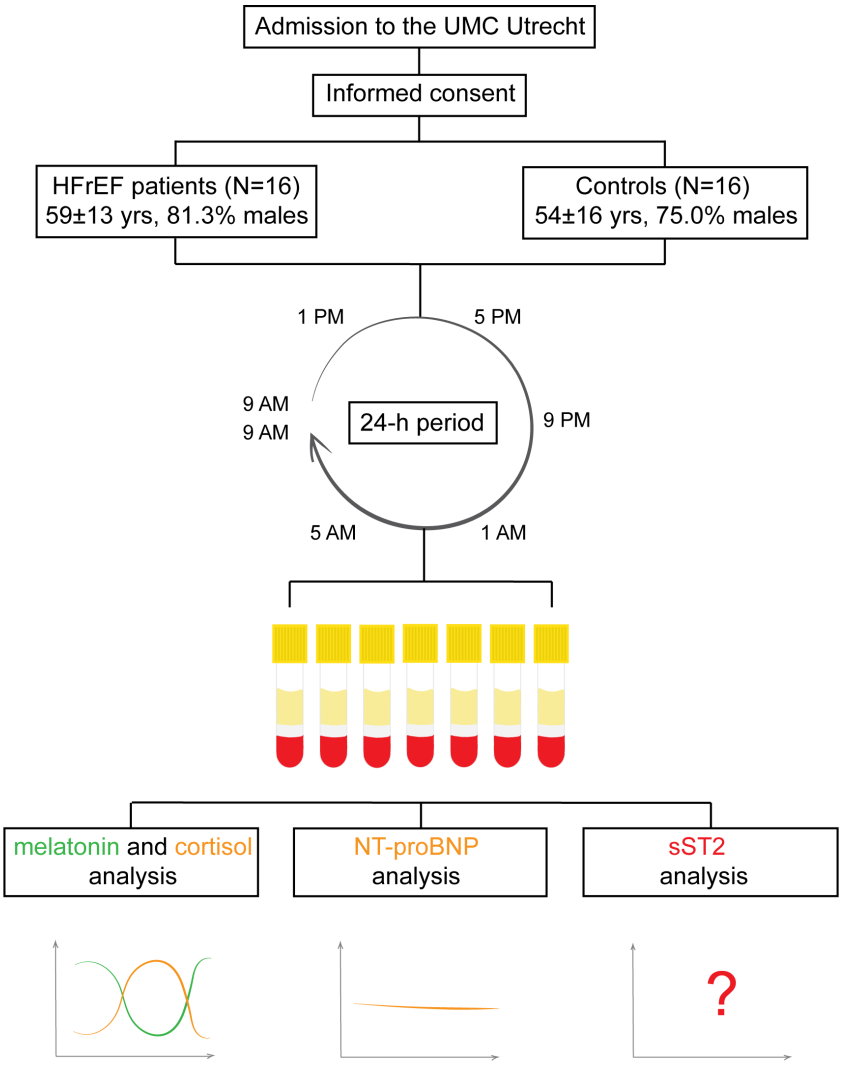

Figure S1 | **Study design overview.** After being admitted to the University Medical Centre Utrecht, study participants provided with the signed informed consent. The study started with 9 AM as a first time-point, after which blood was collected every four hours in a 24-hour period, resulting in seven samples in total per participant. Collected serum was used to measure concentrations of melatonin, cortisol, NT-proBNP and sST2. HFrEF, heart failure with reduced ejection fraction; N, total number of subjects; NT-proBNP, N-terminal pro–B-type natriuretic peptide; sST2, soluble Suppression of Tumorigenicity-2.

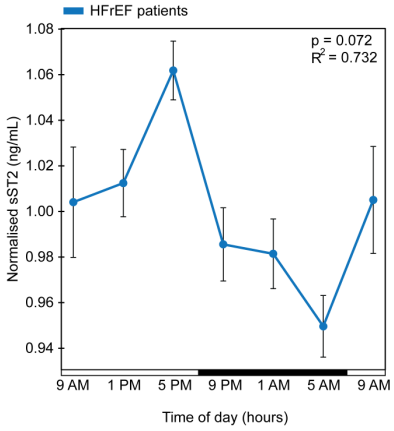

Figure S2 | **Overall 24-hour values of sST2 biomarker in heart failure patients.** Cosinor analysis of normalized sST2 (ng/mL) per each HFrEF patient (mean ± SEM), in a period of 24 hours. Per subject, each time-point was normalized against its mean sST2 value of the entire day (n=16). *P*<0.05 was used as a cut-off for significance. R^2 indicates the proportion of the variance explained by the 24-hour variation. Horizontal bar indicates day (white) and night (black). HFrEF, heart failure with reduced ejection fraction; sST2, soluble Suppression of Tumorigenicity-2.

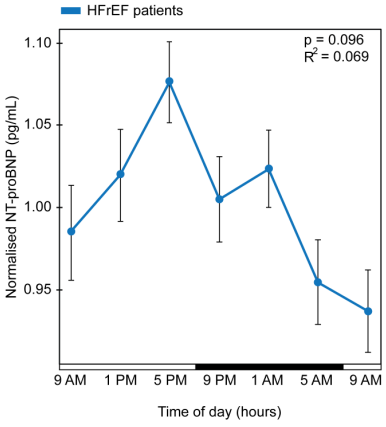

Figure S3 | **Overall 24-hour values of NT-proBNP biomarker in heart failure patients.** Cosinor analysis of normalized NT-proBNP (pg/mL) per each HFrEF patient (mean ± SEM), in a period of 24 hours. Per subject, each time-point was normalized against its mean sST2 value of the entire day (n=16). *P*<0.05 was used as a cut-off for significance. R^2 indicates the proportion of the variance explained by the 24-hour variation. Horizontal bar indicates day (white) and night (black). HFrEF, heart failure with reduced ejection fraction; NT-proBNP, N-terminal pro–B-type natriuretic peptide.

6

CHAPTER

The Importance of Morning CardioMEMS Measurements

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Based on:
Crnko, S. *et al.* Morning pulmonary artery pressure measurements by CardioMEMS are most stable and recommended for pressure trends monitoring. *Neth Heart J* **29**: 409-414 (2021).

Abstract

Aim. The CardioMEMS HF system is used to measure pulmonary artery (PA) pressures of heart failure (HF) patients. The goal of this study was to determine the impact of time in the daily PA pressure measurements, considering variance and influence of circadian rhythms on cardiovascular pathophysiology.

Methods and Results. The study included 10 patients with HF with reduced ejection fraction (LVEF < 40%; NYHA III). Individual daily PA pressures were obtained by CardioMEMS sensors, per protocol, measured up to six times throughout the day, for a period of 5 days. Differences between variation of morning vs. evening PA pressures were compared with Wilcoxon signed-rank test.

Mean PA pressures (mPAP) increased from a morning value of 19.1 ± 2 mmHg (8 AM; mean ± SEM) to 21.3 ± 2 mmHg late in the evening (11 PM; mean ± SEM). Over the course of 5 days, evening mPAP exhibited a significantly higher median coefficient of variation than morning mPAP (14.9 (IQR 7.6 – 21.0) and 7.0 (IQR 5.0 – 12.8), respectively; p=0.01). The same daily pattern of pressure variability was observed in diastolic (p=0.01) and systolic (p=0.04) pressures, with diastolic pressures being more variable than systolic at all time-points.

Conclusions. Morning PA pressure measurements yield more stable values for observing PA trends. Patients should thus be advised to consistently perform their daily PA pressure measurements early in the morning. This will improve reliability and interpretation of the CardioMEMS management, indicating true alterations in the patient's health status, rather than time-of-day dependent variations.

Keywords: Heart Failure, CardioMEMS, Circadian Rhythm, Individualized Therapy, Telemonitoring

Introduction

Heart failure (HF) has become a major socio-economic burden as it is characterized by episodes with acute decompensation, requiring hospitalization, and is associated with increased morbidity and mortality[1,2]. The CardioMEMS system, an implantable wireless pulmonary artery (PA) pressure monitor, was shown to reduce hospitalizations by improving HF management in the CHAMPION trial[3]. Since the 2016 European Society of Cardiology guidelines on HF[4], ambulatory hemodynamic monitoring may be considered (IIb indication) in symptomatic patients with previous HF hospitalisation. Subsequent analyses and data obtained in the clinical setting showed an improved quality of life and reduced morbidity and mortality using ambulatory hemodynamic monitoring[5-8].

Currently, HF patients with a CardioMEMS sensor are instructed by the manufacturer to measure PA pressures in the morning. However, it has not yet been investigated whether morning indeed yields the most precise PA pressure measurements, while some patients may prefer a different time for practical reasons, or may vary the time of their measurement considerably. Additionally, while daily patterns of PA pressures measured in a consistent manner are currently not available, CardioMEMS provides the opportunity to study them. Previous studies on PA pressures in HF suggest the existence of diurnal rhythms[9,10]. Given the emerging importance of these circadian rhythms in cardiovascular pathophysiology[11], we aimed to define the most optimal time of day for measuring PA pressures in HF patients.

Methods

Study design

A total of 10 consecutive patients (NYHA class III), who had received a CardioMEMS sensor according to the current guidelines[4], participated in this study. The study was conducted at the University Medical Center Utrecht and Erasmus University Medical Center in accordance with the declaration of Helsinki and the Medical Research Involving Human Subjects Act. All participants provided written informed consent. Individual chronotypes were self-assessed using the Dutch morningness-eveningness questionnaire (VOA) and PA pressure measurements were extracted from the online system (merlin.net).

Measurements of PA pressures

The CardioMEMS sensor was used to obtain individual daily measurements of

PA pressures at six time-points throughout the day (8 AM, 11 AM, 2 PM, 5 PM, 8 PM, 11 PM) for a period of two consecutive days, followed by three time-points (8 AM, 5 PM, 11 PM) for the next three consecutive days. Measurements were downloaded for analysis using the CardioMEMS online system (merlin.net).

Statistical analysis

The coefficient of variation (CV) was used as a measure of relative variability of mean (mPAP), systolic (sPAP), and diastolic (dPAP) PA pressures, either within- or between-days. The CV was calculated as the ratio of the standard deviation to the mean, and expressed as a percentage. Wilcoxon signed-rank test was used to compare differences between CVs of PA pressures, and Student's t-test was used to compare differences between morning and evening heart rate. A p-value of <0.05 was considered statistically significant. All analyses were performed in IBM SPSS Statistics (v. 23).

Results

Patient characteristics

A total of 10 patients (mean age 59 ± 9 years, 60% male) were enrolled in this study. Patient characteristics are summarized in **Table 1**. All patients were in NYHA class III at the time of CardioMEMS implantation with diuretics and optimal guideline-directed medical therapy.

Diurnal variation of pulmonary artery pressure

Within a 24-h period, in seven out of 10 patients mPAP values increased from morning to evening. The mean mPAP value was 19.1 ± 2 mmHg in the morning (8 AM; mean ± SEM) and 21.3 ± 2 mmHg in the evening (11 PM; mean ± SEM) (**Figure 1A**), thus a relative increase of 11.5% (range: - 6 mmHg = - 28.6% to + 10 mmHg = + 66.7%). The biggest difference was noted in patient 1, with a morning mPAP of 15 mmHg which increased to 25 mmHg at 11 PM.

Next, in order to visualise the overall diurnal patterns of patients with different absolute values, normalised mPAP, sPAP and dPAP were analysed (n=10, **Figure 1B-D**). Again, the same pattern of highest PA pressures was observed late in the evening.

Pulmonary artery pressure is more stable in the mornings than in the evenings

Since clear reproducible differences between morning and evening measurements were observed, we investigated whether it is relevant to strictly

prescribe morning vs. evening PA pressures in daily practice using the between-day CV. Interestingly, the between-day CV of mPAP, sPAP and dPAP was significantly higher in the evening than in the morning measurements (p=0.01, p=0.04, and p=0.01, respectively; **Table 2**). When considering mPAP, the median CV of evening pressures was twice as high compared to morning (14.9 (IQR 7.6 – 21.0) and 7.0 (IQR 5.0 – 12.8), respectively) (**Table 3**). Additionally, the within-day variation was higher than the between-day CV for morning mPAP during 5 consecutive days, illustrating the importance and stability of daily morning measurements (**Figure 2**). sPAP and dPAP followed the same trend (**Table S1**), with dPAP being less stable than mPAP or sPAP

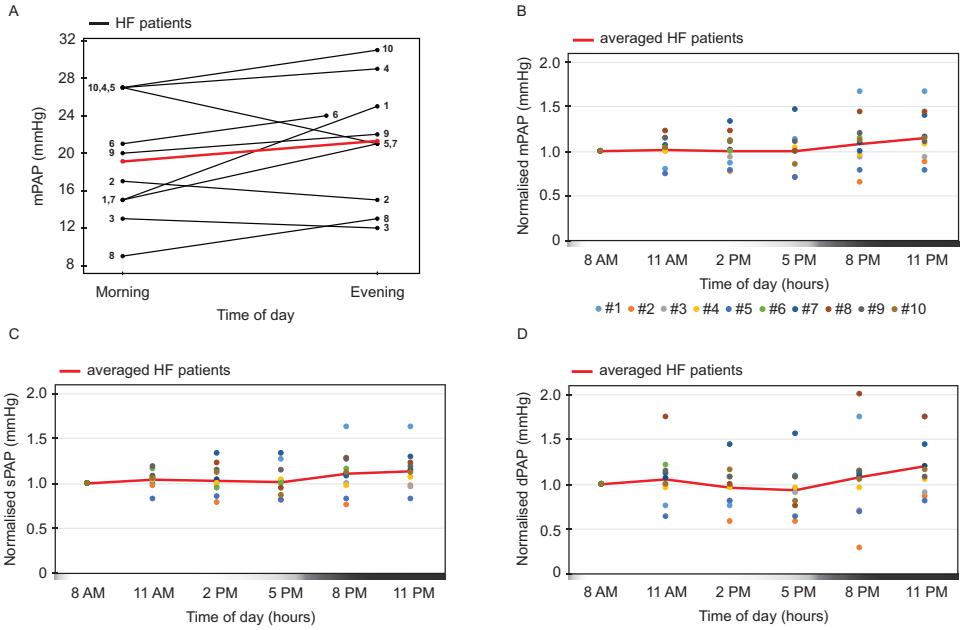

Figure 1 | **Pulmonary artery pressure increases in heart failure patients in the evening. (A)** Morning (8 AM) and evening (11 PM for all patients; except for patient 6, 8 PM) mPAP (mmHg) within-day distribution of 10 individual HF patients. Mean values of morning and evening measurements each are indicated with red line. **(B, C, D)** Within-day distribution of normalised PA pressures (mmHg) in each HF patient (n=6/ patients; except for patient 3 and 6, n=5). Per patient, each measurement was normalised against the value of the first time-point of either **(B)** mPAP, **(C)** sPAP or **(D)** dPAP. Red line represents averaged normalised values of all patients per time-point (n=10; except for time-point 11 AM and 11 PM, n=9). Gradient bar depicts time of day (white=day, black=night). dPAP, diastolic pulmonary artery pressure; HF, heart failure; mPAP, mean pulmonary artery pressure; PAP, pulmonary artery pressure; sPAP, systolic pulmonary artery pressure.

Table 1 | **Patient characteristics at the time of CardioMEMS implantation**

		All subjects (n=10)
Characteristics	Age (years)	59.0 ± 9
	Male sex	60.0
	BMI (kg/m^2)	26.5 ± 3
	eGFR (ml/min/1.73m^2)	52.9 ± 16
	Heart rate (bpm)	69.9 ± 12
	Systolic blood pressure (mmHg)	98.5 ± 12
	Diastolic blood pressure (mmHg)	59.6 ± 9
	Mean arterial pressure (mmHg)	72.6 ± 8
Laboratory assessment	NT-proBNP (pmol/L)	232.2 ± 164
	Sodium (mmol/L)	138.0 ± 2
	Potassium (mmol/L)	4.3 ± 0
	AST (U/L)	34.9 ± 21
	ALT (U/L)	33.2 ± 19
	LD (U/L)	266.9 ± 124
	Gamma-GT (U/L)	44.7 ± 46
	Alkaline phosphatase (U/L)	94.3 ± 45
	Hb (mmol/L)	7.9 ± 1
Comorbidities	Diabetes mellitus	30.0
	Myocardial infarction	50.0
	Atrial flutter/fibrillation	20.0
	Hypertension	10.0
	COPD	0.0
	OSAS	0.0
Cause of cardiomyopathy	iCMP / DCM	50.0 / 50.0
Severity of heart failure	Ejection fraction (%)	23.9 ± 4
	NYHA III (%)	100.0
	HTx	10.0
Medication	Beta blocker	70.0
	RAASi (ARNi)	100.0 (50.0)
	MRA	100.0
	Diuretics	100.0

Values are mean ± standard deviation or percentage. ALT, alanine aminotransferase; ARNi, angiotensin receptor-neprilysin inhibitor; AST, aspartate aminotransferase; BMI, body mass index; COPD, chronic obstructive pulmonary disease; DCM, dilated cardiomyopathy; eGFR, the estimated glomerular filtration rate calculated with the Chronic Kidney Disease Epidemiology Collaboration equation[13]; Gamma-GT, gamma-glutamyl transferase; Hb, haemoglobin; HTx, heart transplantation; iCMP, ischemic cardiomyopathy; LD, lactate dehydrogenase; LVEF, left ventricular ejection fraction; MRA, mineralocorticoid receptor antagonist; NT-proBNP, N-terminal pro b-type natriuretic peptide; NYHA class, New York Heart Association functional classification of heart failure severity[4]; OSAS, obstructive sleep apnoea syndrome; RAASi, renin-angiotensin-aldosterone system inhibitor.

both in mornings and evenings (**Table 2**). Although patients' activity could influence the PA pressures, it does not seem to be the cause of the observed high variation in the evening values, based on the CV of heart rate measurements (6.1 (IQR 4.5 – 12.0) in the morning and 3.2 (IQR 2.6 – 9.2) in the evening, p=0.17; **Table S2**). Intriguingly, patients who exhibited the highest PA pressure variation in the evenings scored as either moderate- or pronounced-morning chronotypes on the VOA questionnaire (data not shown), pointing to the influence of individual diurnal rhythms.

Discussion

In this study, we provided more scientific evidence supporting the current CardioMEMS recommendations to measure PA pressures in the morning. The present data demonstrate the stability of morning PA pressures, suggesting that any observed differences in morning values are more likely reflecting clinically relevant changes in heart failure status, rather than merely natural diurnal variation. Changes in evening PA pressures could be attributed to circadian fluctuations and apparently correct themselves overnight, at least in clinically stable patients.

While diurnal properties of PA pressures were previously explored[9,10], this is the first study to directly assess its clinical relevance in an ambulatory setting. We sought to determine the most optimal time of day for PA pressure measurements based on intra- and inter-days' variation. In order to do so, we opted for a translational setup by omitting time-points during the night (between 12 AM and 6 AM), when maximal PA pressures were previously noted[9].

Table 2 | **Degree of variation between morning and evening PA pressure measurements**

	CV (%) of mPAP (IQR)	CV (%) of sPAP (IQR)	CV (%) of dPAP (IQR)
Morning	7.0 (5.0 – 12.8)	7.0 (5.3 – 10.7)	9.4 (6.6 – 21.2)
Evening	14.9 (7.6 – 21.0)	11.4 (6.1 – 17.1)	15.6 (10.3 – 31.7)
P value	0.01	0.04	0.01

Median CV values are calculated based on measurements of five consecutive days, either in the morning (8 AM; n=5/patient) or in the evening (11 PM; n=5/per patient; except for patient 6, n=2). CV (%) is calculated as the ratio of the standard deviation to the mean. Wilcoxon signed-rank test was used to test differences between morning and evening CV of mPAP, sPAP and dPAP. CV, coefficient of variation; dPAP, diastolic pulmonary artery pressure; IQR, interquartile range; mPAP, mean pulmonary artery pressure; sPAP, systolic pulmonary artery pressure.

Table 3 | **Between-days variation of mPAP (mmHg) in heart failure patients**

Patient number	Time of day	Minimum mPAP (mmHg)	Maximum mPAP (mmHg)	Mean mPAP (mmHg)	CV (%)
1	Morning	15.0	22.0	17.6	15.4
	Evening	12.0	25.0	17.4	30.6
2	Morning	14.0	19.0	16.2	11.9
	Evening	10.0	18.0	13.6	22.4
3	Morning	13.0	14.0	13.2	3.4
	Evening	11.0	16.0	12.6	15.5
4	Morning	24.0	29.0	26.6	6.8
	Evening	26.0	29.0	27.2	4.8
5	Morning	24.0	27.0	25.6	5.2
	Evening	17.0	21.0	19.6	8.5
6	Morning	19.0	23.0	20.8	7.1
	Evening	19.0	24.0	21.5	16.4
7	Morning	15.0	18.0	16.6	6.9
	Evening	18.0	26.0	22.6	14.2
8	Morning	9.0	14.0	12.2	16.8
	Evening	11.0	19.0	14.8	20.5
9	Morning	20.0	22.0	20.6	4.3
	Evening	21.0	22.0	21.4	2.6
10	Morning	26.0	31.0	28.6	7.3
	Evening	25.0	32.0	29.8	9.3

Values are calculated based on measurements of five consecutive days, either in the morning (8 AM; n=5/patient) or in the evening (11 PM; n=5/patient; except for patient 6, n=2). CV (%) is calculated as the ratio of the standard deviation to the mean. CV, coefficient of variation; mPAP, mean pulmonary artery pressure.

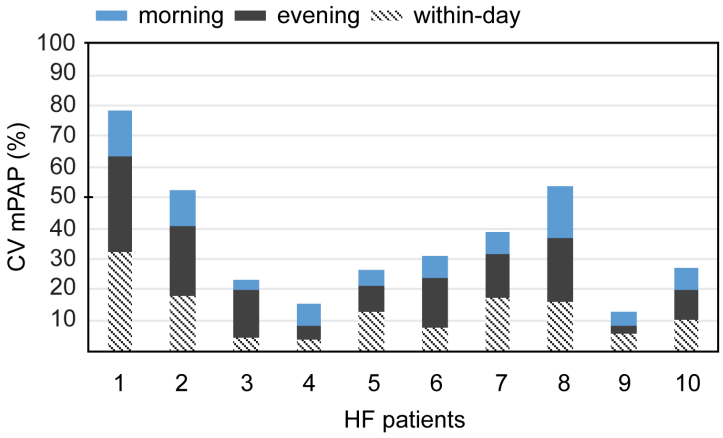

Figure 2 | **Pulmonary artery pressure in heart failure patients varies less when measured in the mornings than in the evenings.** Values are calculated based on measurements of five consecutive days, either in the morning (8 AM; n=5 for each patient) or in the evening (11 PM; n=5/patient; except for patient 6, n=2), or within a 24-h period (n=6/patient; except for patient 3 and 6, n=5). Each bar represents a single HF patient. CV (%) is calculated as the ratio of the standard deviation to the mean. CV, coefficient of variation; HF, heart failure; mPAP, mean pulmonary artery pressure.

In most of the subjects, PA pressures increased in the evening. The evening rise of PA pressures is in line with other studies[9,10], and ascribed to its diurnal nature. Furthermore, over the course of five days, PA pressures exhibited different degrees of time-of-day-dependent variation. Morning measurements were consistently stable in the vast majority of patients, while noticeable fluctuations were usually observed in the evening. Diastolic PA pressure varied more than systolic PA pressure, regardless of the time of day. Pronounced variation in the evenings could be explained by patients' daily activities, e.g. food and fluid daily intake variation, effect of different medications, as well as physical activity. All of these factors influence important physiological processes directly and via sympathetic nerve system or RAAS activation, and can cause PA pressures to fluctuate[12].

Collectively, our data offers a 2-fold evidence to support the current CardioMEMS recommendations to measure PA pressures in the morning. Firstly, we show that PA pressures exhibit a diurnal rhythm, with lowest pressures measured in the morning, and reaching their peak in the evening. Therefore, by measuring PA pressures consistently in the mornings, any changes in pressures could be ascribed to a change in the patient's condition. For example, if the pressure would first be measured in the morning (= lowest PA pressure), with a follow-up measurement in the evening (= highest PA

pressure), it could indicate a deterioration of the patient's condition, while it actually only reflects a mere circadian fluctuation. If the pressure is always measured in the morning, any deviating recording of PA pressures would be a clear indication that an intervention is needed. Secondly, as previously mentioned, PA pressures may be affected by patient's activity during the day, including exercise, and food, fluid, and medication intake. The susceptibility of PA pressures to these daily changes could explain why evening PA pressures were least stable in the majority of subjects during a course of five days. Combined with their diurnal properties, highest and least consistent PA pressures are observed in the evening. Thus, morning PA pressure measurements are more reliable and better reflect the true changes in the patient's condition (e.g. worsening of heart failure), rather than showing the influence of daily activities (e.g. food intake, exercise, etc.) which will reset until the next day. In conclusion, morning PA pressure measurements, as opposed to evening measurements that are prone to external influences, will give a clear indication for intervention if relevant increase in values is observed.

Limitations

Our study included a relatively low number of patients (n=10), however, this was sufficient to show consistent changes in daily PA pressures. A large set of time-points per patient was provided, both within the 24 hours and during the course of five days. This gives a better representation of the individual PA pressure fluctuations, substantiating derived conclusions about morning PA pressure stability.

Conclusion

Given the pronounced variation in ambulatory PA pressures during the day, standardized morning PA pressure measurements will improve reliability and interpretation of the values provided by the CardioMEMS sensor (see **Take home figure**). Clinicians will be able to ascertain which changes of the PA pressures indicate true alterations in the patient's health status (e.g. worsening of HF) and which merely reflect natural diurnal properties.

Acknowledgements

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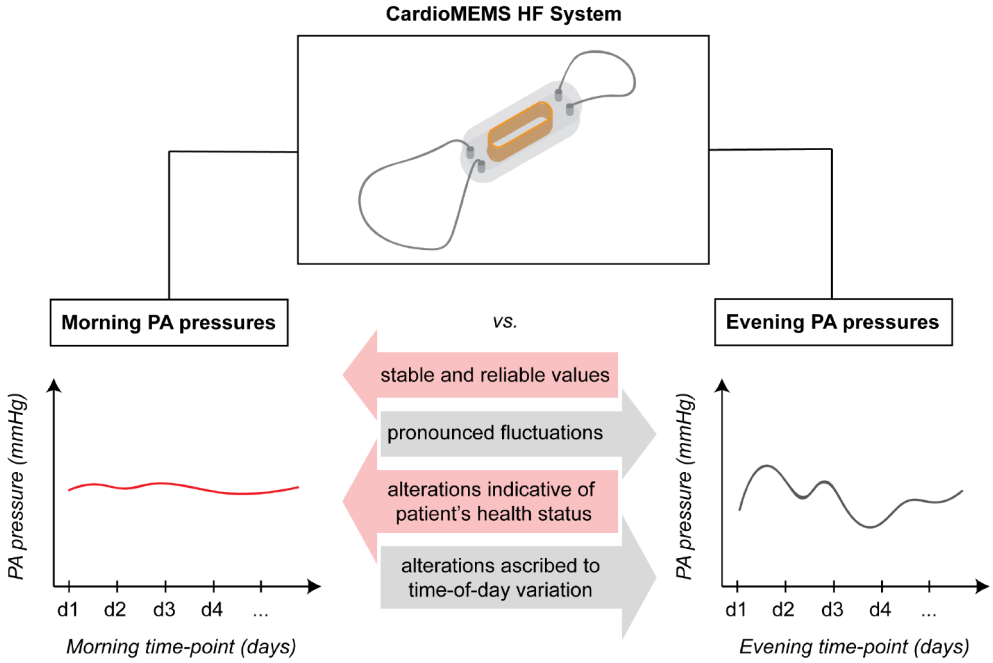

Figure 3 | **Take home figure.** Morning pulmonary artery pressure measurements provided by the CardioMEMS sensor, as opposed to evening measurements, yield more stable and reliable values.

Funding

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Declaration of interest

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References

1. Filippatos, G. & Zannad, F. An introduction to acute heart failure syndromes: definition and classification. *Heart Fail. Rev.* **12**, 87–90 (2007).
2. Mosterd, A. & Hoes, A. W. Clinical epidemiology of heart failure. *Heart* **93**, 1137–1146 (2007).
3. Abraham, W. T. et al. Wireless pulmonary artery haemodynamic monitoring in chronic heart failure: a randomised controlled trial. *Lancet* **377**, 658–666 (2011).
4. Ponikowski, P. et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC) Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur. Heart J.* **37**, 2129–2200 (2016).
5. Cowie, M. R., Simon, M., Klein, L. & Thokala, P. The cost-effectiveness of real-time pulmonary artery pressure monitoring in heart failure patients: a European perspective. *Eur. J. Heart Fail.* **19**, 661–669 (2017).
6. Desai, A. S. et al. Ambulatory Hemodynamic Monitoring Reduces Heart Failure Hospitalizations in 'Real-World' Clinical Practice. *J. Am. Coll. Cardiol.* **69**, 2357–2365 (2017).
7. Givertz, M. M. et al. Pulmonary Artery Pressure-Guided Management of Patients With Heart Failure and Reduced Ejection Fraction. *J. Am. Coll. Cardiol.* **70**, 1875–1886 (2017).
8. Brugts, J. J. et al. A randomised comparison of the effect of haemodynamic monitoring with CardioMEMS in addition to standard care on quality of life and hospitalisations in patients with chronic heart failure : Design and rationale of the MONITOR HF multicentre randomised clinical trial. *Neth. Heart J.* **28**, 16–26 (2020).
9. Gibbs, J. S. et al. Diurnal variation of pulmonary artery pressure in chronic heart failure. *Br. Heart J.* **62**, 30–35 (1989).
10. Brigden, G. & Raftery, E. B. Diurnal variation in pulmonary artery pressure. *J. Sleep Res.* **4**, 37–43 (1995).
11. Crnko, S., Du Pré, B. C., Sluijter, J. P. G. & Van Laake, L. W. Circadian rhythms and the molecular clock in cardiovascular biology and disease. *Nat. Rev. Cardiol.* **16**, 437–447 (2019).
12. Gibbs, J. S., Keegan, J., Wright, C., Fox, K. M. & Poole-Wilson, P. A. Pulmonary artery pressure changes during exercise and daily activities in chronic heart failure. *J. Am. Coll. Cardiol.* **15**, *52–61 (1990).*
13. *Levey, A. S. et* al. A new equation to estimate glomerular filtration rate. *Ann. Intern. Med.* **150**, 604–612 (2009).

Table S1 | **Between-days variation of sPAP and dPAP in heart failure patients**

Patient number	Time of day	Minimum		Maximum		Mean		CV (%)	
		sPAP (mmHg)	dPAP (mmHg)	sPAP (mmHg)	dPAP (mmHg)	sPAP (mmHg)	dPAP (mmHg)	sPAP (mmHg)	dPAP (mmHg)
1	Morning	19.0	12.0	27.0	19.0	23.4	14.2	13.0	19.5
	Evening	19.0	8.0	31.0	21.0	24.0	13.6	20.2	39.1
2	Morning	27.0	5.0	35.0	9.0	30.6	6.4	11.5	26.1
	Evening	23.0	2.0	33.0	8.0	28.0	5.0	14.3	44.7
3	Morning	21.0	9.0	23.0	10.0	22.0	9.4	4.5	5.8
	Evening	19.0	8.0	26.0	11.0	21.4	8.8	12.6	14.8
4	Morning	30.0	20.0	37.0	24.0	33.6	22.4	7.5	6.8
	Evening	32.0	21.0	36.0	24.0	33.8	22.6	4.4	5.9
5	Morning	34.0	14.0	40.0	18.0	37.2	15.8	6.4	9.4
	Evening	28.0	9.0	33.0	14.0	31.2	12.0	6.6	15.6
6	Morning	27.0	13.0	34.0	16.0	31.4	13.8	8.6	9.4
	Evening	27.0	12.0	36.0	16.0	31.5	14.0	20.2	20.2
7	Morning	27.0	9.0	31.0	12.0	29.0	10.4	5.5	12.9
	Evening	33.0	12.0	42.0	17.0	37.8	15.0	10.1	15.6
8	Morning	18.0	4.0	23.0	9.0	20.8	6.8	10.4	26.3
	Evening	21.0	5.0	31.0	11.0	25.2	8.6	16.0	29.2
9	Morning	27.0	14.0	30.0	16.0	28.6	14.8	4.0	5.7
	Evening	30.0	14.0	32.0	15.0	31.0	14.8	3.2	3.0
10	Morning	44.0	19.0	51.0	23.0	47.6	21.0	6.2	7.5
	Evening	42.0	17.0	52.0	23.0	48.8	21.4	8.5	11.7

Values are calculated based on measurements of five consecutive days, either in the morning (8 AM; n=5/patient) or in the evening (11 PM; n=5/patient; except for patient 6, n=2). CV (%) is calculated as the ratio of the standard deviation to the mean. CV, coefficient of variation; dPAP, diastolic pulmonary artery pressure; mPAP, mean pulmonary artery pressure; sPAP, systolic pulmonary artery pressure.

Table S2 | **Between-days variation of heart rate in heart failure patients**

Patient number	Time of day	Maximum HR (bpm)	Minimum HR (bpm)	Mean HR (bpm)	CV (%) HR (bpm)	*P* value Morning vs. evening HR
1	Morning	105.0	89.0	97.6	7.0	0.85
	Evening	103.0	95.0	98.2	3.5	
2	Morning	65.0	57.0	61.4	4.7	0.26
	Evening	63.0	53.0	58.4	7.1	
3	Morning	65.0	60.0	62.8	3.1	0.03
	Evening	69.0	65.0	66.8	2.7	
4	Morning	102.0	76.0	85.8	11.7	0.84
	Evening	90.0	79.0	84.4	5.3	
5	Morning	88.0	77.0	81.0	5.2	0.00
	Evening	69.0	65.0	67.0	2.4	
6	Morning	94.0	71.0	76.6	12.8	0.73
	Evening	89.0	86.0	87.5	2.4	
7	Morning	61.0	56.0	58.0	4.0	0.49
	Evening	76.0	54.0	60.6	15.5	
8	Morning	86.0	66.0	75.6	9.4	0.40
	Evening	81.0	75.0	78.4	2.9	
9	Morning	88.0	79.0	81.8	4.7	0.54
	Evening	83.0	78.0	80.2	2.7	
10	Morning	65.0	44.0	49.4	17.8	0.21
	Evening	67.0	43.0	51.6	17.6	

Values are calculated based on measurements of five consecutive days, either in the morning (8 AM; n=5/patient) or in the evening (11 PM; n=5/patient; except for patient 6, n=2). CV (%) is calculated as the ratio of the standard deviation to the mean. *P* values indicate differences between morning and evening HR values per patient, calculated with Student's t-test. CV, coefficient of variation; HR, heart rate.

7 CHAPTER

Pirfenidone has Anti-fibrotic Effects in a Tissue-engineered Model of Human Cardiac Fibrosis

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[*]Equal contribution

Based on:
Bracco Gartner, TCL. *et al.* Pirfenidone has anti-fibrotic effects in a tissue-engineered model of human cardiac fibrosis. (submitted)

Abstract

Aim. A fundamental process in the development and progression of heart failure is fibrotic remodelling, characterized by excessive deposition of extracellular matrix proteins in response to injury. Currently, therapies that effectively target and reverse cardiac fibrosis are lacking, warranting novel therapeutic strategies and reliable methods to study their effect.
Methods and Results. In this study, a multi-cellular mechanically tuneable 3D *in vitro* model of human cardiac fibrosis is presented, consisting of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) and human foetal cardiac fibroblasts (hfCF) embedded in a gelatine methacryloyl (GelMA) hydrogel. Stimulation with exogenous TGF-β1 results in an evident fibrotic response, reflected by the elevated expression of pro-fibrotic genes, detected by quantitative real-time PCR, immunofluorescence imaging and targeted proteomics. Pirfenidone serves as a test-drug with supposed anti-fibrotic effects to verify the drug-testing potential of this model. In this study, pirfenidone exhibits anti-fibrotic effects but does not reverse all TGF-β1 induced pro-fibrotic changes.
Conclusions. Overall, this study reports the development of a multi-cellular mechanically tuneable 3D *in vitro* model of human cardiac fibrosis. It provides proof-of-concept of the drug testing potential of this platform and yields new insights into the mechanism of action of the novel anti-fibrotic drug pirfenidone.

Keywords: Cardiac Fibrosis, Tissue-engineering, Disease Modeling, Pirfenidone, Targeted Proteomics, 3D Cell Culture

Introduction

On a tissue level, heart failure is characterized by cardiomyocyte hypertrophy and apoptosis, and cardiac fibrosis.[1] The main effector cell driving cardiac fibrosis is the cardiac fibroblast, which remodels the cardiac extracellular matrix (ECM) upon physiological environmental stimuli. Although cardiac fibroblasts play a pivotal role in the initially beneficial wound healing response by depositing collagens to protect the structural integrity of the cardiac ECM[2], perpetual activation of cardiac fibroblasts during prolonged pathological stress exposure leads to ongoing deposition and accumulation of fibrous ECM material, which eventually causes cardiac dysfunction, most notably diastolic dysfunction, and increased susceptibility for lethal cardiac dysrhythmias.[3] Current treatments for heart failure aim at reducing signs and symptoms, but do not target cardiac fibrosis directly.[4]

Although many factors are known to be involved, cardiac fibrosis is primarily regulated by transforming growth factor beta (TGF-β). This growth factor activates cardiac fibroblasts into secretory myofibroblasts by inducing formation of α-smooth muscle actin (α-SMA)-containing stress-fibers and promoting expression of ECM genes, most notably increasing collagen and periostin levels.[5] Although TGF-β is a key factor, treating cardiac fibrosis by targeting TGF-β directly has so far not yielded satisfactory results.[4,6]

The current challenge of targeting cardiac fibrosis directly reveals both our incomplete understanding of this complex pathophysiological process and the need to improve the development of novel cardiovascular drugs.[4,7,8] An important issue in cardiovascular drug development is the large share of compounds that seem promising in preclinical animal research but fail to show efficacy in human clinical trials.[9] To address this challenge, three-dimensional (3D) *in vitro* models of the human heart have been created using tissue-engineering techniques.[10] Earlier work by our group established the feasibility of mimicking cardiac fibrosis *in vitro* and has investigated the anti-fibrotic effect of cardiac progenitor cells in 3D tissue-engineered constructs containing human cardiac fibroblasts.[11,12]

The use of 3D tissue-engineered constructs with a physiological ECM stiffness is essential for reliably studying cardiac fibrosis *in vitro*, as cardiac cell behavior is known to be influenced through mechanosensitive pathways.[13] Cardiomyocytes have been shown to contract best on hydrogels with an elastic modulus in the physiological range and induced pluripotent stem cell-derived cardiomyocytes (iPS-CMs) show enhanced maturation and contraction patterns when cultured in a 3D environment which recapitulates the human heart.[14,15] Furthermore, cardiac fibroblasts are known to spontaneously

transdifferentiate into their active pro-fibrotic form when exposed to a high substrate stiffness, but to stay quiescent in a 3D environment.[16] As such, using a 3D cell culture system with mechanical properties that mimic the human heart seems an important next step to study cardiac fibrosis and develop new anti-fibrotic treatments.

A promising approach with anti-fibrotic effects, clinically used to treat idiopathic pulmonary fibrosis, is pirfenidone.[17] Although several preclinical studies have shown its anti-proliferative effect on fibroblasts and decreased deposition of collagens in numerous organs, the precise molecular targets and mechanism of action of pirfenidone are unknown.[18-21] The extent of cardiac fibrosis was reduced in rodent models of myocardial infarction[22] and pressure overload[23-25], but to date, the effects of pirfenidone on human cardiac fibrosis are largely unknown.

In this study, we report the optimization of our previously developed 3D tissue-engineered *in vitro* model of human cardiac fibrosis by using a co-culture of iPS-CMs and primary cardiac fibroblasts in a mechanically tunable hydrogel. We demonstrate the feasibility of using this human cardiac fibrosis model as a drug-testing platform, by evaluating the effects of a new anti-fibrotic drug – pirfenidone – on the transcriptomic and proteomic level and providing new insights into its mechanism of action.

Methods

Gelatin methacryloyl fabrication

Gelatine methacryloyl (GelMA) was prepared as reported previously.[33] Briefly, gelatine type A from porcine skin (Sigma-Aldrich) was dissolved in PBS (10% w/v) under continuous stirring for 20 minutes at 60°C. Gelatine molecules were modified by adding methacryloyl side groups through dropwise addition of 8% (v/v) methacrylic anhydride at 50°C for 3 hours. The reaction was stopped by adding PBS. The GelMA solution was then dialyzed in 12-14 kDa dialysis tubing for a week. Afterwards, the GelMA solution was put through a 22 μm filter, lyophylized for a week and stored at -80°C until further use. H-NMR was used to confirm the degree of methacrylation.

Hydrogel characterization

The elastic modulus of GelMA hydrogels was assessed through a micro-indentation test, applying unconfined compression at a constant rate (0.1 mm/s) up to a strain of 70% at room temperature (RT). The elastic modulus was then calculated from the linear region of the stress-strain curve.

Cell sources

Healthy foetal cardiac tissue was transferred to the laboratory for experimental use after planned abortions. Parental consent was given for all foetal material involved and the protocol has been approved by the Medical Ethical Committees of the University Medical Center Utrecht and Leiden University Medical Center, as previously described.[45] All procedures are in accordance with the declaration of Helsinki (on ethical principles for medical research involving human subjects) and the declaration of Taipei (on ethical considerations regarding health databases and biobanks).[46,47]

Human foetal cardiac fibroblast (hfCF) isolation and culture

Single cell suspension of heart tissue was plated overnight on uncoated standard tissue culture plastic to allow fibroblasts to adhere. Human foetal cardiac fibroblasts (hfCF) were cultured using fibroblast medium, consisting of Dulbecco's Modified Eagle Medium (DMEM, Gibco 41965), supplemented with 10% foetal bovine serum (FBS, Biowest S1810) and 1% penicillin/streptomycin (Gibco 15140122). HfCF were expanded in culture, passaged at a confluency of 90% and harvested for experimental use at passage 4-8. Cells were maintained at 5% CO_2, 20% O_2, 37°C, in a humidified atmosphere.

Human induced pluripotent stem cell-derived cardiomyocyte (iPS-CM) differentiation and culture

All iPS cell lines were provided by the European Bank for Induced Pluripotent Stem Cells. iPS cells were cultured on 10 μg/cm² Matrigel-coated plates (Corning 356231) and maintained in E8 medium (Gibco A1517001). When iPS cells reached confluency (~70-80%) they were either split using 0.5 mM EDTA (Invitrogen 15575020) or differentiated towards cardiomyocytes. Differentiation was started using CHIR-99021 (Selleck Chemicals S2924) in heparin medium (DMEM/F12 (Gibco 31331) containing 1:100 chemically defined lipid concentrate (Gibco 11905031), 213 μg/mL L-ascorbic acid (Sigma-Aldrich A8960), 1.5 IU/mL heparin (LEO 9005496) and 1% penicillin/streptomycin). After 48 hours, the medium was replaced by heparin medium containing 2 μM WNT-C59 (R&D Systems 5148). Another 48 hours later, the medium was replaced by heparin medium. From day 7 until day 10 the medium was replaced by insulin medium (DMEM/F12 containing 1:100 chemically defined lipid concentrate, 213 μg/mL L-ascorbic acid, human recombinant insulin (Sigma-Aldrich I9278) and 1% penicillin/streptomycin). Starting on day 10, the cells were purified using purification medium (RPMI 1640 L-glutamine without glucose (Gibco 11879), 1:100 chemically defined lipid concentrate, 213 μg/mL L-ascorbic acid, 21 μg/mL human recombinant

insulin, 3.5 μM sodium-DL-lactate (Santa Cruz Biotechnology 301818) and 1% penicillin/streptomycin). On day 15 the cells were replated using Tryple Select 10x (Thermo Fisher Scientific A1217702), after which they were maintained in RPMI 1640 L-glutamine (Gibco 21875) containing 0.5x B27 (Thermo Fisher Scientific 17504001).

Tissue-engineering cardiac tissue constructs

GelMA was used as a scaffold material for 3D culture of hfCF and iPS-CM (**Figure 1A**). First, photo-initiator (PI, Irgacure 2959) was dissolved in PBS in a concentration of 0.1% (w/v). GelMA was added to the PI-solution in concentrations of 7.5% and 10% (w/v). Simultaneously, hfCF and iPS-CM had been harvested and the cells were resuspended in the GelMA polymer solution. A 30 μL drop of cell-laden GelMA polymer solution was placed between two 450 μm high spacers and covered by a glass slide. Crosslinking of the polymer solution was then induced by UV-light (wavelength 365 nm, 5.6 W/cm^2 for 50 seconds), resulting in cardiac tissue constructs (CTC). CTC were washed in PBS once, put in non-adhesive well plates for suspension culture and maintained at 5% CO_2, 20% O^2, 37°C, in a humidified atmosphere. CTC were maintained in DMEM containing 10% KnockOut serum replacement (Gibco 10828028) and 1% penicillin/streptomycin.

Experimental conditions

CTC were engineered at day 0 and allowed to self-organize during a week until synchronous beating was observed. On day 7, experimental conditions were started (TGF-β1 and/or pirfenidone versus control) and the constructs were harvested on day 14 for subsequent analysis. Transforming growth factor beta 1 (TGF-β1, Peprotech 100-21C) was used in a concentration of 2 ng/mL to induce a fibrotic response, as reported in earlier work.[11,12] Pirfenidone (Cayman Chemical 13986) was used to treat fibrosis in a concentration of 1 mg/mL, in accordance with work of other groups.[18,21,48] Medium was renewed every two days.

Viability assay

Cell viability in CTC was assessed using the Live/Dead Viability kit for mammalian cells (Life Technologies). CTC were washed twice with PBS and subsequently incubated for 30 minutes at RT in a 2 μM calcein-AM and 4 μM ethidium homodimer-1 solution. After incubation, CTC were washed with PBS and immediately imaged using a Leica SP8X confocal microscope. Z-stacks (step size 10 μm) were made at three random sites per CTC and quantified using ImageJ software.

RNA isolation and cDNA synthesis

CTC were frozen at -80°C in 1 mL TriPure isolation reagent (Roche). After thawing, the samples were homogenized using ceramic beads (1.4 mm zirconium oxide beads, Precellys) and a beadbeater. The lysate was transferred to a new Eppendorf tube, which was centrifuged at 12.000 g to remove debris. Chloroform was added to the supernatant, vortexed and subsequently centrifuged at 12.000 g. The aqueous layer was loaded on an RNA isolation column (NucleoSpin RNA columns, Macherey-Nagel) with 70% ethanol. RNA was isolated using manufacturer's instructions, which included DNase treatment (RNase-free DNase set, Qiagen). Isolated RNA was quantified using a DS-11 spectrophotometer (DeNovix) and 100 ng was taken to synthesize cDNA (qScript cDNA synthesis kit, QuantaBio).

Polymerase chain reaction (PCR)

Quantitative real-time PCR (RT-qPCR) was performed in a BioRad CFX Connect, using SYBR Green (QuantaBio) and specific primers for genes of interest, including GAPDH, periostin, α-SMA and COL1a1. Primer sequences can be found in **Table S1**. Relative expression of genes was quantified using the 2^{-ddCt} method.[49]

Histology and immunofluorescence staining

CTC were washed twice in PBS and fixed in 4% paraformaldehyde (Santa Cruz Biotechnology 281692) for 25 minutes. CTC were partly dehydrated in a 30% (w/v) sucrose solution overnight at 4°C before being embedded in TissueTek OCT Compound (Sakura 4583). Cryosections of 7 μm were made using a cryotome (Thermo fisher Cryostar NX70). The slides were dried for 1h at RT and rehydrated with PBS for 10 minutes. Samples were permeabilized using 0.1% triton (Sigma-Aldrich X-100) for 10 minutes, washed thrice for 5 minutes using 0.5% Tween-80 (Millipore 817061) and blocked using 5% BSA (Millipore 10735086001) for 30 minutes. Slides were washed again and subsequently incubated with primary antibodies (**Table S2**) diluted in 5% BSA for 90 minutes at RT. After washing, secondary antibodies were combined with 1 μg/mL Hoechst 33342 (Invitrogen H1399) and incubated for 60 minutes at RT. Afterwards, slides were washed with PBS thrice for 5 minutes and sealed with Fluoromount-G (Invitrogen 00495802). Slides were imaged using a Leica SP8X confocal microscope and analysed using ImageJ software.

Protein isolation and targeted proteomics

CTC were washed twice in PBS, after which each hydrogel was incubated at 37°C in TrypLE Select (Gibco 12604) for 30 minutes. To completely degrade

the CTC, Liberase TH (Roche 05401135001) dissolved in HBSS (Gibco 24020) was added and the lysate was incubated for another 30 minutes at 37°C. Protein content of the lysate was analysed using the BCA Protein Assay Kit (Thermo Fisher 23225) and targeted proteomics was performed using Cardiovascular Panel 3 of Olink Proteomics (Uppsala, Sweden).

Statistics

Results were analysed using Graphpad Prism software (version 7.02, La Jolla, California, USA). Means are reported with the standard error of the mean (SEM), unless indicated otherwise. Paired two-tailed t-tests were used to compare the means of two groups and a two-way ANOVA for repeated measures with a Tukey's multiple comparisons post-hoc test was used to compare the means of multiple groups. A p-value <0.05 was considered statistically significant. Figures were created using Adobe Illustrator and BioRender.com.

Results

A 3D co-culture of cardiac fibroblasts and iPS-derived cardiomyocytes in a GelMA hydrogel results in a functional cardiac tissue construct

In this study, we aimed to use an *in vitro* model of human cardiac fibrosis using GelMA, hfCF and iPS-CM (**Figure 1A**). First, the mechanical characteristics of 5, 7.5, 10 and 12.5% GelMA were tested by micro-indentation and revealed elastic moduli of 1.4 to 24.2 kPa (**Figure 1B**), which is in line with previous reports.[26] The elastic modulus of 7.5% GelMA was 6.9 ± 1.4 kPa and mimics the stiffness of the healthy heart.[27] Therefore, in order to reliably represent the *in vivo* elastic modulus, and based on the excellent cell viability within the construct, this GelMA concentration was selected for all subsequent experiments involving cardiac cells.

Furthermore, to enable essential cell-cell contact and coupling throughout the CTC, we varied the cell density and the ratio between iPS-CM and hfCF and monitored synchronous beating. Cell densities of 10 million cells per mL, 28 million cells per mL and 50 million cells per mL were compared. Simultaneously, different ratios between iPS-CM and hfCF were used, in which constructs contained 70, 80, 90 or 100% iPS-CM and 30, 20, 10 and 0% hfCF, respectively (**Figure 1C**). CTC with 100% and 90% iPS-CM did not demonstrate synchronous contractions (**Video S1**), irrespective of cell density used. For CTC containing 10 million cells per mL, no synchronized contractions were noted either (**Video S2**), irrespective of cell ratios used. However, the CTC consisting of 28 and 50 million cells per mL with 70 or 80%

iPS-CM did show synchronized beating throughout the entire construct (**Video S3 and S4**).

As commonly seen for tissue engineered constructs, cells need additional time to become fully functional after fabrication.[10,28,29] In this study, we refer to this period as the maturation phase. The iPS-CM started contracting individually within two to five days after embedding and would progress to contracting synchronously throughout the CTC within one or two additional days. However, only when cell concentrations of 28 or 50 million cells per mL were used with 70 or 80% iPS-CM. CTC started contracting synchronously earlier when a cell concentration of 50 million cells per mL was used and were consistently doing so before day 6. Furthermore, CTC showed excellent cell viability until 14 days after fabrication (**Figure 1D and 1E**), with an average cell viability of 80% on day 1, 93% on day 7 and 97% on day 14. All subsequent experiments were therefore performed in CTC containing 70% iPS-CM with a cell density of 50 million cells per mL.

TGF-β1 induces a fibrotic response in cardiac tissue constructs

To examine the potential of CTC to serve as an *in vitro* model for human cardiac fibrosis, TGF-β1 was added to the culture medium from day 7 onwards in a concentration of 2 ng/mL (**Figure 2A**). This resulted in an increase in expression of well-established fibrotic markers on day 14; α-SMA ($p=0.002$), periostin (POSTN, $p=0.001$), collagen type 1 (COL1a1, $p=0.049$), and collagen type 3 (COL3, $p=0.035$) as measured by RT-qPCR (**Figure 2B**). Immunofluorescence imaging confirmed the increase in periostin (27% vs 83%, $p<0.0001$) and α-SMA (13% vs 31%, $p=0.051$) expression after stimulation with TGF-β1 (**Figure 2C and 2D**). To further investigate the fibrotic effects of TGF-β1 on CTC, we performed targeted proteomics in which a preset panel of 92 cardiovascular disease-related proteins was measured in CTC lysates. 27 of these proteins were detected in our CTC upon TGF-β1 stimulation (**Figure S1**). TGF-β1 caused an increase in expression of key fibrotic proteins COL1a1 ($p=0.0003$, **Figure 3**), matrix metalloproteinase-2 (MMP2, $p=0.008$) and osteoprotegerin (OPG, $p=0.021$), and in heart failure-associated proteins insulin-like growth factor binding protein-7 (IGFBP7, $p=0.036$) and growth differentiation factor-15 (GDF-15, $p=0.08$). Also, upon TGF-β1 stimulation, cardiac hypertrophy-associated protein phospholipase C (PLC) and epithelial cell adhesion molecule (Ep-CAM), involved in epithelial-mesenchymal transition (EMT), showed a trend towards increased expression ($p=0.054$ and $p=0.061$, respectively). Furthermore, expression of tumour necrosis factor superfamily member 6 (FAS) was increased ($p=0.041$), as were three plasminogen related proteins, including plasminogen activator inhibitor

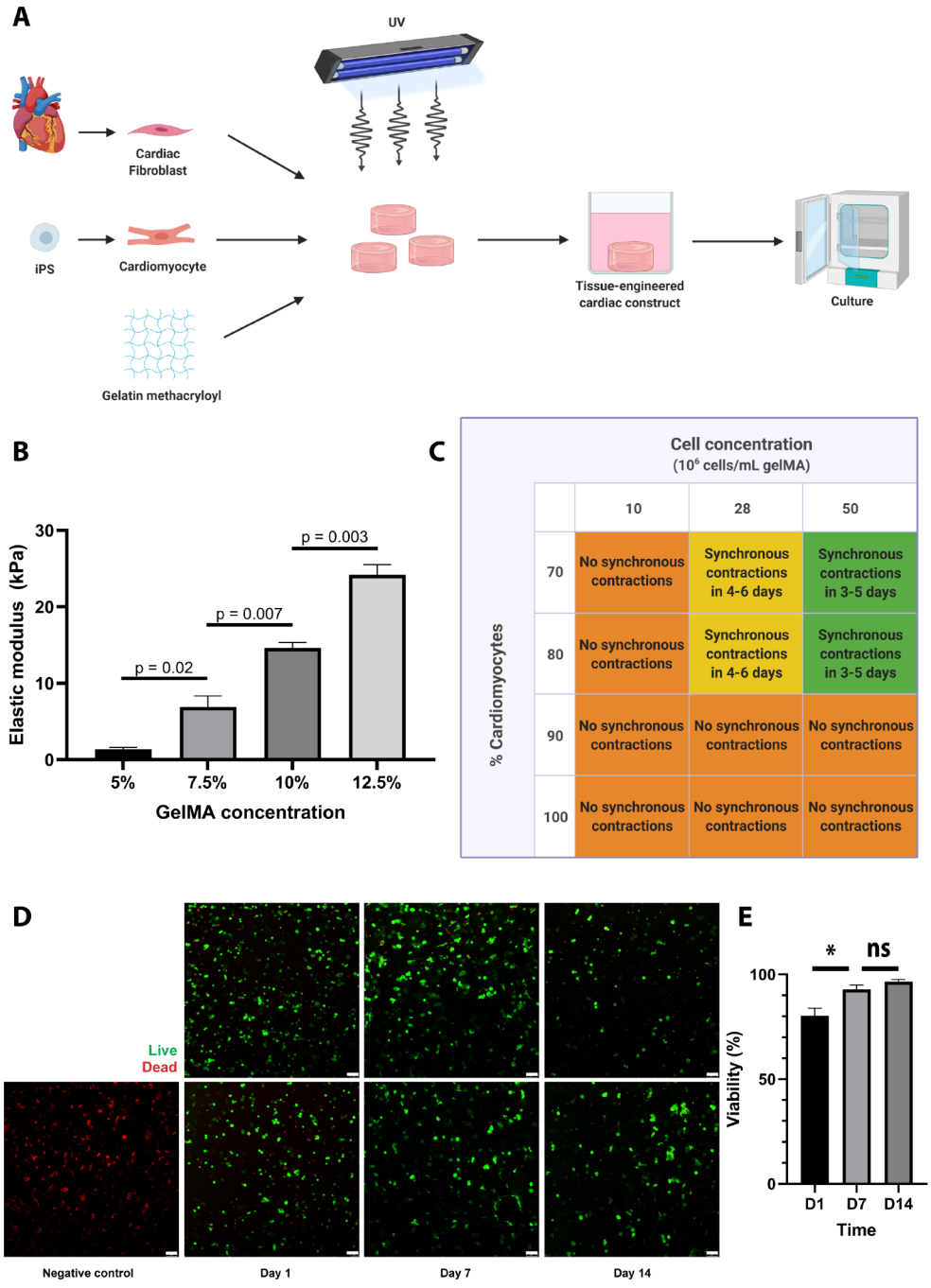


Figure 1 | **A 3D co-culture of cardiac fibroblasts and iPS-derived cardiomyocytes in a GelMA hydrogel results in a functional cardiac tissue construct. (A)** Schematic overview of the tissue engineering process. Primary cardiac fibroblasts and iPS-CM were

suspended in a GelMA polymer solution and subsequently cross-linked by exposure to UV-light (wavelength 365 nm). The resulting cell-laden hydrogels were washed in PBS to remove uncrosslinked polymers and were then placed in culture medium in non-adhesive well plates for suspension culture. **(B)** Mechanical characterization of the hydrogels, showing the elastic modulus (in kPa) for different GelMA concentrations as measured by microindentation testing (n=3 per condition). Statistical analysis was performed using a one-way ANOVA with Sidak's multiple comparison test. Data are represented as mean ± SEM. **(C)** Schematic overview of the outcomes of combined cell density and cell ratio optimization experiments. Synchronous contractions throughout the cardiac tissue constructs were only observed when a cell concentration of 28 or 50 million cells per mL was used, with a cardiomyocyte percentage of 70 or 80. In other conditions, individual cells were contracting, but synchronous contractions throughout the cardiac tissue construct would not develop. **(D, E)** Cells remain viable in 7.5% GelMA until 14 days in culture, as demonstrated by immunofluorescence microscopy after live-dead staining on day 1, day 7 and day 14 after cardiac tissue construct fabrication (n=3). Scale bars, 75 μm. GelMA, gelatine methacryloyl; iPS-CM, induced pluripotent stem cell-derived cardiomyocytes.

(PAI, p=0.033), urokinase-type plasminogen activator (uPA, p=0.021) and urokinase receptor (U-PAR, p=0.005). Overall, TGF-β1 induces distinct pro-fibrotic and heart failure related changes, establishing CTC as a relevant cardiac fibrosis model.

Pirfenidone has distinct anti-fibrotic effects in cardiac tissue constructs

The anti-fibrotic drug pirfenidone was added to conditions with and without TGF-β1 and these were compared to respective controls (**Figure 4A**). Pirfenidone resulted only in a reduction of periostin, COL1a1 and COL3 mRNA expression ($p<0.001$, $p=0.011$, $p=0.002$) in the absence of exogenous TGF-β1 (**Figure 4B**). In the presence of exogenous TGF-β1, pirfenidone did not have an anti-fibrotic effect on the explored mRNAs ($p=0.55$, $p=0.99$, $p=0.30$). Activation status of fibroblasts, explored via α-SMA expression or immunofluorescence imaging was not significantly affected by pirfenidone in either condition ($p=0.63$ with exogenous TGF-β1 and $p=0.10$ without exogenous TGF-β1). However, periostin expression decreased in our human CTC upon pirfenidone treatment (64% vs 55%, $p=0.03$) (**Figure 4C and 4D**).

To confirm these observations and explore which specific effects can be seen upon pirfenidone exposure, we repeated the targeted proteomics approach. 27 of these proteins were detected in CTC lysates (**Figure S2**). Pirfenidone caused a decrease in collagen type 1, MMP2 and OPG expression in both the presence and the absence of TGF-β1 ($p<0.001$, $p=0.068$, $p=0.034$, **Figure 5**). Both heart failure associated IGFBP7 expression ($p=0.02$) and

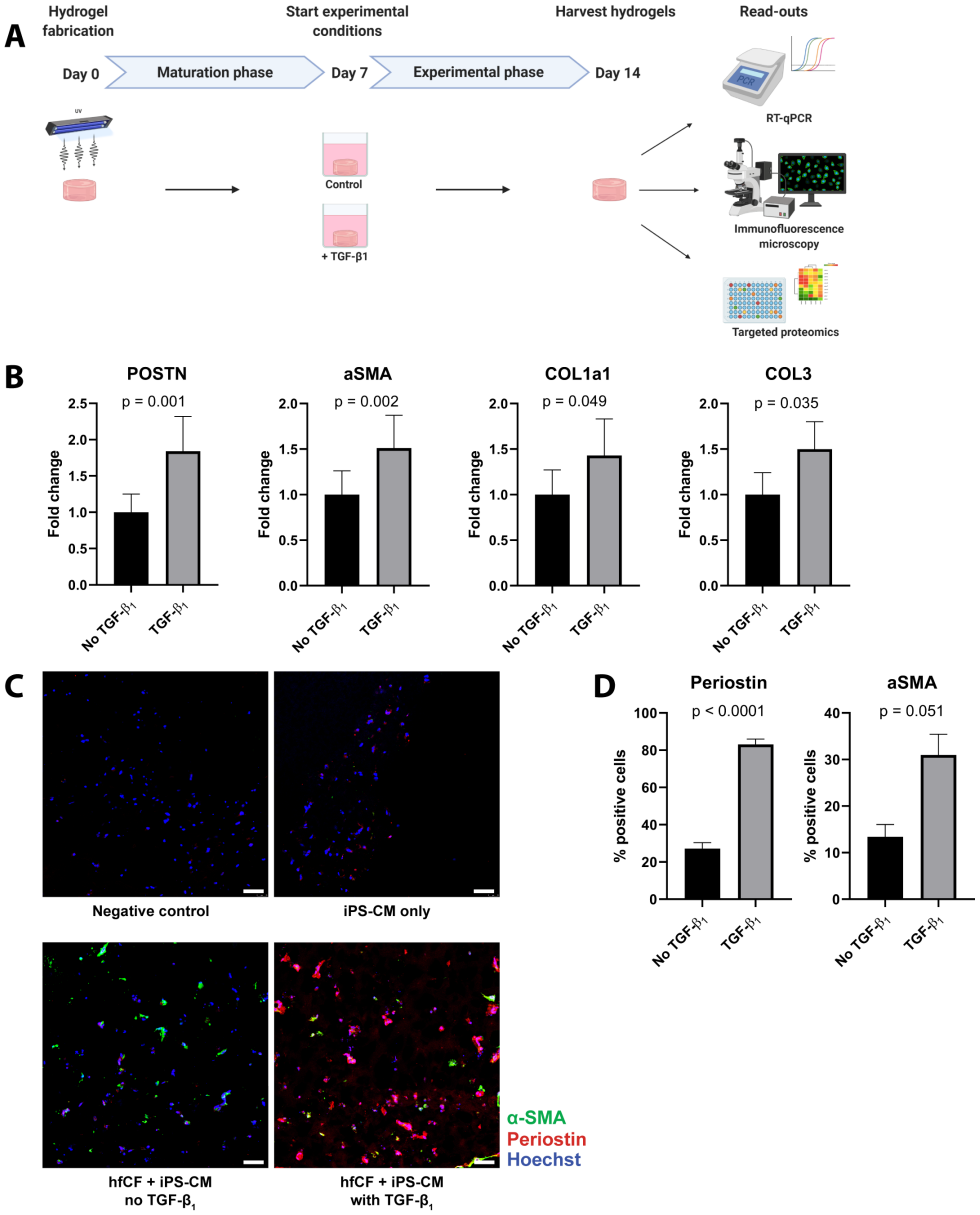


Figure 2 | **TGF-β1 induces a fibrotic response in cardiac tissue constructs. (A)** Timeline of the experiment. Cardiac tissue constructs were fabricated at day 0 and cultured until day 7. On day 7, experimental conditions were started. Two experimental conditions were created: TGF-β1 was added in the first group in a concentration of 2 ng/mL, the other group served as controls. On day 14, the constructs were harvested for analysis, consisting of RT-qPCR, immunofluorescence staining and targeted proteomics. **(B)** TGF-β1 stimulation results in an increased expression of fibrotic genes POSTN, αSMA, COL1a1 and COL3, as demonstrated by RT-qPCR (n=13). Data are represented

as mean relative expression (compared to GAPDH) ± SEM. Statistical analysis was performed using paired two-tailed Student's t-test. **(C, D)** Immunofluorescence staining shows an increase in α-SMA (green) and periostin (red) expression in tissue-engineered cardiac constructs stimulated with TGF-β1 (n=4). Scale bars, 50 μm. Statistical analysis was performed using paired two-tailed Student's t-test. αSMA, alpha-smooth muscle actin; COL1a1, collagen type 1; collagen type 3, COL3; POSTN, periostin; TGF-β1, transforming growth factor beta 1.

cardiac hypertrophy-associated PLC expression (p=0.041) showed a similar decrease. However, pirfenidone did not cause a decrease in expression of heart failure-related protein GDF-15, nor did it significantly influence the expression of plasminogen-related proteins PAI, uPA and U-PAR which were all upregulated by TGF-β1 stimulation.

Overall, pirfenidone demonstrated distinct anti-fibrotic effects in our CTC, but did not reverse all TGF-β-induced changes.

Discussion

This study demonstrates that a tissue-engineered 3D *in vitro* model of human cardiac fibrosis can be used as a drug screening platform to investigate anti-fibrotic properties of new cardiovascular drug candidates. Using this human cardiac fibrosis model, we investigated the effects of pirfenidone, an anti-fibrotic drug that showed promising results in pre-clinical animal models of cardiac fibrosis[23,25,30] and is already used clinically in idiopathic pulmonary fibrosis.[17] This study demonstrated that pirfenidone can have favourable anti-fibrotic effects *in vitro* in human cardiac fibrosis as well, but does not undo all of the TGF-β1-induced changes in cardiac cell behaviour.

Employing a 3D cell-culture system for this study was essential, as these have been proven superior to cell monolayers when mimicking the functions of living tissues.[31,32] In this study, GelMA was utilized as the hydrogel of choice due to its favourable and well-established properties for 3D cell-culture.[26,33] Most importantly, GelMA has a controllable and tuneable stiffness; via modification of the methacrylation degree, gel concentration and exposure to UV-light, its mechanical properties can be adjusted to suit the cell type in question.[33]

Furthermore, ECM characteristics have been shown to strongly influence cellular functionality and behaviour.[34] As demonstrated here, hiPSC-CM embedded in GelMA hydrogels were fully functional and seemed to be interconnected, exhibiting synchronous beating throughout the constructs. Two main cardiac cell types were used here, namely iPS-derived

cardiomyocytes and cardiac fibroblasts; however, based on this successful co-culture, our system could allow for the integration of other relevant cell types as well, such as endothelial or immune cells. The importance of multicellular constructs is shown in this study, where cardiomyocytes needed to be surrounded by a certain number of cardiac fibroblasts in order to functionally connect and to start beating synchronously. This is in line with other studies in the field, which show that cardiomyocytes, in co-culture with cardiac fibroblasts, have a more mature phenotype, align better with their environment, and start beating synchronously.[10,29,35]

Upon continuous TGF-β1 stimulation, an evident fibrotic response was observed on both the transcriptomic and proteomic level. Expression of major pro-fibrotic genes α-SMA, POSTN, COL1a1 and COL3 was significantly elevated compared to controls, which is in line with previously published studies.[11,28] While in previous fibrosis research the focus was mainly on RNA or microRNA expression, we focused on the protein level and for the first time utilizing a targeted proteomics approach in which a panel of 92 cardiovascular disease-related proteins was measured in a human *in vitro* model of cardiac fibrosis. TGF-β1 induced the expression of key fibrotic proteins collagen type 1 and MMP2, but also less well-known heart failure related proteins, such as OPG, IGFBP7, PAI, uPA, and U-PAR.

uPA is a serine protease which is associated with tissue remodelling and cell migration. When bound to its cell surface receptor U-PAR, it has extracellular proteolytic activity and can activate MMPs.[36] In a recent study, uPA and U-PAR, along with PAI, have been identified as strong predictors of adverse cardiovascular outcomes in chronic heart failure.[37]

PAI has been known to be upregulated by TGF-β, but has also been proposed to be cardioprotective in rodent models, making its exact role in cardiac fibrosis unclear.[38,39] A recent study clarifies this controversial matter. It identifies PAI as a molecular switch which controls the heart's TGF-β axis through a cardiomyocyte-specific feedforward mechanism in which PAI induces TGF-β production and cardiac fibrosis.[40] The cardiomyocyte specificity of this mechanism stresses the importance of including cardiomyocytes in cardiac fibrosis *in vitro* models.

Interestingly, the upregulation of some of these cardiac fibrosis and heart failure related proteins could be treated by administration of pirfenidone, confirming the status of pirfenidone as a potential cardioprotective anti-fibrotic drug. However, not all the TGF-β1-induced changes in protein expression were reversed. Whereas COL1a1, MMP2, OPG and IGFBP7 expression were reduced by pirfenidone treatment, the plasminogen-related proteins PAI, uPA and U-PAR were not affected. This is in line with a recent study in engineered

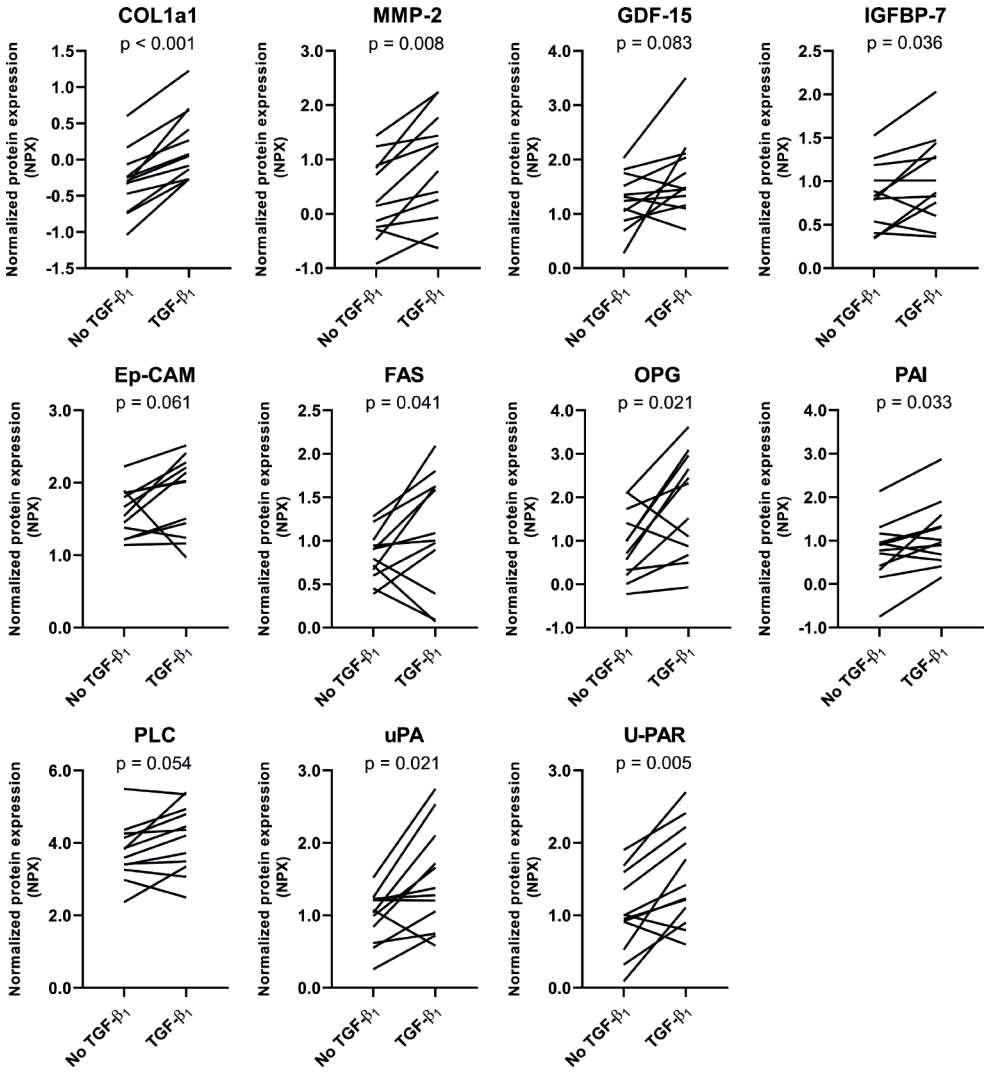
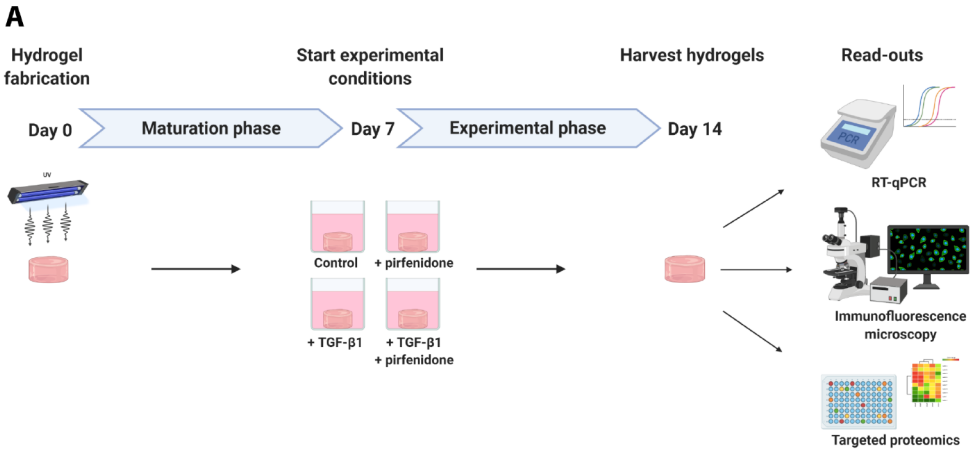
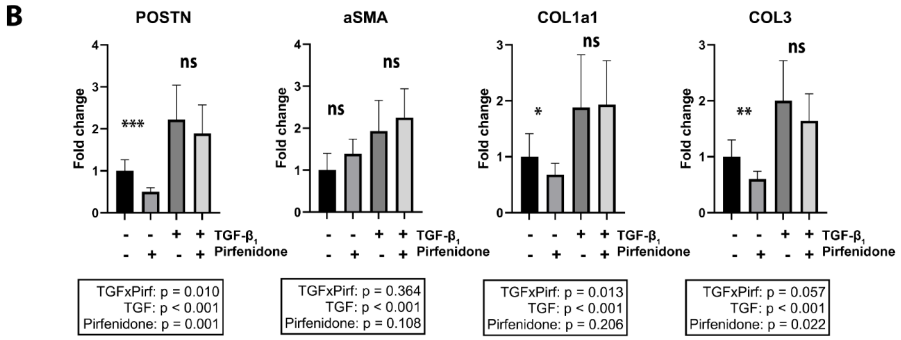
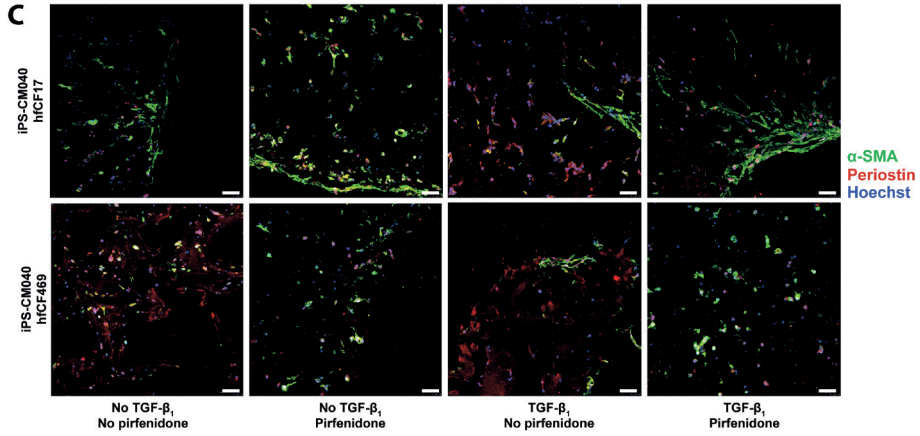


Figure 3 | **Targeted proteomics confirms the TGF-β1-induced fibrotic response in tissue-engineered cardiac constructs.** TGF-β1 stimulation results in an increased expression of several fibrosis-related proteins. Data are represented as mean normalized protein expression (compared to internal control) per experiment (n=12). Statistical analysis was performed using paired two-tailed Student's t-test. COL1a1, collagen type 1; Ep-CAM, epithelial cell adhesion molecule; FAS, tumour necrosis factor superfamily member 6; GDF-15, growth differentiation factor-15; IGFBP7, insulin-like growth factor binding protein-7; MMP2, matrix metalloproteinase-2; OPG, osteoprotegerin; PAI, plasminogen activator inhibitor; PLC, protein phospholipase C; TGF-β1, transforming growth factor beta 1; uPA, urokinase-type plasminogen activator; U-PAR, urokinase receptor.


A
Hydrogel fabrication
Start experimental conditions
Harvest hydrogels
Read-outs
Day 0
Maturation phase
Day 7
Experimental phase
Day 14
UV
Control
+ pirfenidone
+ TGF-β1
+ TGF-β1 + pirfenidone
RT-qPCR
Immunofluorescence microscopy
Targeted proteomics


B
POSTN
aSMA
COL1a1
COL3
Fold change
ns
***
*
**
TGF-β1
Pirfenidone
TGFxPirf: p = 0.010
TGF: p < 0.001
Pirfenidone: p = 0.001
TGFxPirf: p = 0.364
TGF: p < 0.001
Pirfenidone: p = 0.108
TGFxPirf: p = 0.013
TGF: p < 0.001
Pirfenidone: p = 0.206
TGFxPirf: p = 0.057
TGF: p < 0.001
Pirfenidone: p = 0.022


C
iPS-CM040 hfCF17
iPS-CM040 hfCF469
α-SMA
Periostin
Hoechst
No TGF-β1 No pirfenidone
No TGF-β1 Pirfenidone
TGF-β1 No pirfenidone
TGF-β1 Pirfenidone

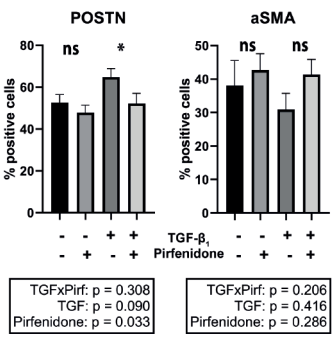
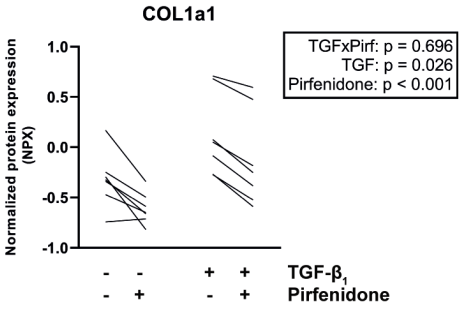
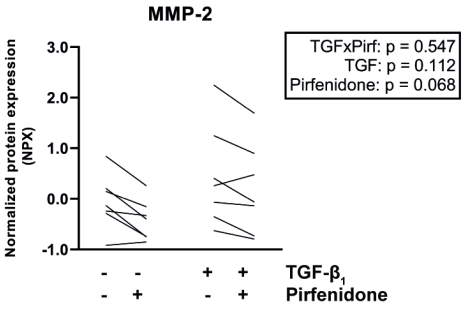


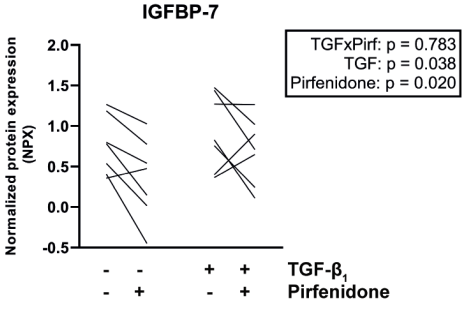
Figure 4 | **Pirfenidone has distinct anti-fibrotic effects in cardiac tissue constructs. (A)** Timeline of the experiment. Cardiac tissue constructs were fabricated at day 0 and cultured under regular circumstances until day 7. On day 7, experimental conditions were started. Four experimental conditions were created, in which TGF-β1 (2 ng/mL) and/or pirfenidone (1 mg/mL) were added to the culture medium and a group without TGF-β1 and pirfenidone served as controls. On day 14, the constructs were harvested for analysis, consisting of RT-qPCR, immunofluorescence staining and targeted proteomics. **(B)** Pirfenidone causes a decrease in POSTN, COL1a1 and COL3 expression in control conditions, but does not affect αSMA expression, as assessed with RT-qPCR (n=13). Data are represented as mean relative expression (compared to GAPDH) ± SEM. Statistical analysis was performed using repeated measures two-way ANOVA. The interaction effect and the main effects are reported in the statistical box underneath the graph, the simple main effects are reported in the graph itself. **(C, D)** Immunofluorescence staining shows a decrease in POSTN (red) expression after pirfenidone treatment of cardiac tissue constructs, whereas α-SMA (green) was unaffected (n=11). Each row shows a separate experiment with different cell lines included. Scale bars, 50 μm. Statistical analysis was performed using repeated measures two-way ANOVA. The interaction effect and the main effects are reported in the statistical box underneath the graph. aSMA, alpha-smooth muscle actin; COL1a1, collagen type 1; collagen type 3, COL3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; POSTN, periostin; TGF-β1, transforming growth factor beta 1.

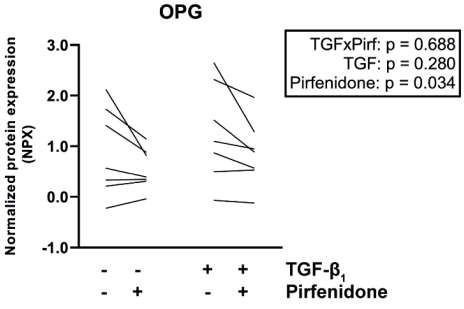
human heart tissues which showed a reduction in gene expression in several fibrotic genes upon pirfenidone treatment, but not in α-SMA expression.[28] A possible explanation for this observation is that pirfenidone interferes in the canonical TGF-β pathway but not in the non-canonical TGF-β pathways (**Figure 6**).

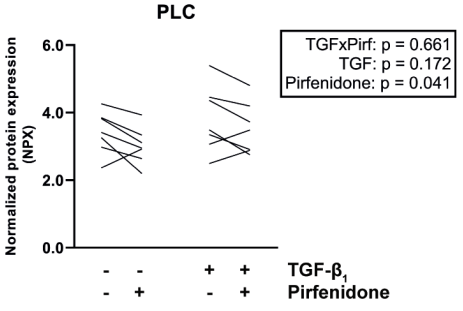
A convincing body of evidence in preclinical research supports the ndeavour of the recently started PIROUETTE trial which studies the anti-fibrotic properties of pirfenidone in HFpEF patients, even though the exact mechanism of action of pirfenidone has not been elucidated yet.[41,42] *In vitro* models of human cardiac fibrosis could play an important role in clarifying

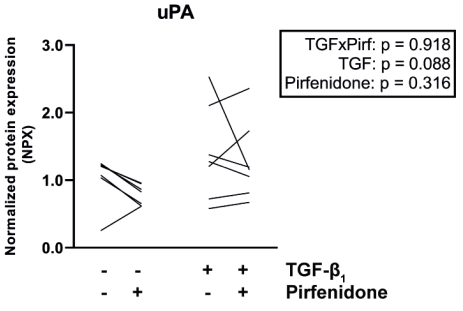

COL1a1
Normalized protein expression (NPX)
1.0
0.5
0.0
-0.5
-1.0
TGFxPirf: p = 0.696
TGF: p = 0.026
Pirfenidone: p < 0.001
- - + + TGF-β1
- + - + Pirfenidone

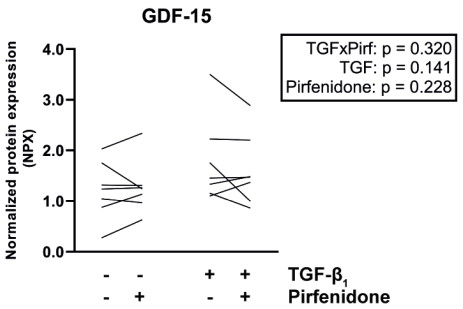

MMP-2
Normalized protein expression (NPX)
3.0
2.0
1.0
0.0
-1.0
TGFxPirf: p = 0.547
TGF: p = 0.112
Pirfenidone: p = 0.068
- - + + TGF-β1
- + - + Pirfenidone

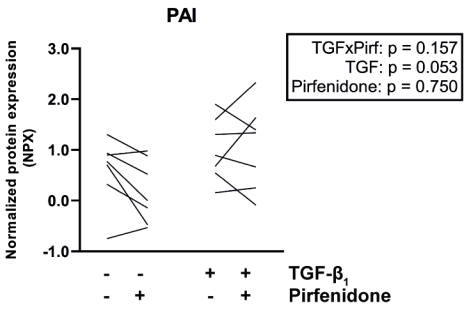

IGFBP-7
Normalized protein expression (NPX)
2.0
1.5
1.0
0.5
0.0
-0.5
TGFxPirf: p = 0.783
TGF: p = 0.038
Pirfenidone: p = 0.020
- - + + TGF-β1
- + - + Pirfenidone


OPG
Normalized protein expression (NPX)
3.0
2.0
1.0
0.0
-1.0
TGFxPirf: p = 0.688
TGF: p = 0.280
Pirfenidone: p = 0.034
- - + + TGF-β1
- + - + Pirfenidone


PLC
Normalized protein expression (NPX)
6.0
4.0
2.0
0.0
TGFxPirf: p = 0.661
TGF: p = 0.172
Pirfenidone: p = 0.041
- - + + TGF-β1
- + - + Pirfenidone


uPA
Normalized protein expression (NPX)
3.0
2.0
1.0
0.0
TGFxPirf: p = 0.918
TGF: p = 0.088
Pirfenidone: p = 0.316
- - + + TGF-β1
- + - + Pirfenidone


GDF-15
Normalized protein expression (NPX)
4.0
3.0
2.0
1.0
0.0
TGFxPirf: p = 0.320
TGF: p = 0.141
Pirfenidone: p = 0.228
- - + + TGF-β1
- + - + Pirfenidone


PAI
Normalized protein expression (NPX)
3.0
2.0
1.0
0.0
-1.0
TGFxPirf: p = 0.157
TGF: p = 0.053
Pirfenidone: p = 0.750
- - + + TGF-β1
- + - + Pirfenidone

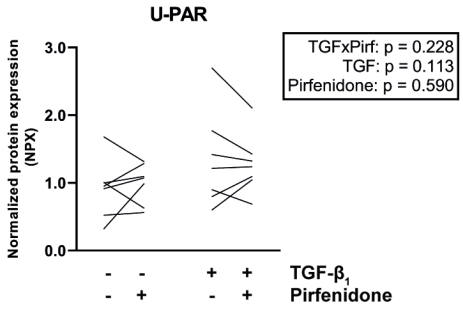


Figure 5 | **Targeted proteomics reveals that pirfenidone does not undo all TGF-β1-induced pro-fibrotic effects.** Pirfenidone causes a decrease in COL1a1 expression in both fibrotic and control conditions. In other fibrosis- and heart failure-related proteins, different effects can be seen. Data are represented as mean normalized protein expression (compared to internal control) per experiment (n=7). Statistical analysis was performed using repeated measures two-way ANOVA. The interaction effect and the main effects are reported in the statistical box on the right side of the graph. COL1a1, collagen type 1; GDF-15, growth differentiation factor-15; IGFBP7, insulin-like growth factor binding protein-7; MMP2, matrix metalloproteinase-2; OPG, osteoprotegerin; PAI, plasminogen activator inhibitor; PLC, protein phospholipase C; TGF-β1, transforming growth factor beta 1; uPA, urokinase-type plasminogen activator; U-PAR, urokinase receptor.

the molecular basis of the results found in this clinical study, as it is difficult to study the effects of pirfenidone treatment in the cardiac tissue of the included patients.

The rapid increase of new heart failure cases urges a necessary shift in the current approaches. Although conventional monolayer cell-culture systems and animal studies gave us valuable information about cardiac physiology and the changes occurring during pathological remodelling, these models do not facilitate the translation of effective therapeutics to the clinical arena when it comes to targeting cardiac fibrosis. Advanced human *in vitro* models could bridge this gap by providing us with the necessary tissue complexity without the disadvantages of interspecies differences.

In this study we engineered a 3D *in vitro* model which recapitulates human cardiac fibrosis. By successfully incorporating hiPSC-CM derived from various different donors, we opened a window of opportunity towards a more personalized approach to tackling heart failure. With the recent successful creation of hiPSC-cardiac fibroblasts[43,44], the future 3D fibrosis models will be able to completely mimic patient-specific situations, allowing for tailored drug-testing. Our model can be used for identification of differential fibrosis-related

transcriptomic and proteomic profiles in diseased and healthy cells, as well as to screen and test novel anti-fibrotic therapeutics, proof-of-principle of which we provided with pirfenidone. Furthermore, by using this tunable 3D cell culture system, new therapeutic targets could be found, ultimately contributing to the development of interventions that could prevent or reverse fibrotic changes in the failing heart.

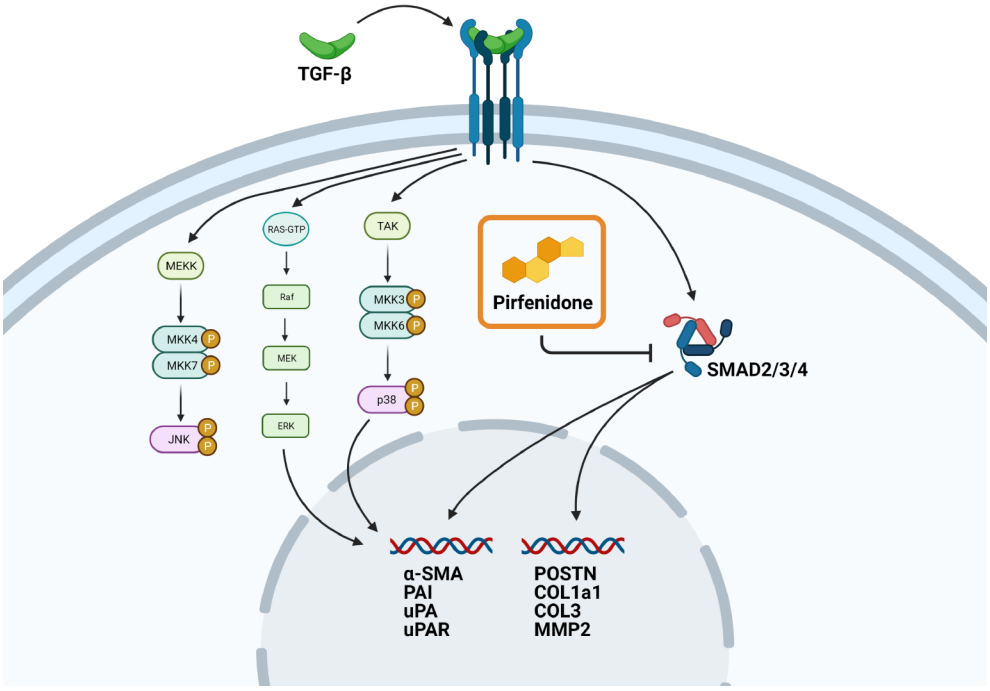


Figure 6 | **Proposed mechanism of action of pirfenidone.** Schematic showing the proposed role of pirfenidone in CTC. Upregulation of pro-fibrotic gene expression through the canonical TGF-β pathway is blocked by pirfenidone, but upregulation of pro-fibrotic gene expression through the non-canonical pathway is still possible.

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Declaration of interest

Conflicts of interest: none.

References

1. Hill, J. A. & Olson, E. N. Cardiac plasticity. *N. Engl. J. Med.* **358**, 1370-1380 (2008).
2. Gourdie, R. G., Dimmeler, S. & Kohl P. Novel therapeutic strategies targeting fibroblasts and fibrosis in heart disease. *Nat. Rev. Drug. Discov.* **15**, 620 -638 (2016).
3. Ponikowski, P. et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC) Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur. Heart J.* **37**, 2129–2200 (2016).
4. de Boer, R. A. et al. Towards better definition, quantification and treatment of fibrosis in heart failure. A scientific roadmap by the Committee of Translational Research of the Heart Failure Association (HFA) of the European Society of Cardiology. *Eur. J. Heart. Fail.* **21**, 272-285 (2019).
5. Forte, E., Furtado, M. B. & Rosenthal, N. The interstitium in cardiac repair: role of the immune-stromal cell interplay. *Nat. Rev. Cardiol.* 2018, **15**, 601-616 (2018).
6. Leask, A. Getting to the heart of the matter: new insights into cardiac fibrosis. *Circ. Res.* **116**, 1269-1276 (2015).
7. Hwang, T. J., Lauffenburger, J. C., Franklin, J. M. & Kesselheim, A. S. Temporal trends and factors associated with cardiovascular drug development, 1990 to 2012. *JACC Basic Transl. Sci.* **1**, 301-308 (2016).
8. Hwang, T. J. & Kesselheim, A. S. Challenges in the development of novel cardiovascular therapies. *Clin. Pharmacol. Ther.* **102**, 194-196 (2017).
9. Rongen, G. A. & Wever, K. E. Cardiovascular pharmacotherapy: Innovation stuck in translation. *Eur. J. Pharmacol.* **759**, 200-204 (2015).
10. Saini, H., Navaei, A., van Putten, A. & Nikkhah, M. 3D cardiac microtissues encapsulated with the co-culture of cardiomyocytes and cardiac fibroblasts. *Adv. Healthc. Mater.* **4**, 1961-1971 (2015).
11. Bracco Gartner, T. C. L. et al. Anti-fibrotic effects of cardiac progenitor cells in a 3D-model of human cardiac fibrosis. *Front. Cardiovasc. Med.* **6**, 52 (2019).
12. Sadeghi, A. H. et al. Engineered 3D cardiac fibrotic tissue to study fibrotic remodeling. *Adv. Healthc. Mater.* **6** (2017).
13. Bracco Gartner, T. C. L. et al. Advanced *in vitro* modeling to study the paradox of mechanically induced cardiac fibrosis. *Tissue Eng. Part C: Methods* **27**, 100-114 (2021).
14. Engler, A. J. et al. Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. *J. Cell. Sci.* **121**, 3794-3802 (2008).
15. Ronaldson-Bouchard, K. et al. Advanced maturation of human cardiac tissue grown from pluripotent stem cells. *Nature* **556**, 239-243 (2018).
16. Tomasek, J. J., Gabbiani, G., Hinz, B., Chaponnier, C. & Brown, R. A. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat. Rev. Mol. Cell. Biol.* **3**, 349-363 (2002).
17. Noble, P. W. et al. Pirfenidone in patients with idiopathic pulmonary fibrosis (CAPACITY): two randomised trials. *The Lancet* **377**, 1760-1769 (2011).
18. Jin, J. et al. Pirfenidone attenuates lung fibrotic fibroblast responses to transforming growth factor-β1. *Respir. Res.* **20** (2019).
19. Zanotti, S. et al. Anti-fibrotic effect of pirfenidone in muscle derived-fibroblasts from

Duchenne muscular dystrophy patients. *Life Sciences* **145**, 127-136 (2016).
20. Lin, X., Yu, M., Wu, K., Yuan, H. & Zhong, H. Effects of pirfenidone on proliferation, migration, and collagen contraction of human tenon's fibroblasts *in vitro*. *Invest. Ophthalmol. Vis. Sci.* **50**, 3763-3770 (2009).
21. Shi, Q. et al. *In vitro* effects of pirfenidone on cardiac fibroblasts: proliferation, myofibroblast differentiation, migration and cytokine secretion. *PLoS ONE* **6**, e28134 (2011).
22. Nguyen, D. T., Ding, C., Wilson, E., Marcus, G. M. & Olgin, J. E. Pirfenidone mitigates left ventricular fibrosis and dysfunction after myocardial infarction and reduces arrhythmias *Heart Rhythm* **7**, 1438-1445 (2010).
23. Wang, Y., Wu, Y., Chen, J., Zhao, S. & H. Li. Pirfenidone attenuates cardiac fibrosis in a mouse model of TAC-induced left ventricular remodeling by suppressing NLRP3 inflammasome formation. *Cardiology* **126** (2013).
24. Yamazaki, T. et al. Yoshiyama. The antifibrotic agent pirfenidone inhibits angiotensin II-induced cardiac hypertrophy in mice. *Hypertens. Res.* **35**, 34-40 (2012).
25. Yamagami, K. et al. Pirfenidone exhibits cardioprotective effects by regulating myocardial fibrosis and vascular permeability in pressure-overloaded hearts. *Am. J. Physiol. Heart Circ. Physiol.* **309**, H512-H522 (2015).
26. Nichol, J. W., Koshy, S. T., Bae, H., Hwang, C. M. & Yamanlar, S. & Khademhosseini, A. Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials* **31**, 5536-5544 (2010).
27. Berry, M. F. et al. Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance. *Am J Physiol Heart Circ Physiol* **290**, H2196-H2203 (2006).
28. Mastikhina, O. et al. Human cardiac fibrosis-on-a-chip model recapitulates disease hallmarks and can serve as a platform for drug testing. *Biomaterials* **233**, 119741 (2020).
29. Beauchamp, P. et al. 3D co-culture of hiPSC-derived cardiomyocytes with cardiac fibroblasts improves tissue-like features of cardiac spheroids. *Front. Mol. Biosci.* **7** (2020).
30. Schaefer, C. J., Ruhrmund, D. W., Pan, L., Seiwert, S. D. & Kossen, K. Antifibrotic activities of pirfenidone in animal models. *Eur. Respir. Rev.* **20**, 85-97 (2011).
31. Pampaloni, F., Reynaud, E. G. & Stelzer, E. H. K. The third dimension bridges the gap between cell culture and live tissue. *Nat. Rev. Mol. Cell. Biol.* **8**, 839-845 (2007).
32. Zuppinger, C. 3D culture for cardiac cells. *Biochim. Biophys. Acta* **1863**, 1873-1881 (2016).
33. Loessner, D. et al. Functionalization, preparation and use of cell-laden gelatin methacryloyl-based hydrogels as modular tissue culture platforms. *Nat. protoc.* **11**, 727-746 (2016).
34. Humphrey, J. D., Dufresne, E. R. & Schwartz, M. A. Mechanotransduction and extracellular matrix homeostasis. *Nat. Rev. Mol. Cell. Biol.* **15**, 802-812 (2014).
35. Hussain, A., Collins, G., Yip, D. & Cho, C. H. Functional 3-D cardiac co-culture model using bioactive chitosan nanofiber scaffolds. *Biotechnol. Bioeng.* **110**, 637-647 (2013).
36. Hohensinner, P. J. et al. Urokinase plasminogen activator protects cardiac myocytes from oxidative damage and apoptosis via hOGG1 induction. *Apoptosis* **22**, 1048-1055 (2017).

37. van Den Berg, V. J. et al. Longitudinally measured fibrinolysis factors are strong predictors of clinical outcome in patients with chronic heart failure: the Bio-SHiFT study. *Thromb. and Haemost.* **119**, 1947-1955 (2019).
38. Macfelda, K. et al. Plasminogen activator inhibitor 1 expression is regulated by the inflammatory mediators interleukin-1alpha, tumor necrosis factor-alpha, transforming growth factor-beta and oncostatin M in human cardiac myocytes. *J. Mol. Cell. Cardiol.* **34**, 1681-1691 (2002).
39. Ghosh, A. K., Murphy, S. B., Kishore, R. & Vaughan. D. E. Global gene expression profiling in PAI-1 knockout murine heart and kidney: molecular basis of cardiac-selective fibrosis. *PLoS ONE* **8**, e63825 (2013).
40. Flevaris, P. et al. Plasminogen activator inhibitor type I controls cardiomyocyte transforming growth factor-β and cardiac fibrosis. *Circulation* **136**, 664-679 (2017).
41. Lewis, G. A. et al. Pirfenidone in heart failure with preserved ejection fraction-rationale and design of the PIROUETTE trial. *Cardiovasc. Drugs. Ther.* **33**, 461-470 (2019).
42. Aimo, A. et al. Pirfenidone is a cardioprotective drug: Mechanisms of action and preclinical evidence. *Pharmacol. Res.* **155**, 104694 (2020).
43. Zhang, J. et al. Enhancing and controlling valley magnetic response in MoS_2/WS_2 heterostructures by all-optical route. *Nat. Commun.* **10**, 4226 (2019).
44. Zhang, H. et al. Generation of quiescent cardiac fibroblasts from human induced pluripotent stem cells for *in vitro* modeling of cardiac fibrosis. *Circ. Res.* **125**, 552-566 (2019).
45. Smits, A. M. et al. Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an *in vitro* model for studying human cardiac physiology and pathophysiology. *Nat. Protoc.* **4**, 232-243 (2009).
46. World Medical Association. Declaration of Helsinki **79** (2001).
47. World Medical Association. Declaration of Taipei on ethical considerations regarding health databases and biobanks (2016).
48. Cui, Y. et al. Pirfenidone inhibits cell proliferation and collagen I production of primary human intestinal fibroblasts. *Cells* **9**, 775 (2020).
49. Sluijter, J. P. G., Smeets, M. B., Velema, E., Pasterkamp, G. & de Kleijn, D. P. V. Increased collagen turnover is only partly associated with collagen fiber deposition in the arterial response to injury. *Cardiovasc. Res.* **61**, 186-195 (2004).

Supporting information

Table S1 | **Primers used for quantitative real-time polymerase chain reaction (qPCR)**

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	*ACAGTCAGCCGCATCTTC*	*GCCCAATACGACCAAATC*
α-SMA	*AGCCCAGCCAAGCACTG*	*CAAAGCCGGCCTTACAGAG*
COL1a1	*TGCCATCAAAGTCTTCTGC*	*CATACTCGAACYGGAATCCATC*
COL3	*AGGGGAGCTGGCTACTTCTC*	*GGACTGACCAAGATGGGAA*
POSTN	*TGCCCTGGTTATATGAGAATGGAAG*	*GATGCCCAGAGTGCCATAAACA*

α-SMA, alpha-smooth muscle actin; COL1a1, collagen type 1; COL3, collagen type 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; POSTN, periostin.

Table S2 | **Antibodies used for immunofluorescence imaging**

Target	Manufacturer	Cat.#	Host	Dilution
Anti-α-SMA	Sigma Aldrich	A2547	Mouse	1:25
Anti-POSTN	Abcam	Ab92460	Rabbit	1:25
Anti-mouse 488	Invitrogen	A11029	Goat	1:200
Anti-rabbit 568	Invitrogen	A11036	Goat	1:200

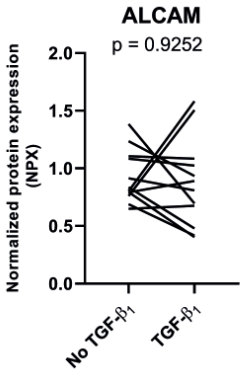
α-SMA, alpha-smooth muscle actin; POSTN, periostin.

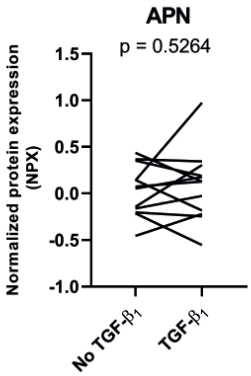
Video S1. No synchronized contractions in cardiac tissue constructs when no cardiac fibroblasts are present (cell density of 28 million cells per mL, 100% iPS-CM, 0% hfCF, GelMA 7.5%, imaging at day 7).

Video S2. No synchronized contractions in cardiac tissue constructs with a cell density of 10 million cells per mL at day 6 (70% iPS-CM, 30% hfCF, GelMA 7.5%).

Video S3. Synchronized contractions in cardiac tissue constructs with a cell density of 28 million cells per mL at day 6 (70% iPS-CM, 30% hfCF, GelMA 7.5%).

Video S4. Synchronized contractions in cardiac tissue constructs with a cell density of 50 million cells per mL at day 6 (70% iPS-CM, 30% hfCF, GelMA 7.5%).


ALCAM
p = 0.9252
Normalized protein expression (NPX)
2.0
1.5
1.0
0.5
0.0
No TGF-β1
TGF-β1


APN
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0.0
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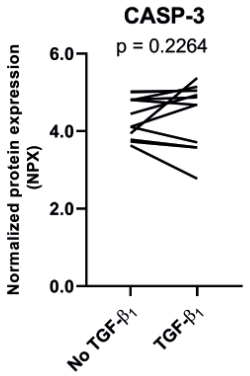
-1.0
No TGF-β1
TGF-β1


CASP-3
p = 0.2264
Normalized protein expression (NPX)
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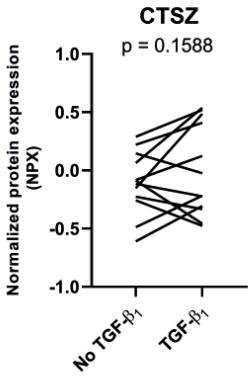
4.0
2.0
0.0
No TGF-β1
TGF-β1


CTSZ
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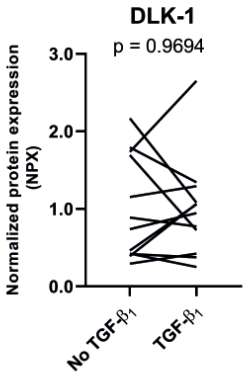
-1.0
No TGF-β1
TGF-β1


DLK-1
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Normalized protein expression (NPX)
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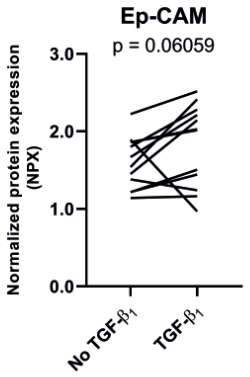
2.0
1.0
0.0
No TGF-β1
TGF-β1


Ep-CAM
p = 0.06059
Normalized protein expression (NPX)
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2.0
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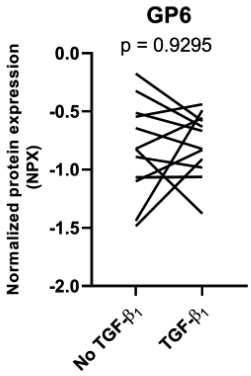
0.0
No TGF-β1
TGF-β1


GP6
p = 0.9295
Normalized protein expression (NPX)
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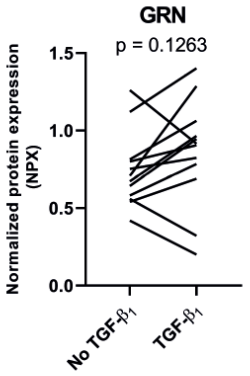
-0.5
-1.0
-1.5
-2.0
No TGF-β1
TGF-β1


GRN
p = 0.1263
Normalized protein expression (NPX)
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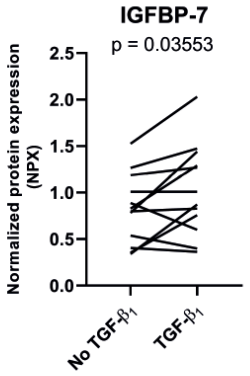
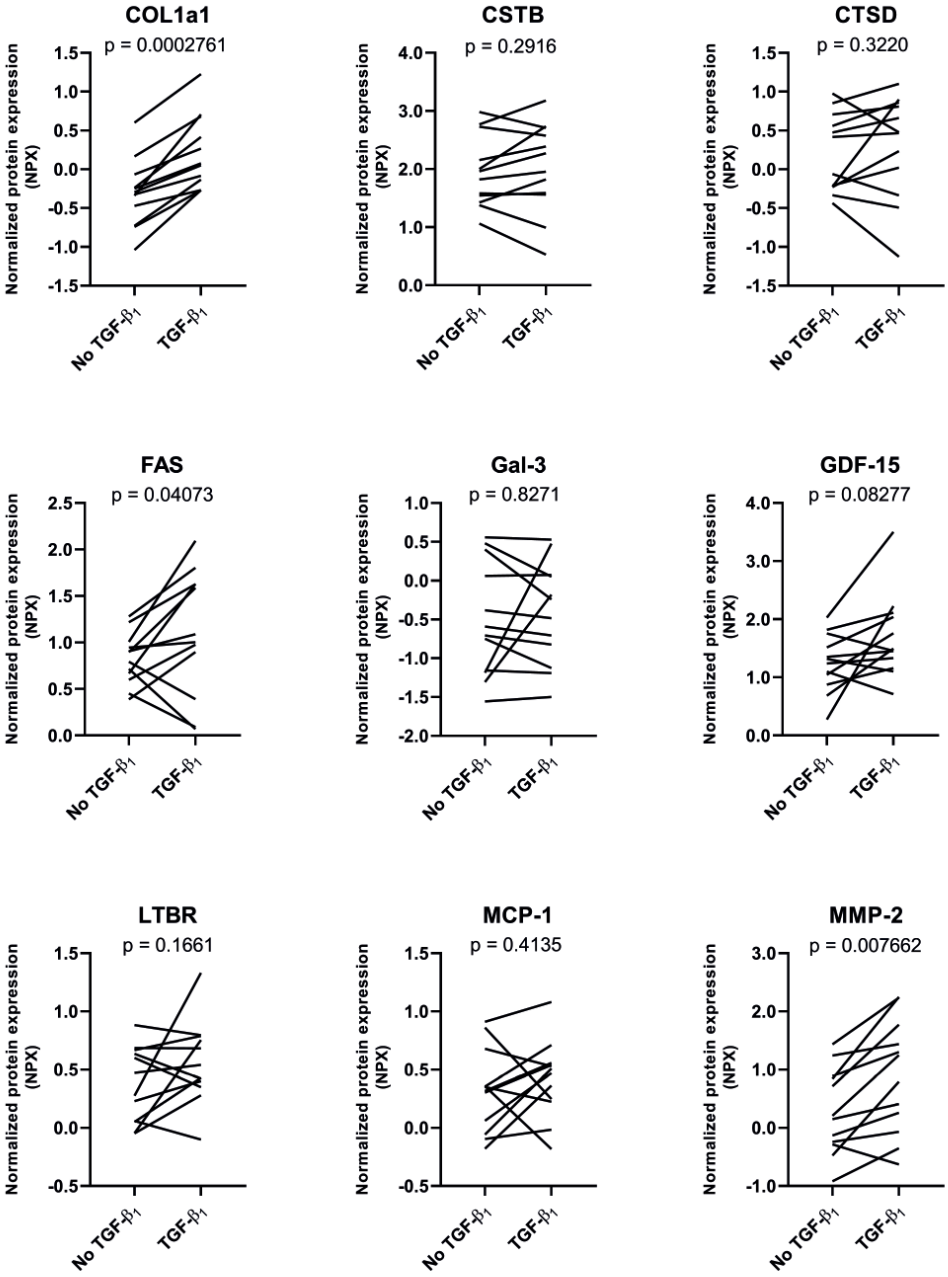
1.0
0.5
0.0
No TGF-β1
TGF-β1


IGFBP-7
p = 0.03553
Normalized protein expression (NPX)
2.5
2.0
1.5
1.0
0.5
0.0
No TGF-β1
TGF-β1


COL1a1
p = 0.0002761
CSTB
p = 0.2916
CTSD
p = 0.3220
FAS
p = 0.04073
Gal-3
p = 0.8271
GDF-15
p = 0.08277
LTBR
p = 0.1661
MCP-1
p = 0.4135
MMP-2
p = 0.007662
Normalized protein expression (NPX)
No TGF-β1
TGF-β1

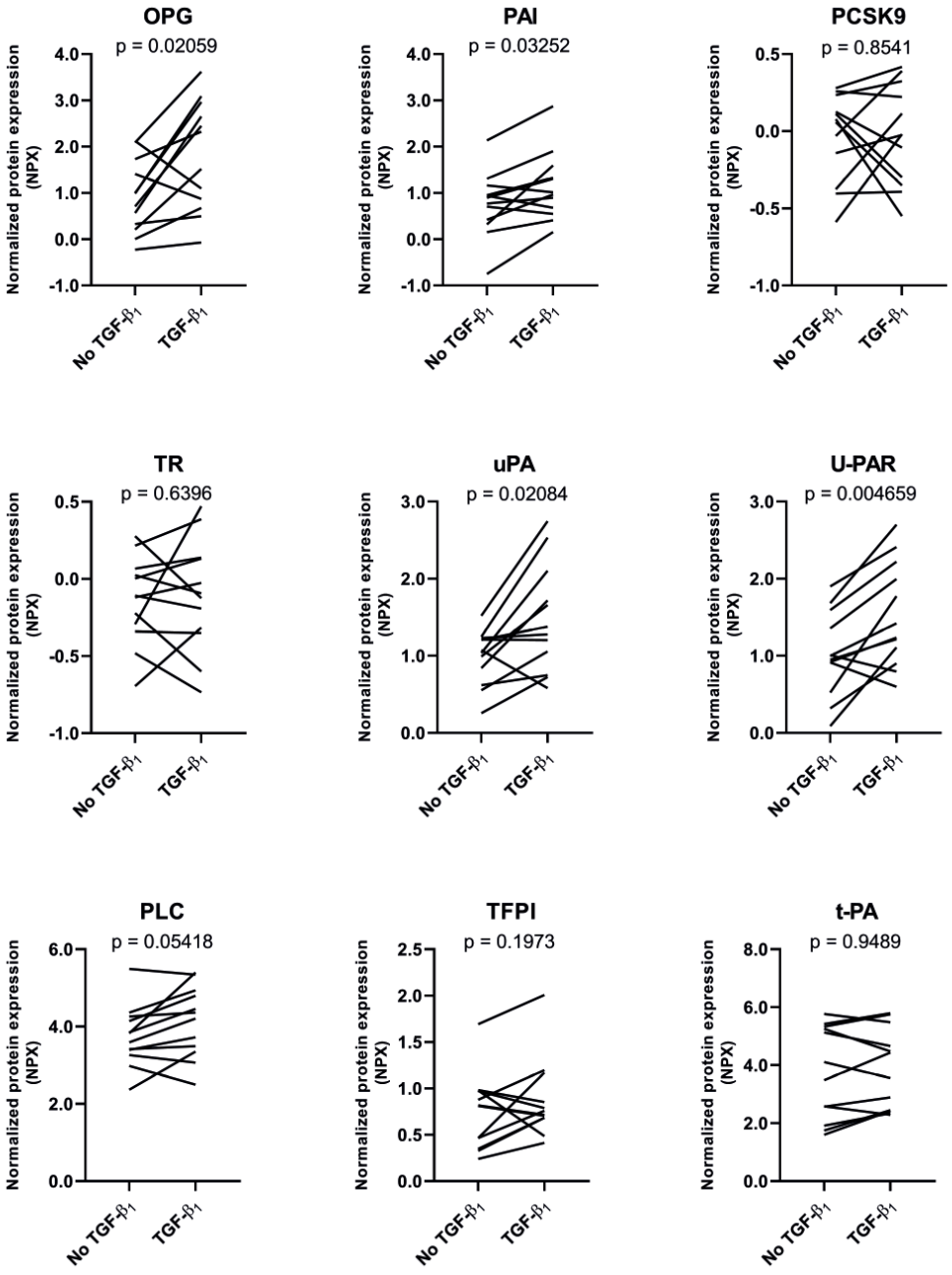
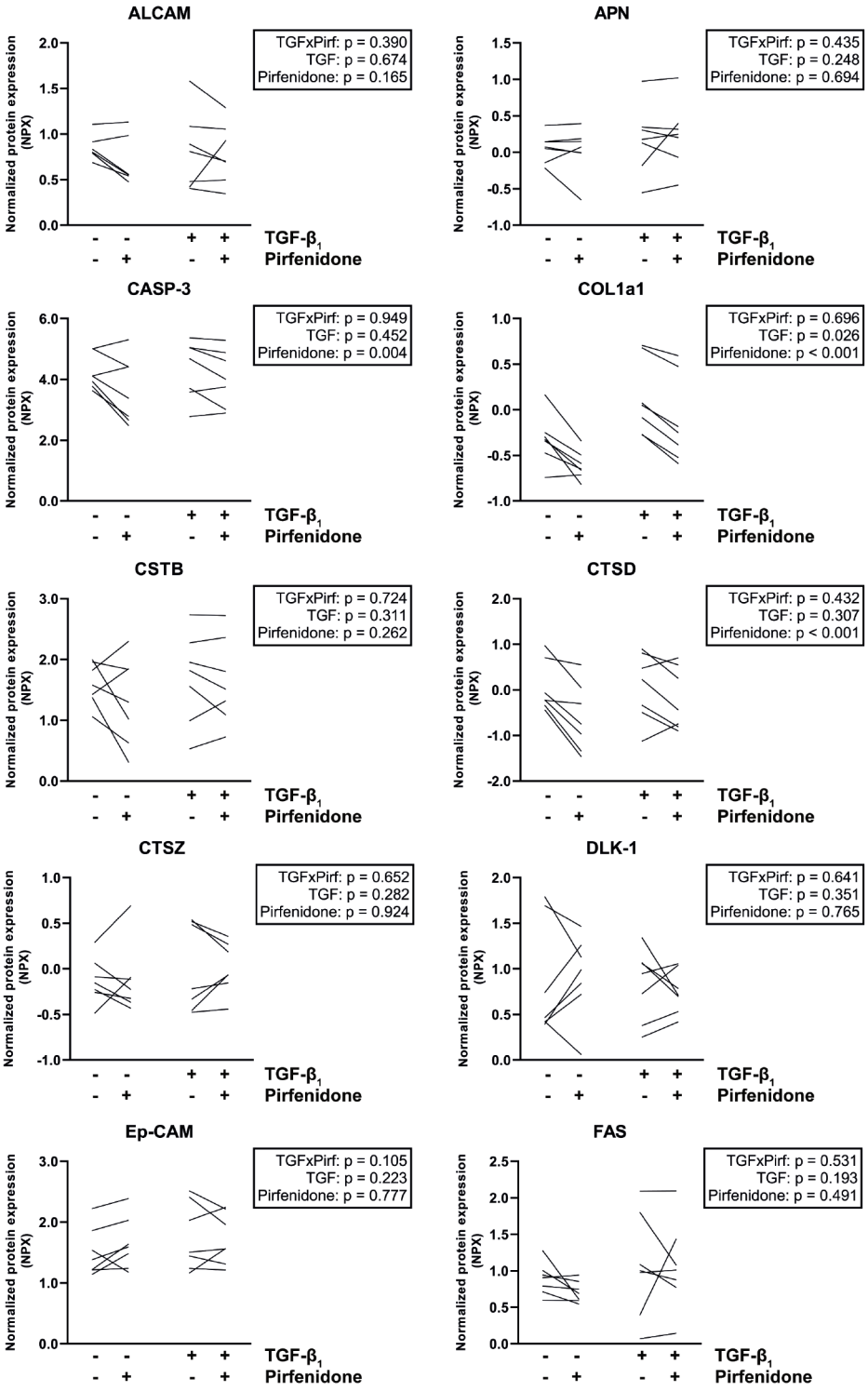
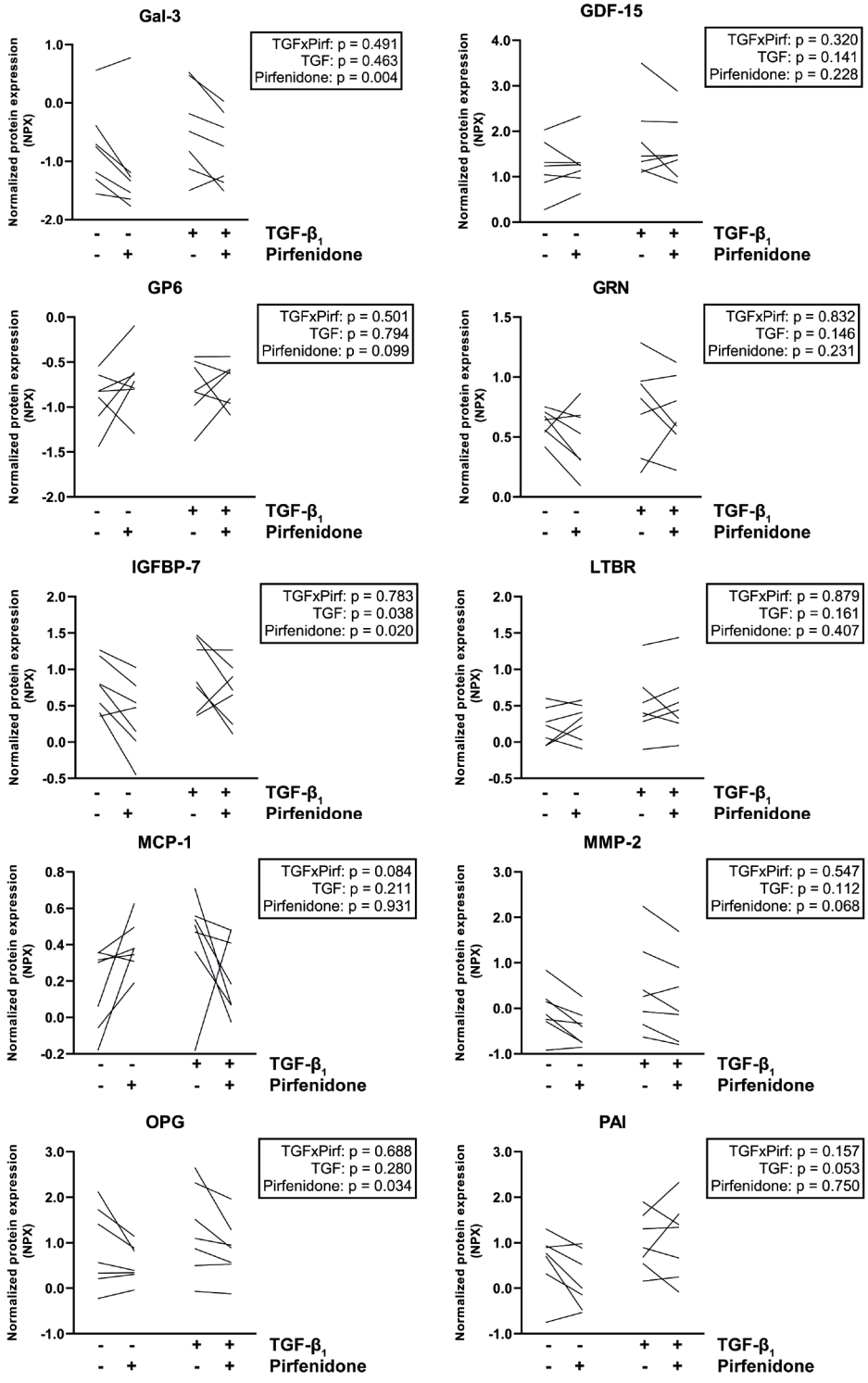


Figure S1 | **Targeted proteomics of fibrosis in tissue-engineered cardiac constructs.** Normalized protein expression in fibrotic and control conditions for all proteins in Olink Cardiovascular panel 3 that exceeded the level of detection (n=12). Data are represented as mean normalized protein expression (compared to internal control) per experiment. Statistical analysis was performed using paired two-tailed Student's t-test.


ALCAM
TGFxPirf: p = 0.390
TGF: p = 0.674
Pirfenidone: p = 0.165
APN
TGFxPirf: p = 0.435
TGF: p = 0.248
Pirfenidone: p = 0.694
CASP-3
TGFxPirf: p = 0.949
TGF: p = 0.452
Pirfenidone: p = 0.004
COL1a1
TGFxPirf: p = 0.696
TGF: p = 0.026
Pirfenidone: p < 0.001
CSTB
TGFxPirf: p = 0.724
TGF: p = 0.311
Pirfenidone: p = 0.262
CTSD
TGFxPirf: p = 0.432
TGF: p = 0.307
Pirfenidone: p < 0.001
CTSZ
TGFxPirf: p = 0.652
TGF: p = 0.282
Pirfenidone: p = 0.924
DLK-1
TGFxPirf: p = 0.641
TGF: p = 0.351
Pirfenidone: p = 0.765
Ep-CAM
TGFxPirf: p = 0.105
TGF: p = 0.223
Pirfenidone: p = 0.777
FAS
TGFxPirf: p = 0.531
TGF: p = 0.193
Pirfenidone: p = 0.491
Normalized protein expression (NPX)
TGF-β1
Pirfenidone


Gal-3
TGFxPirf: p = 0.491
TGF: p = 0.463
Pirfenidone: p = 0.004
GDF-15
TGFxPirf: p = 0.320
TGF: p = 0.141
Pirfenidone: p = 0.228
GP6
TGFxPirf: p = 0.501
TGF: p = 0.794
Pirfenidone: p = 0.099
GRN
TGFxPirf: p = 0.832
TGF: p = 0.146
Pirfenidone: p = 0.231
IGFBP-7
TGFxPirf: p = 0.783
TGF: p = 0.038
Pirfenidone: p = 0.020
LTBR
TGFxPirf: p = 0.879
TGF: p = 0.161
Pirfenidone: p = 0.407
MCP-1
TGFxPirf: p = 0.084
TGF: p = 0.211
Pirfenidone: p = 0.931
MMP-2
TGFxPirf: p = 0.547
TGF: p = 0.112
Pirfenidone: p = 0.068
OPG
TGFxPirf: p = 0.688
TGF: p = 0.280
Pirfenidone: p = 0.034
PAI
TGFxPirf: p = 0.157
TGF: p = 0.053
Pirfenidone: p = 0.750
Normalized protein expression (NPX)
TGF-β1
Pirfenidone

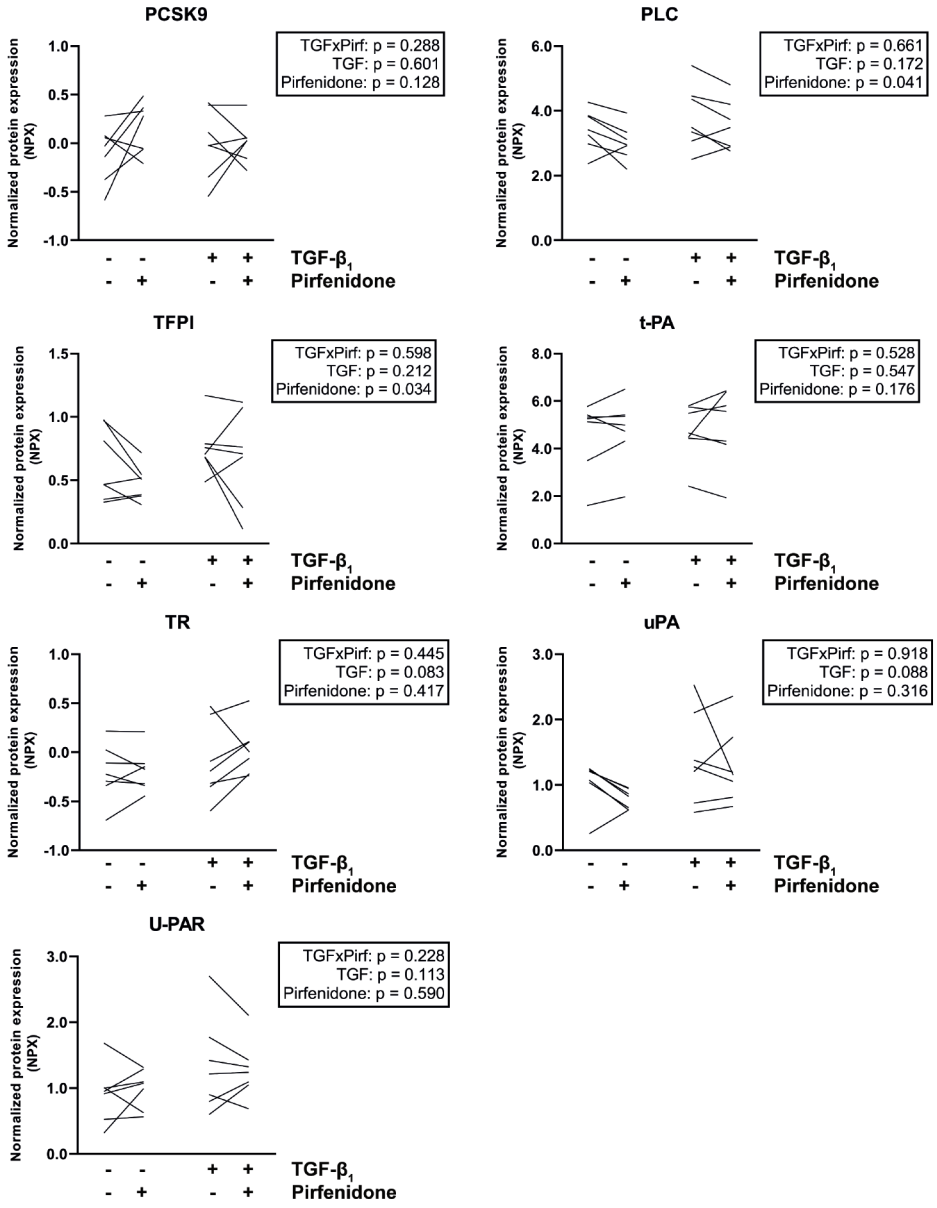


Figure S2 | **Targeted proteomics of anti-fibrotic treatment in tissue-engineered cardiac constructs.** Normalized protein expression following pirfenidone treatment in fibrotic and control conditions for all proteins in Olink Cardiovascular panel 3 that exceeded the level of detection (n=7). Data are represented as mean normalized protein expression (compared to internal control) per experiment. Statistical analysis was performed using repeated measures two-way ANOVA. The interaction effect and the main effects are reported in the statistical box on the right side of the graph.

CHAPTER 8

Summary and Outlook

Introduction

Cardiovascular diseases (CVDs) are a major health problem, accounting for 31% of all deaths worldwide.[1] Among CVDs, heart failure is one of the most prevalent diseases, affecting more than 23 million people globally.[2] Each person has a lifetime risk of 20% to develop heart failure, which is especially prominent above 65 years of age.[2] With a prognosis of only 5 years, heart failure rivals many common types of cancers.[3]

Heart failure can be classified into: heart failure with reduced ejection fraction (HFrEF; EF < 40%), heart failure with preserved ejection fraction (HFpEF; EF > 50%), and more recently introduced heart failure with mid-range or mildly reduced ejection fraction (HFmrEF; EF between 40 and 49%).[4] Improved treatments of heart failure lead to the reduction of mortality and hospitalisation, mostly in patients with HFrEF. However, it remains an incurable disease with very poor prognosis. One-year mortality rates for hospitalised heart failure patients still remains high at 17%, with no reduction in mortality of patients with HFpEF and HFmrEF thus far.[4] Although new promising therapies have recently emerged,[5] heart transplantation remains the only cure for the end-stage heart failure, however severe shortage of available donor organs is a big issue.[6] In the Netherlands alone less than 40 heart transplantations are performed annually,[7] which is not nearly sufficient for the growing number of heart failure cases.

Owing to the devastating prognosis of patients diagnosed with heart failure, with no available cure, it is of utmost importance that new approaches to understand and tackle this disease are developed. This thesis offers two different dimensions from which heart failure can be approached, each bringing unique advantages to the field and representing a way of bridging the gap between research and clinical arena (**Figure 1**). By adding the dimension of time (4D) – in terms of circadian rhythms and daily fluctuations – and by using advanced 3D *in vitro* models that adequately recapitulate native human myocardium, we gained novel insights into this deadly disease.

Circadian rhythms: bringing the concept of time to the medicine

Circadian rhythms in a nutshell

Circadian rhythms are biological rhythms with a period of approximately 24 h that allow organisms to prepare for the fluctuations brought on by day–night cycles. In essence, they align internal biological functions of organisms with external, environmental changes, thus adapting their physiology and behaviour accordingly. Although ubiquitous in nature,

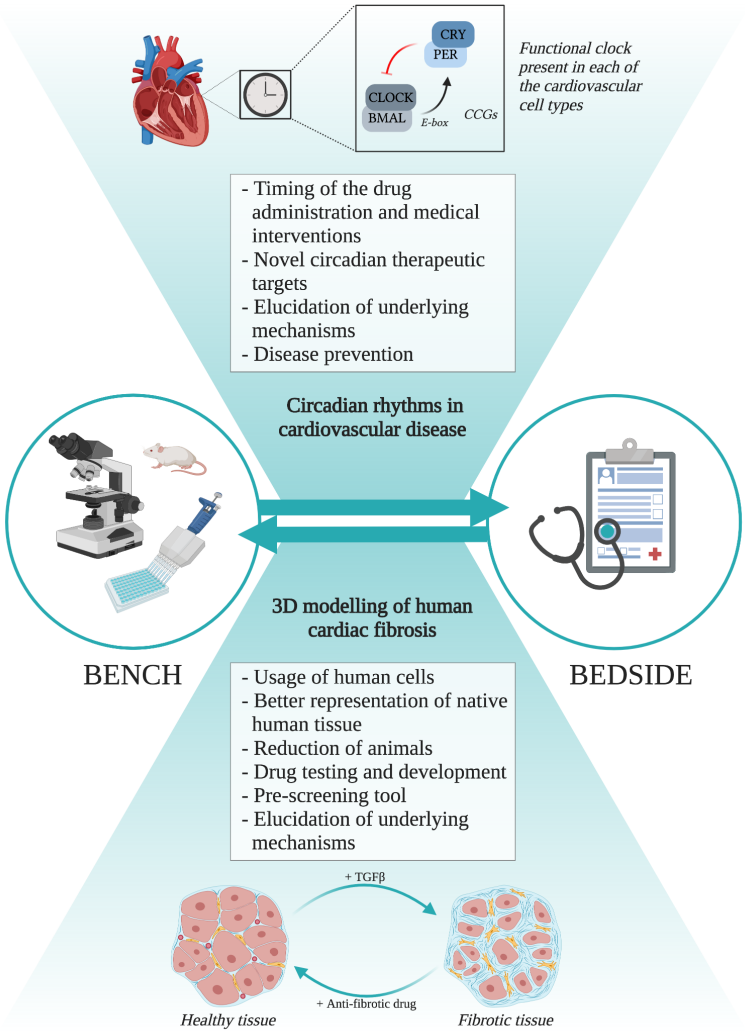


Figure 1 | **From bedside to bench and back:** Dimensions of time (circadian rhythms) and space (3D modelling) as a way of bridging the gap. Created with BioRender.com.

circadian rhythms gained wider recognition only in 2017, when Jeffrey C. Hall, Michael Rosbash and Michael W. Young received the Nobel Prize for their finding of the molecular machinery underpinning the biological clock.[8-10] Following their discovery in the 1980s, these scientists provided us with crucial and fundamental insights into circadian rhythms. They enabled future research that would ultimately link circadian rhythms to human physiology and disease.

Today it is known that circadian rhythms influence almost every aspect of human physiology, spanning from body temperature and hormonal

homeostasis to cardiovascular function, liver metabolism and, amongst others the immune system.[11,12] Furthermore, circadian rhythms are linked to various diseases, including cancer, infection, and diseases of the nervous system.[13] The in-depth explanation of circadian biology, and its role and importance in cardiovascular (patho)physiology, which was the main focus of this thesis, was discussed in **Chapter 2**. Circadian rhythms have been found in every cell type within the cardiovascular system, influencing function in many ways: from vascular tone, heart rate, blood pressure, signalling, to cardiac metabolism and thrombus formation. Environmental changes, diseases, and unhealthy behaviour, often challenge these rhythms. Various animal studies showed that the disrupted environmental rhythms lead to developing cardiomyopathy, cardiac fibrosis, and systolic dysfunction, and ultimately cardiovascular death. Aside from being the cause of the disease, this desynchrony between animals and their surroundings can also aggravate the state of disease, leading to a worse health outcome. In humans, this disruption is seen as a misalignment of external stimuli (day–night cycle) and the intrinsic circadian clock. The causes of this misalignment range from everyday exposure to artificial light in the evening (e.g. via television, smartphones, and computer screens) to more severe sleep disorders, jetlag, and working in shifts.

Circadian rhythms are an integral part of biological processes in all tissues, and their dysfunction has important roles in diseases. Hence, new techniques for identifying circadian rhythms and for understanding their underlying mechanisms in the diseases are needed to develop innovative circadian-based strategies for disease prevention, management, and ultimately cure.

Lessons from the periphery

Since most of the circadian-related research is performed in animal or *in vitro* studies, in **Chapter 3** we sought to determine which of the human peripheral tissue sources could be used to directly and more reliably reflect circadian changes in various disease states. Most of the human organs of interest for studying these rhythms, such as the brain, liver, and heart, are not available for repeated sampling. Therefore, surrogate tissues – blood, hair and beard follicle, and oral mucosa – were used instead. Sampling from these tissues is also proven to accurately reflect the molecular changes of circadian milieu in various disease states. At the same time, they are easily obtained, and without leaving the tissue permanently scarred or damaged after sampling (as opposed to, for example, organ biopsies). Circadian rhythms can be found in virtually every tissue in the body, and finding suitable sources to study peripheral rhythms in humans directly at the source by using human tissue samples could

bear an immense importance for discovering new insights about human (patho)physiology. Novel insights could lead to discovering new links between the disrupted clock and the disease in question, and finding new circadian biomarkers or innovative therapeutic targets. Furthermore, since efficacy and side effects of clinical therapies may be time-dependent, and the circadian clock can modify and confound clinical research, it is of importance to have readily available ways to determine the circadian phase of the subjects. Overall, profiling of peripheral clock gene expression can be used to determine functional circadian rhythms, as well as to recognize the causes, environmental or endogenous, and consequences of deviating rhythms, as seen in the example of shift workers.[14,15] By replacing the working schedule from day to night, thereby disrupting day-night cycling, shift workers experience serious health problems due to the disrupted circadian clock. As a result, shift work is associated with an increased incidence of metabolic syndrome, myocardial infarction, ischaemic stroke and premature death.[16-18] These changes in the circadian clock were observed at the molecular level by utilising previously mentioned peripheral sources. Having the possibility to determine the cause of the problem allows for the development of strategies that could ameliorate those negative effects. In the case of shift workers, this could mean adjustments of light exposure, taking regular naps during the night, or by resetting the clock with strict food intake schedules.[19,20]

Although we were unable to detect any clear rhythmicity of the core clock genes in the blood of HFrEF patients and controls, possibly due to the interference of various cell types present in the whole blood (**Chapter 3**), in **Chapter 4** we did establish their circadian profile using serum melatonin and cortisol measurements. Since circadian rhythms influence many important processes observed in heart failure (e.g. neurohormones relevant in heart failure exhibit a diurnal variation;[21] insomnia increases risk of incident heart failure and heart failure patients often suffer from insomnia[22]), we sought to determine whether its functionality remains intact in heart failure. We investigated the expression of the main endocrine products of the central clock, namely melatonin and cortisol, and found that the central clock output is dampened in heart failure patients. Although both hormones followed the expected diametrically opposite 24-hour pattern in concentration changes (with melatonin peaking during the night and cortisol during the day), their diurnal variation was significantly lower when compared to controls without heart failure. Furthermore, this dampening corresponded to the high prevalence of non-dipping blood pressure and heart rate pattern, while the molecular peripheral clock remained intact, as observed directly in heart tissue of two independent animal models of heart failure. This study further confirms

the intertwined relationship between the circadian rhythms and cardiovascular diseases, and more specifically heart failure, emphasizing the importance of taking circadian rhythms into account both in research and therapy of heart failure. The melatonin and cortisol release are under the direct regulation of the central clock, therefore any changes observed in their rhythmicity also reflect changes in the functionality of the central clock itself. This opens a myriad of opportunities to tackle heart failure and improve the quality of life and survival of patients suffering from this disease. These range from developing new circadian interventions, such as hormonal supplementation, to using non-dipping blood pressure and heart rate in order to develop prevention strategies and improve prognosis.

Clinical impact of circadian variation

In **Chapter 5** and **Chapter 6** we show in two examples the important reach to the clinic that circadian rhythms can have. Biomarkers are crucial components of clinical decision making and monitoring of disease states in general, and are increasingly used in clinical heart failure trials as an inclusion criterion, surrogate endpoint, and target for therapy.[23] Soluble suppression of tumorigenicity-2 (sST2) recently emerged as a biomarker with great prognostic power, serving as a tool for predicting outcomes of patients already diagnosed with heart failure, as well as to optimise their therapy.[24] The cut-off value of 35 ng/mL is currently applied for chronic heart failure patients, meaning that those with serum concentration of sST2 above 35 ng/mL will have a less favourable outcome in terms of e.g. risk of mortality and hospitalization.[24] However, this value was determined without taking into account the possible 24-hour variation of sST2 concentration. In **Chapter 5** we showed that sST2 exhibits diurnal fluctuation, reaching its peak in the afternoon, with the lowest concentration during the night-time. Therefore, physiological diurnal variation of sST2 concentration may lead to the misinterpretation of its values, based on the time of day when blood sampling took place. The maximum range spread of sST2 concentration in all of our subjects was 34.2% and, even in our relatively small cohort of patients, we observed that within the same day two patients had sST2 values both above and under 35 ng/mL. The importance of this phenomenon could be seen when using sST2 concentration to monitor and adjust the heart failure therapy. For example, if a first measurement in the afternoon is above 35 ng/mL, and a follow-up measurement in the morning is below this value, it may indicate that patient's condition is improving and that current therapy is yielding a good therapeutic response. However, since morning sST2 values are usually lower than those in the afternoon, the decrease may also result from mere normal circadian fluctuation and showcase

a misinterpretation of improvement in the patient's condition. sST2 is not an isolated case of biomarkers exhibiting this 24-hour variation. As mentioned before, circadian rhythms have a great impact on the human physiology in general, and specifically on the cardiovascular system.[25] Therefore, it is not surprising that certain cardiovascular-related biomarkers, including cardiac troponin T,[26] are also under its influence.

In **Chapter 6** we provided yet another example of the influence that circadian rhythms can have on clinical decision-making. We investigated individual daily pulmonary artery pressures obtained by CardioMEMS sensors in order to determine the optimal time of day for measuring these pressures in heart failure patients. The importance of this implantable wireless pulmonary artery pressure monitor lies in improved heart failure management, leading to reduced hospitalisations of heart failure patients.[27] Although the diurnal variation of pulmonary artery pressure was previously investigated,[28,29] suggesting that it indeed fluctuates within a 24-hour period, it has not yet been confirmed when this daily measurement should take place in order to provide pulmonary artery pressures with high precision. We used a less invasive and less cumbersome technique to measure these pressures during the normal everyday activity of the patients, as opposed to catheter-based systems which required a hospitalisation of the patients throughout the duration of the study. CardioMEMS sensors are implanted in the pulmonary artery only once, during a minimally invasive outpatient procedure, and allow wireless monitoring of the pressures at any time and from any location. We confirmed the existence of diurnal variation of pulmonary artery pressures, with increasing values in the evening, and showed that pulmonary artery pressures should be measured in the morning. Firstly, as seen in sST2, it is of importance to always measure the pressures at the same time of the day. Due to the circadian rhythms, a first measurement in the morning would yield a lower value, while a follow-up measurement in the evening yielding higher values could suggest a deterioration of patient's condition, while actually merely reflecting its diurnal property. Secondly, not only were the pulmonary artery pressures lower in the morning, but were also consistently more stable in the course of a 5-day period. Pulmonary artery pressures may be influenced by physical activity, food and fluid intake, and different medications,[30] therefore causing inconsistent variations of daily evening pressures. Due to the noticeable between-days fluctuations seen in the evening, any clinically relevant changes in the patient's condition, such as worsening of heart failure, will likely be observed only in the morning, giving a clear indication that an intervention is needed. Evening pressures, on the other hand, reflect the behavioural choices of patients (e.g. exercise and food intake) rather than the

patient's health status. Collectively, if the pulmonary artery pressure is always measured in the morning, any deviating recording would be a clear indication that an intervention is needed.

The results of these studies indicate that circadian rhythms have a far-reaching influence in the clinic. Clinicians should therefore be cautious when interpreting patient data, especially when it comes to novel biomarkers or read-outs for which possible circadian fluctuation has not been established yet. In order not to misinterpret the treatment effect, over- and underestimate the prognostic warning signs, or confound clinical trial outcomes, it is crucial to take into account at what time the patient's sample was taken and to keep the serial measurements consistent at all time.

Perfect timing: Circadian medicine and translational aspects of circadian rhythms

Circadian rhythms are a fundamental part of our biology, underlying many important physiological and pathophysiological processes. They can be envisioned as a plethora of tiny clock mechanisms scattered throughout all tissues in the body, perfectly coordinating timing of various biological processes. However, nowadays, these biological rhythms are very often challenged with our increasingly 24-7 lifestyles. Unhealthy behaviour, including working and eating during the night, and disease, ultimately lead to a misalignment between the internal clock and the external environment. Circadian disruption entails severe health consequences, observed as the development of numerous cardiovascular, metabolic, cognitive and mood disorders. Furthermore, it can also serve as an early warning sign of the disease, as observed in some neurodegenerative processes.[31] Throughout this thesis, we showed that circadian rhythms can indeed be used to improve clinical interventions, diagnosis and prognosis of heart failure patients, and offered an overview of sources suitable to study these rhythms both in human health and disease.

The utilisation of circadian rhythms can be two-fold: they can be used as a tool to study, improve, and develop new therapies, but can also serve as a therapeutic target themselves. Chronotherapy entails the adjustment of treatments based on the different times of the circadian cycle, thus minimising adverse side effects and maximising therapeutic effects. This method of treatment dates back to the 1970s and cancer research, where mice responded better to a treatment given in adjustment to a 24-hour cycle.[32] Today, several medications could be used in this manner, including calcium-channel blockers, angiotensin-converting enzyme inhibitors and aspirin.[33,34] Unfortunately, in practice, these medications are still prescribed randomly throughout the day,

although it has been shown that the risk-to-benefit ratio of angiotensin-converting enzyme-inhibition exhibits 24-hour variation in hypertensive patients.[35] Timing in chemotherapy also proved to be crucial for reducing adverse side effects and maximising therapeutical efficiency.[36,37] Indeed, we found rhythmicity in stress tolerance of human SCA1+ cells[38] and neonatal rat cardiomyocytes[39] to doxorubicin, an anti-cancer drug with known cardiotoxic side effects, thus mimicking the time-of-day-dependent responses of patients to ischemia-reperfusion-based injury.[40] Aside from the circadian variability of pharmacokinetics and pharmacodynamics of various drugs, adjusting the timing of medical procedures could also yield beneficial effects. Montaigne *et al.*[41] showed that there is a greater risk of cardiac damage and major cardiac events after a morning heart surgery, in comparison to the afternoon surgery. Subsequent analysis revealed that this was due to the intrinsic morning–afternoon variation in hypoxia–reoxygenation tolerance, correlated with high morning expression of clock gene REV-ERBα which inhibited the protective effect of CDKN1a/p21 protein. Although no daytime variation was found in similar retrospective studies,[42] the study by Montaigne *et al.*[41] is the only study with a prospective arm thus far. In the multi-centre retrospective study, Nemeth *et al.*[42] found no evidence of the impact of time of day on operative mortality or postoperative complications. However, due to the missing data, this study could not provide specifics on myocardial injury for each patient, such as troponin T levels, and there were some differences in practice procedures across multiple including hospitals. Although it is plausible to assume that circadian rhythms have an effect on the surgical procedures, based on their immense roles in cardiovascular physiology and pathophysiology, further studies are needed to confirm and clarify how circadian patterns impact individual patient risk. Conversely, circadian rhythms can be used as therapeutic targets themselves, as seen in the example of REV-ERB.[43,44] Additionally, the incidence, development and outcome of the disease are also linked to the circadian clock, and their treatment and analysis of the outcome can be tailored to a particular time of day. Incidence of acute myocardial infarctions,[45] sudden cardiac death,[46] and arrhythmias,[47] peaks in the morning, but also other diseases such as asthma, acute and chronic inflammations, allergies and malignancies show 24-hour fluctuations in symptoms and presentation.[48]

Lastly, not only do shift work and jet lag have detrimental effects on human health. Perhaps unexpectedly, circadian rhythms can be disturbed in the places where healing is supposed to take place – in hospitals. Circadian rhythms, in terms of 24-hour fluctuation of blood pressure, heart rate, core temperature, hormone secretion, and activity, were disrupted in patients in the

intensive care unit, possibly impeding their recovery.[49] The cause of this disruption lies in the (lack of) exposure of patients to various input signals, such as parenteral feeding, dim artificial light, frequent wakening in the night by the nursing staff, and an absence of activity or social interaction owing to the bed rest or sedation. Having this in mind, the field of human-centric circadian lightning emerged in several hospitals and nursing homes as an alternative solution for keeping natural circadian rhythms intact while patients are in recovery. The main idea entails tuning the indoor lightning to mimic the gradual colour changes of the sun observed during the day: from bright, blueish morning light to amber at dusk, and ultimately to as dark as possible, but taking into account the work of the hospital staff. The neurosurgical unit in Aarhus University Hospital and neurological unit in Glostrup in Denmark represent some of the many hospitals, psychiatric wards and nursing homes throughout Scandinavia utilising such an environment. Although more scientific evidence is required to determine the exact effect on patients' outcomes, according to the hospitals this stimulating environment benefits patients in terms of improved quality of sleep, mood, fatigue, and depression.[50] A recent study in a psychiatric hospital unit showed that the blue-depleted lightning in the evening improves sleep and supresses melatonin levels less when compared to normal lightning, pointing to potential benefits of installing similar circadian lightning systems in other hospitals.[51] Another important example was provided in the preclinical study of Alibhai *et al.*[52], where disrupted diurnal rhythm immediately after myocardial infarction impaired cardiac healing and worsened maladaptive remodelling. This study suggested that the disruption of circadian rhythms, as seen in cardiac care or intensive care units, may have an adverse long-term effect on the patient's health outcome.

Time is a crucial consideration in the treatment of cardiovascular but also many other diseases. Various adverse effects could potentially be avoided if clock disruption in our everyday lives, but also in the hospitals, would be minimised. With the increasing scientific knowledge of the importance that the circadian rhythms hold and the rapid pace with which this field is growing, we deem it necessary that the newly proposed field of circadian medicine be implemented in the everyday clinical setting.[53] By bridging the clinical practice with circadian rhythms, our understanding of the underlying disease mechanisms will be improved, timing of the drug administration or other therapeutic interventions refined, and new potential circadian therapeutic targets for certain diseases unravelled.

Three-dimensional (3D) engineered tissues for cardiac disease modelling

Another dimension explored in this thesis entails the usage of 3D *in vitro* models for disease modelling and as platforms for testing and validating therapeutics. Cardiac fibrosis is the main hallmark of heart failure, with no available cure so far. One of the major issues regarding the cardiovascular drug development is the failure of translating preclinical discoveries into the clinical arena, largely due to the inter-species differences.[54] Although animal studies are still very much necessary to appropriately address the complexity of the human organism, early *in vitro* proof-of-concept studies directly on human material are needed to distinguish which compounds are feasible to enter further pre-clinical trials. In **Chapter 7**, we engineered a mechanically tuneable 3D *in vitro* model of human cardiac fibrosis as one approach to tackle these hurdles.

The main strength of an *in vitro* model also represents its main weakness: it is a simplified system in comparison to the animal models, or even the targeted patient. However, it allows for easier tweaking of its properties, controlled environment and the usage of human-derived cells, while reducing the number of sacrificed animals. When compared to conventional cellular monolayers, 3D cell-culture models are proven to be superior, especially when it comes to mimicking fibrotic remodelling.[55,56] Cardiac fibroblasts are exceptionally sensitive to material stiffness and spontaneously differentiate to their active myofibroblast form if cultured on culture plastic, as is the case for conventional 2D monolayers. In this study, we used human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes and primary cardiac fibroblasts, embedded in gelatine methacryloyl (GelMA) hydrogels. Due to its tuneable stiffness, GelMA allowed for cardiac fibroblasts to remain in their quiescent state until the fibrotic remodelling was initiated. Using such model confirmed the anti-fibrotic activity of pirfenidone, which is currently used in a clinical trial for treating cardiac fibrosis in HFpEF patients.[57] Ours and similar 3D models could illuminate the molecular basis of the results of this clinical trial which otherwise could not be tackled due to the difficulties of studying treatment effects directly in cardiac tissue of patients. These models could also serve as a pre-screening tool for other compounds before even entering clinical studies.

Combining the dimension of space and time could further yield superior cardiac 3D constructs, as described in a recent review of ours.[58] Taking the importance of circadian rhythms in human physiology into account, the authors propose to tweak the cell composition and biochemical and mechanical cues of the extracellular matrix (ECM) as a first step of optimising cardiac constructs, with a goal to obtain rhythms with optimal

amplitude for each cardiac cell type. This way, the *in vitro* cardiac constructs are tuned in both time and space, thus more reliably reflecting the native cardiac microenvironment. The rationale for these studies can be found in several examples linking cardiovascular diseases with ECM abnormalities,[59-61] as well as associating abnormalities in matrix with dampened molecular circadian output.[62,63] 3D matrices are also important for retaining rhythmical properties of cardiac cells, as seen in our study on SCA1+ cells.[38] Cell dissociation using trypsinization or collagenase treatment resulted in disrupted circadian oscillation of SCA1+ cells cultured on 2D monolayers. However, when loaded in matrices, cells oscillated as they did before the enzymatic treatment while not requiring such detachment procedures for further downstream applications (e.g. cell therapy). Overall, we showed that there is an overwhelming amount of evidence that circadian rhythms play an immense role in human (patho)physiology. In addition, merging the knowledge of time with the development of novel 3D cellular constructs will yield more reliable and representative *in vitro* human tissues.

Time for a change: one-size-fits-all approach no longer fits cardiovascular diseases

A new era in science is dawning. Throughout this thesis, we have shown that the time for a change in science is ripe and necessary in order to keep pace with ever-growing diseased numbers emerging from the societal changes. In this work, two dimensions are introduced into the cardiovascular field, but can be applied to other research areas as well: dimension of time and dimension of space, in terms of circadian rhythms and 3D *in vitro* modelling, respectively. However, these dimensions, although an important addition to current scientific practice by themselves, can be further broken down into different categories which need to be addressed.

Personalised or precision medicine emerged as a concept in medicine in which a person's genes, lifestyle and environment are used to individually tailor their health care. Sex, age and ethnic differences can be seen as special categories when discussing precision medicine. In **Chapter 2**, we laid out the various examples where *one-size* approach is not applicable and where differences between individuals need to be taken into account when researching circadian rhythms in cardiovascular disease. Shortly, studies have shown that free-running circadian period differs between African-American and European-American individuals,[64] while African-American individuals have a greater incidence of cardiovascular disease than European-American, and experience more nocturnal hypertension (non-dippers).[65] Furthermore, ageing changes circadian rhythms on different levels: older individuals

experience a shift towards a morning chronotype in terms of cognitive performance and alertness, their sleep quality and quantity deteriorate, their main hormonal hallmarks of circadian rhythms – melatonin and cortisol – are dampened with shifted peak, and progressive dampening of metabolic rhythms is observed, leading to a development of risk factors for cardiovascular diseases (e.g. diabetes, dyslipidaemia and hypertension).[66,67] A flurry of recent studies show that heart diseases manifest differently in males and females, with an important underlying role of circadian rhythms.[68-70] Males and females have a different resilience to cardiovascular diseases as well as different pathophysiological outcomes.[70] Alibhai *et al.* indeed found that female sex protects against clock-mediated accelerated pathological remodelling of the aging heart, which is likely mediated by ovarian hormones. In a recent editorial, which is in an **addendum to this discussion**, we further discuss this role of the biological sex in the age-dependent cardiomyopathy. Although these broadly defined differences are important categories that should be taken into account both in the clinic and research, even among the patients with the same sex, age and ethnicity the individual differences should be distinguished and recognised. As we recently described,[71] targeting circadian rhythms can open new therapeutic avenues by adjusting individual circadian rhythms of patients: restoring/resynchronising circadian rhythm (e.g. blood pressure dipping at night), or using light therapy and exogenous melatonin to prevent cardiovascular disorders by limiting the disruption of circadian rhythms. Furthermore, in the laboratory setting, utilising hiPSCs, as described in **Chapter 7**, not only represents a unique tool to study specific human diseases and develop models for therapeutic testing, but also opens an immense opportunity to advance medical therapy by personalising the field of regenerative medicine.

Through the multidimensional looking glass

Until now, looking for affected pathways and drug targets for heart failure has been done in a static way. However, by comparing the diseased and healthy condition at a single time-point, important differences can be overlooked. By pinpointing differentially expressed circadian profiles in heart failure patients and healthy controls, new druggable targets and circadian biomarkers can be revealed, and early warning signs of the disease recognized. This can be done by using peripheral, minimally invasive human tissues as a reliable source of individual circadian rhythms. Furthermore, inconsistent serial measurements and the disregard for the time when the patient's sample was taken could lead to the misinterpretation of the treatment effect, and over- and underestimation of the prognostic warning signs. Since efficacy and side effects of clinical

therapies may also be time-dependent, disregarding the component of time can potentially confound clinical trial outcomes. Aside from circadian rhythms, *in vitro* studies can be improved by using 3D models to more reliably mimic healthy cardiac tissue, as well as investigate underlying disease pathways and potential therapeutic targets for cardiac fibrosis.

In conclusion, this thesis underscored the importance of broadening the perspectives of the scientific research in the cardiovascular field by introducing dimensions of time and space. Broadly speaking, observations made in the clinical arena shape hypotheses which are then tested and validated at the bench, finally making their way back to the patient's bedside in a form of novel therapies or recommendations for patient care (**Figure 1**). This journey should be paved with novel 3D constructs, more reliably representing human tissues, and a consideration of time (in terms of circadian rhythms), with complementing knowledge of both scientists and clinicians. Overall, through the work in this thesis, we set the stage for novel dimensions of diagnostic, prognostic and therapeutic approaches in heart failure. We show that time and space are a prerequisite for future research and prompt researchers and clinicians to take them into consideration when unravelling this deadly disease (**Figure 2**).

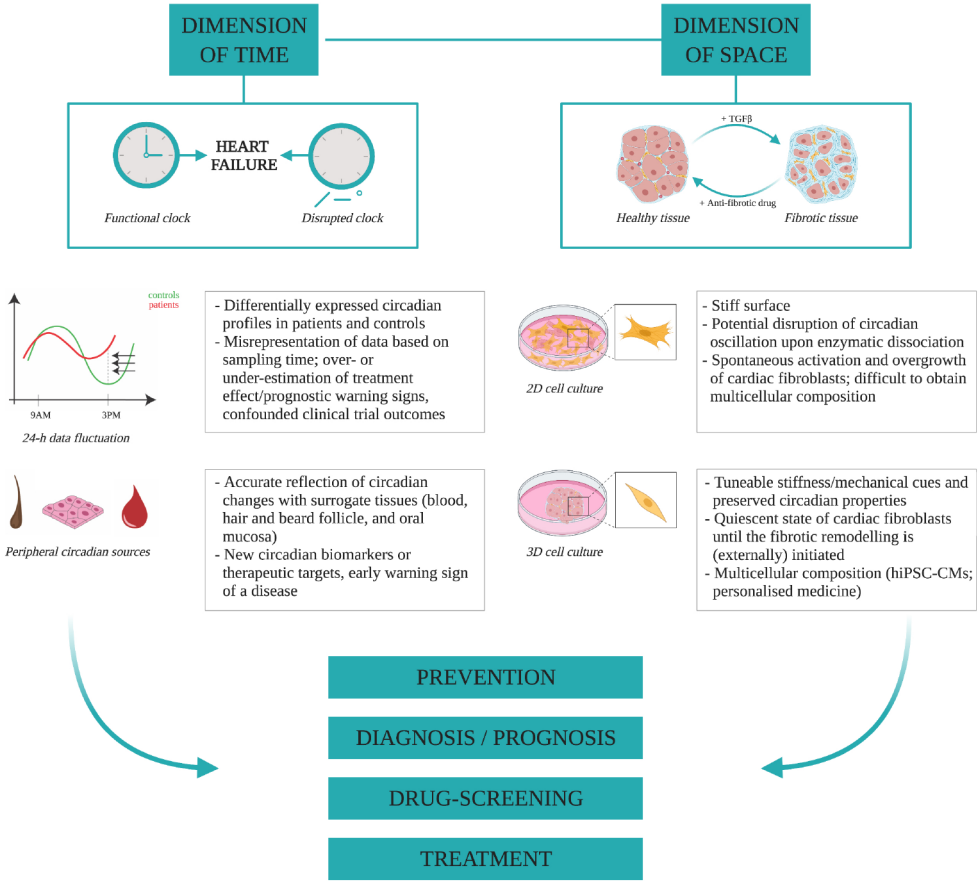


Figure 2 | **Through the multidimensional looking glass:** Novel perspectives on heart failure. Created with BioRender.com.

References

1. WHO. Cardiovascular Diseases. https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds) (2020).
2. Bui, A. L., Horwich, T. B. & Fonarow, G. C. Epidemiology and risk profile of heart failure. *Nat. Rev. Cardiol.* **8**, 30–41 (2011).
3. Stewart, S., MacIntyre, K., Hole, D. J., Capewell, S. & McMurray, J. J. More 'malignant' than cancer? Five-year survival following a first admission for heart failure. *Eur. J. Heart Fail.* **3**, 315–322 (2001).
4. Ponikowski, P. et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC)Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur. Heart J.* **37**, 2129–2200 (2016).
5. Levin, R. et al. Reverse remodeling induced by Sacubitril/Valsartan. *J. Am. Coll. Cardiol.* **73**, 702 (2019).
6. Fuchs, M. et al. Does the heart transplant have a future? Eur. J. Cardio-Thorac. Surg. Off. J. Eur. Assoc. *Cardio-Thorac. Surg.* **55**, i38–i48 (2019).
7. Nederlandse Transplantatie Stichting. Organen: jaarcijfers. https://www.transplantatiestichting.nl/publicaties-en-naslag/cijfers-over-donatie-en-transplantatie/organen-jaarcijfers.
8. Siwicki, K. K., Eastman, C., Petersen, G., Rosbash, M. & Hall, J. C. Antibodies to the period gene product of Drosophila reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* **1**, 141–150 (1988).
9. Bargiello, T. A., Jackson, F. R. & Young, M. W. Restoration of circadian behavioural rhythms by gene transfer in Drosophila. *Nature* **312**, 752–754 (1984).
10. Hardin, P. E., Hall, J. C. & Rosbash, M. Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. *Nature* **343**, 536–540 (1990).
11. Durgan, D. J. & Young, M. E. The cardiomyocyte circadian clock: emerging roles in health and disease. *Circ. Res.* **106**, 647–658 (2010).
12. Scheiermann, C., Kunisaki, Y. & Frenette, P. S. Circadian control of the immune system. *Nat. Rev. Immunol.* **13**, 190–198 (2013).
13. Xie, Y. et al. New Insights Into the Circadian rhythm and its related diseases. *Front. Physiol.* **10**, 682 (2019).
14. Koshy, A., Cuesta, M., Boudreau, P., Cermakian, N. & Boivin, D. B. Disruption of central and peripheral circadian clocks in police officers working at night. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **33**, 6789–6800 (2019).
15. Akashi, M. et al. Noninvasive method for assessing the human circadian clock using hair follicle cells. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 15643–15648 (2010).
16. Esquirol, Y. et al. Shift work and cardiovascular risk factors: new knowledge from the past decade. *Arch. Cardiovasc. Dis.* **104**, 636–668 (2011).
17. Vyas, M. V. et al. Shift work and vascular events: systematic review and meta-analysis. *BMJ* **345**, e4800 (2012).
18. Roenneberg, T. & Merrow, M. The circadian clock and human health. *Curr. Biol. CB* **26**, R432-443 (2016).
19. Neil-Sztramko, S. E., Pahwa, M., Demers, P. A. & Gotay, C. C. Health-related

interventions among night shift workers: a critical review of the literature. *Scand. J. Work. Environ. Health* **40**, 543–556 (2014).
20. Purnell, M. T., Feyer, A. M. & Herbison, G. P. The impact of a nap opportunity during the night shift on the performance and alertness of 12-h shift workers. *J. Sleep Res.* **11**, 219–227 (2002).
21. Sole, M. J. & Martino, T. A. Diurnal physiology: core principles with application to the pathogenesis, diagnosis, prevention, and treatment of myocardial hypertrophy and failure. *J. Appl. Physiol. Bethesda Md 1985* **107**, 1318–1327 (2009).
22. Laugsand, L. E., Strand, L. B., Platou, C., Vatten, L. J. & Janszky, I. Insomnia and the risk of incident heart failure: a population study. *Eur. Heart J.* **35**, 1382–1393 (2014).
23. Ibrahim, N. E., Gaggin, H. K., Konstam, M. A. & Januzzi, J. L. Established and emerging roles of biomarkers in heart failure clinical trials. *Circ. Heart Fail.* **9**, (2016).
24. Boisot, S. et al. Serial sampling of ST2 predicts 90-day mortality following destabilized heart failure. *J. Card. Fail.* **14**, 732–738 (2008).
25. Crnko, S., Du Pré, B. C., Sluijter, J. P. G. & Van Laake, L. W. Circadian rhythms and the molecular clock in cardiovascular biology and disease. *Nat. Rev. Cardiol.* **16**, 437–447 (2019).
26. Klinkenberg, L. J. J. et al. Circulating Cardiac Troponin T Exhibits a Diurnal Rhythm. *J. Am. Coll. Cardiol.* **63**, 1788–1795 (2014).
27. Abraham, W. T. et al. Wireless pulmonary artery haemodynamic monitoring in chronic heart failure: a randomised controlled trial. *Lancet Lond. Engl.* **377**, 658–666 (2011).
28. Gibbs, J. S. et al. Diurnal variation of pulmonary artery pressure in chronic heart failure. *Br. Heart J.* **62**, 30–35 (1989).
29. Brigden, G. & Raftery, E. B. Diurnal variation in pulmonary artery pressure. *J. Sleep Res.* **4**, 37–43 (1995).
30. Gibbs, J. S., Keegan, J., Wright, C., Fox, K. M. & Poole-Wilson, P. A. Pulmonary artery pressure changes during exercise and daily activities in chronic heart failure. *J. Am. Coll. Cardiol.* **15**, 52–61 (1990).
31. Tranah, G. J. et al. Circadian activity rhythms and risk of incident dementia and mild cognitive impairment in older women. *Ann. Neurol.* **70**, 722–732 (2011).
32. Haus, E. et al. Increased tolerance of leukemic mice to arabinosyl cytosine with schedule adjusted to circadian system. *Science* **177**, 80–82 (1972).
33. Smolensky, M. H., Hermida, R. C., Ayala, D. E., Tiseo, R. & Portaluppi, F. Administration-time-dependent effects of blood pressure-lowering medications: basis for the chronotherapy of hypertension. *Blood Press. Monit.* **15**, 173–180 (2010).
34. Racca, C. et al. Aspirin intake in the morning is associated with suboptimal platelet inhibition, as measured by serum Thromboxane B2, during infarct-prone early-morning hours. *Platelets* **30**, 871–877 (2019).
35. Hermida, R. C., Ayala, D. E., Mojón, A. & Fernández, J. R. Bedtime dosing of antihypertensive medications reduces cardiovascular risk in CKD. *J. Am. Soc. Nephrol.* **22**, 2313–2321 (2011).
36. Lévi, F. A. et al. Chronomodulated versus fixed-infusion-rate delivery of ambulatory chemotherapy with oxaliplatin, fluorouracil, and folinic acid (leucovorin) in patients with colorectal cancer metastases: a randomized multi-institutional trial. *J. Natl. Cancer Inst.* **86**, 1608–1617 (1994).
37. Lévi, F., Zidani, R. & Misset, J. L. Randomised multicentre trial of chronotherapy with

oxaliplatin, fluorouracil, and folinic acid in metastatic colorectal cancer. International Organization for Cancer Chronotherapy. *Lancet Lond. Engl.* **350**, 681–686 (1997).
38. Du Pré, B. C. et al. SCA1+ cells from the heart possess a molecular circadian clock and display circadian oscillations in cellular functions. *Stem Cell Rep.* **9**, 762–769 (2017).
39. du Pré, B. C. et al. Neonatal rat cardiomyocytes as an in vitro model for circadian rhythms in the heart. *J. Mol. Cell. Cardiol.* **112**, 58–63 (2017).
40. Durgan, D. J. et al. Ischemia/reperfusion tolerance is time-of-day-dependent: Mediation by the cardiomyocyte circadian clock. *Circ. Res.* **106**, 546–550 (2010).
41. Montaigne, D. et al. Daytime variation of perioperative myocardial injury in cardiac surgery and its prevention by Rev-Erbα antagonism: a single-centre propensity-matched cohort study and a randomised study. *Lancet Lond. Engl.* **391**, 59–69 (2018).
42. Nemeth, S., Schnell, S., Argenziano, M., Ning, Y. & Kurlansky, P. Daytime variation does not impact outcome of cardiac surgery: Results from a diverse, multi-institutional cardiac surgery network. *J. Thorac. Cardiovasc. Surg.* (2019).
43. Woldt, E. et al. Rev-erb-α modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy. *Nat. Med.* **19**, 1039–1046 (2013).
44. Sulli, G. et al. Pharmacological activation of REV-ERBs is lethal in cancer and oncogene induced senescence. *Nature* **553**, 351–355 (2018).
45. Muller, J. E. et al. Circadian variation in the frequency of onset of acute myocardial infarction. *N. Engl. J. Med.* **313**, 1315–1322 (1985).
46. Muller, J. E. et al. Circadian variation in the frequency of sudden cardiac death. *Circulation* **75**, 131–138 (1987).
47. Portaluppi, F. & Hermida, R. C. Circadian rhythms in cardiac arrhythmias and opportunities for their chronotherapy. *Adv. Drug Deliv. Rev.* **59**, 940–951 (2007).
48. Smolensky, M. H. et al. Diurnal and twenty-four hour patterning of human diseases: acute and chronic common and uncommon medical conditions. *Sleep Med. Rev.* **21**, 12–22 (2015).
49. Paul, T. & Lemmer, B. Disturbance of circadian rhythms in analgosedated intensive care unit patients with and without craniocerebral injury. *Chronobiol. Int.* **24**, 45–61 (2007).
50. Chromaviso Chroma Zenit Circadian Lighting - Research and evidence | Chromaviso. https://chromaviso.com/en/circadian-lighting/research-in-circadian-lighting/.
51. Vethe, D. et al. The evening light environment in hospitals can be designed to produce less disruptive effects on the circadian system and improve sleep. *Sleep* (2020).
52. Alibhai, F. J. et al. Short-term disruption of diurnal rhythms after murine myocardial infarction adversely affects long-term myocardial structure and function. *Circ. Res.* **114**, 1713–1722 (2014).
53. Panda, S. The arrival of circadian medicine. *Nat. Rev. Endocrinol.* **15**, 67–69 (2019).
54. Rongen, G. A. & Wever, K. E. Cardiovascular pharmacotherapy: Innovation stuck in translation. *Eur. J. Pharmacol.* **759**, 200–204 (2015).
55. Pampaloni, F., Reynaud, E. G. & Stelzer, E. H. K. The third dimension bridges the gap between cell culture and live tissue. *Nat. Rev. Mol. Cell Biol.* **8**, 839–845 (2007).
56. Zuppinger, C. 3D culture for cardiac cells. *Biochim. Biophys. Acta* **1863**, 1873–1881 (2016).
57. Lewis, G. A. et al. Pirfenidone in heart failure with preserved ejection fraction - Rationale and design of the PIROUETTE Trial. *Cardiovasc. Drugs Ther.* **33**, 461–470 (2019).

58. Chirico, N., Van Laake, L. W., Sluijter, J. P. G., van Mil, A. & Dierickx, P. Cardiac circadian rhythms in time and space: The future is in 4D. *Curr. Opin. Pharmacol.* **57**, 49–59 (2021).
59. Burgess, M. L., McCrea, J. C. & Hedrick, H. L. Age-associated changes in cardiac matrix and integrins. *Mech. Ageing Dev.* **122**, 1739–1756 (2001).
60. Bouzeghrane, F., Reinhardt, D. P., Reudelhuber, T. L. & Thibault, G. Enhanced expression of fibrillin-1, a constituent of the myocardial extracellular matrix in fibrosis. *Am. J. Physiol. Heart Circ. Physiol.* **289**, H982-991 (2005).
61. Murray, D. B., Gardner, J. D., Brower, G. L. & Janicki, J. S. Effects of nonselective endothelin-1 receptor antagonism on cardiac mast cell-mediated ventricular remodeling in rats. *Am. J. Physiol. Heart Circ. Physiol.* **294**, H1251-1257 (2008).
62. Williams, J. et al. Epithelial and stromal circadian clocks are inversely regulated by their mechano-matrix environment. *J. Cell Sci.* **131** (2018).
63. Yang, N. et al. Cellular mechano-environment regulates the mammary circadian clock. *Nat. Commun.* **8** (2017).
64. Eastman, C. I., Suh, C., Tomaka, V. A. & Crowley, S. J. Circadian rhythm phase shifts and endogenous free-running circadian period differ between African-Americans and European-Americans. *Sci. Rep.* **5**, 8381 (2015).
65. Yamasaki, F., Schwartz, J. E., Gerber, L. M., Warren, K. & Pickering, T. G. Impact of shift work and race/ethnicity on the diurnal rhythm of blood pressure and catecholamines. *Hypertens.* **32**, 417–423 (1998).
66. Hood, S. & Amir, S. The aging clock: circadian rhythms and later life. *J. Clin. Invest.* **127**, 437–446 (2017).
67. Pagani, L. et al. Serum factors in older individuals change cellular clock properties. *Proc. Natl. Acad. Sci.* **108**, 7218–7223 (2011).
68. Savarese, G. & D'Amario, D. Sex Differences in Heart Failure. *Adv. Exp. Med. Biol.* **1065**, 529–544 (2018).
69. Alibhai, F. J. et al. Female ClockΔ19/Δ19 mice are protected from the development of age-dependent cardiomyopathy. *Cardiovasc. Res.* **114**, 259–271 (2018).
70. Glen Pyle, W. & Martino, T. A. Circadian rhythms influence cardiovascular disease differently in males and females: role of sex and gender. *Curr. Opin. Physiol.* **5**, 30–37 (2018).
71. Crnko, S., Cour, M., Van Laake, L. W. & Lecour, S. Vasculature on the clock: Circadian rhythm and vascular dysfunction. *Vascul. Pharmacol.* **108**, 1–7 (2018).

8

CHAPTER

Addendum: New Dimensions in Circadian Clock Function: the Role of Biological Sex

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Based on:
Crnko, S. *et al.* New dimensions in circadian clock function: the role of biological sex. *Cardiovasc Res* **114**, 203–204 (2018).

This editorial refers to 'Female *CLOCK*$^{\Delta 19/\Delta 19}$ mice are protected from the development of age-dependent cardiomyopathy' by F. J. Alibhai *et al.*, pp. 259–271.

Cardiovascular physiology and pathophysiology are both profoundly influenced by 24-h (circadian or diurnal) rhythms, ranging from daily heart rate and blood pressure variations[1] to onset and outcome of myocardial infarction.[2] With at least 10% of genes in the intact heart showing significant time-of-day dependent oscillations,[3] it does not strike as a surprise that disrupting diurnal rhythms presents a major risk of developing cardiovascular disease.[4,5] The importance of investigating 24-h rhythms in the cardiovascular context is emerging especially since the main cellular clockwork mechanism, including key clock genes *BMAL1/2*, *CLOCK*, *CRY1/2* and *PER1/2/3*,[6] has now been identified within all cardiovascular tissues.[7]

In this issue of Cardiovascular Research, Alibhai *et al.*[8] bring a unique set of data to the field by exploring biological sex as an important player in the development of age-dependent cardiomyopathy in *CLOCK*$^{\Delta 19/\Delta 19}$ mice. In these mutant mice the *CLOCK* gene is ubiquitously mutated resulting in a protein incapable of proper heterodimerization with BMAL and thereby lacking the capacity of transcriptional activation of *PER*. As such, the transcriptional-translational feedback loop is severely disturbed and mice with this mutation lack circadian clock function in all their cell types. In a previous study, the authors observed the development of age-dependent cardiac dysfunction in male *CLOCK*$^{\Delta 19/\Delta 19}$ mice,[9] confirming a previous finding linking the circadian clock with aging.[10]

In this study Alibhai *et al.*[8] tackled the intriguing question whether female sex protects against clock-mediated accelerated pathological remodelling of the aging heart. The authors investigated several metabolic changes and differences between the male and female wild-type and *CLOCK*$^{\Delta 19/\Delta 19}$ mice. They describe that even with the mutated *CLOCK*, in comparison to male, female mice do not develop age-dependent cardiac disease until a very late age of 21 months, when they show milder signs of cardiac aging. The phenotype observed in male mice, comprising increased heart weight, cardiomyocyte hypertrophy, dilation, reduced myogenic responsiveness, impaired contractility and interstitial fibrosis, was mitigated by female biological sex. Ovariectomized female *CLOCK*$^{\Delta 19/\Delta 19}$ mice on the other hand did develop cardiac dilation, glucose intolerance and reduced cardiac cytochrome c oxidase; i.e. the same phenotype as observed in male *CLOCK*$^{\Delta 19/\Delta 19}$ mice, corroborating the protective role of the female sex hormones along with the importance of *CLOCK* in age-dependent

cardiomyopathy.

Several questions arise from the work of Alibhai *et al.*[8] For example, the next interesting step of investigation would be to determine the exact mechanism of the protectiveness of female biological sex. Metabolism is likely to be a key player in the interaction with oestrogens. Zhu *et al.*[11] showed that by interacting with the intrinsic circadian clock in adipose tissue, oestrogen prevents abnormal lipid accumulation caused by circadian disruption. However, the exact mechanisms of estrogen-protecting role are complex and not yet fully understood. Furthermore, along with exploring the mechanisms and signalling pathways responsible for the protective role of female biological sex in age-dependent cardiomyopathy, it would be of interest to investigate whether the same phenomena can be observed in other heart diseases with known influence of circadian oscillations, such as myocardial infarction.

Notably, this work reinforces the importance of studying sex-specific responses to internal or external factors influencing aging and organ function as recently reviewed by Ventura-Clapier *et al.*[12] Thus, the use of animals of both sexes should be promoted in basic research to investigate deeper the contribution of hormonal and non-hormonal actors (sex-specific gene expression) underlying sex differences.[13] In the present context, as the important roles of circadian rhythms in cardiovascular (patho)physiology are emerging,[14] new platforms to analyse them are being developed. Embryonic stem cell-derived cardiomyocytes,[15] stem cell antigen 1-positive (SCA1+) cells (SPCs)[16] and neonatal rat cardiomyocytes[17] are relevant *in vitro* models regarding both molecular and functional influence of circadian rhythms in the heart. *In vivo* rhythmicity can therefore be recapitulated, allowing for the physiological and disrupted cardiac clock to be monitored. The influence of biological sex in clock-mediated cardiotropic mechanisms could be studied using these recently established *in vitro* and *in vivo* systems by taking into account the sex of the cell donor and mimicking hormonal influences in culture conditions. This will advance our understanding of sex-specific responses to circadian rhythm dysregulation affecting the cardiac system and pave the way to the sex-tailored therapy (**Figure 1**).

In conclusion, this is the first time that interaction of ovarian hormones and circadian rhythms has been shown to influence cardiac aging. Since the majority of studies in the field of circadian rhythms are done in males, the findings of Alibhai *et al.*[8] highlight the importance of taking the influence of biological sex into the account when it comes to studying circadian rhythms and heart diseases. Taken together, insights provided within this research on difference between male and female *CLOCK*$^{\Delta 19/\Delta 19}$ mice may elucidate novel potential circadian targets and interventions in cardiovascular therapy.

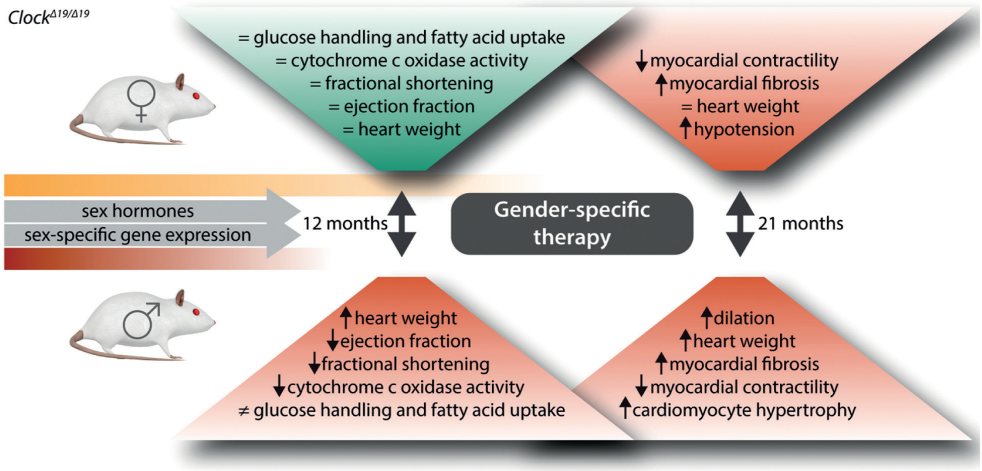

Figure 1 | **Clock disruption has differential effects in males and females, envisioning the potential and need for more personalized therapies.** An impaired heart function begins to develop only from 21 months in female *CLOCK*$^{\Delta 19/\Delta 19}$ mice, while male *CLOCK*$^{\Delta 19/\Delta 19}$ mice exhibit adverse cardiac changes from early age, suggesting the protective role of female biological sex in the development of age- and clock-dependent cardiomyopathy. All data are shown in comparison with WT mice.

Declaration of interest

None declared.

References

1. Degaute, J. P., van de Borne, P., Linkowski, P. & Van Cauter, E. Quantitative analysis of the 24-hour blood pressure and heart rate patterns in young men. *Hypertension* **18**: 199–210 (1991).
2. Bulluck, H. et al. Circadian variation in acute myocardial infarct size assessed by cardiovascular magnetic resonance in reperfused STEMI patients. *Int. J. Cardiol.* **230**: 149–154 (2017).
3. Martino, T. et al. Day/night rhythms in gene expression of the normal murine heart. *J. Mol. Med.* **82**: 256–264 (2004).
4. Alibhai, F. J., Tsimakouridze, E. V., Reitz, C. J., Pyle, W. G. & Martino, T. A. Consequences of circadian and sleep disturbances for the cardiovascular system. *Can. J. Cardiol.* **31**: 860–872 (2015).
5. Vyas, M. V. et al. Shift work and vascular events: systematic review and meta-analysis. *Bmj* **345**: e4800 (2012).
6. Lowrey, P. L. & Takahashi, J. S. Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annu. Rev. Genom. Hum. Genet.* **5**: 407–441 (2004).
7. Davidson, A. J., London, B., Block, G. D. & Menaker, M. Cardiovascular tissues contain independent circadian clocks. *Clin. Exp. Hypertens.* **27**: 307–311 (2005).
8. Alibhai, F. J. et al. Female Clock$^{\Delta 19/\Delta 19}$ mice are protected from the development of age-dependent cardiomyopathy. *Cardiovasc. Res.* **114**: 259–271 (2018).
9. Alibhai, F. J. et al. Disrupting the key circadian regulator CLOCK leads to age-dependent cardiovascular disease. *J. Mol. Cell. Cardiol.* **105**: 24–37 (2017).
10. Sato, S. et al. Circadian reprogramming in the liver identifies metabolic pathways of aging. *Cell* **170**: 664–677.e11 (2017).
11. Zhu, L. et al. Estrogens prevent metabolic dysfunctions induced by circadian disruptions in female mice. *Endocrinology* **156**: 2114–2123 (2015).
12. Ventura-Clapier, R. et al. Sex in basic research: concepts in the cardiovascular field. *Cardiovasc. Res.* **113**: 711–724 (2017).
13. Merz AA & Cheng S. Sex differences in cardiovascular ageing. *Heart* **102**: 825–831 (2016).
14. Martino TA & Young ME. Influence of the cardiomyocyte circadian clock on cardiac physiology and pathophysiology. *J. Biol. Rhythms.* **30**: 183–205 (2015).
15. Dierickx, P. et al. Circadian networks in human embryonic stem cell-derived cardiomyocytes. *EMBO Rep.* **18**: 1199–1212 (2017).
16. Du Pré, B. C. et al. SCA1+ cells from the heart possess a molecular circadian clock and display circadian oscillations in cellular functions. *Stem Cell Rep.* **9**: 762–769 (2017).
17. Du Pré, B. C. et al. Neonatal rat cardiomyocytes as an *in vitro* model for circadian rhythms in the heart. *J. Mol. Cell. Cardiol.* **112**: 58–63 (2017).

APPENDIX A

Nederlandse Samenvatting

Acknowledgements

List of Publications

Curriculum Vitae

Nederlandse samenvatting

Met een aandeel van 31% in het wereldwijde aantal sterfgevallen vormen hart- en vaatziekten een groot gezondheidsprobleem. Wereldwijd leiden meer dan 23 miljoen mensen aan hartfalen, waarmee het een van de meest voorkomende hart- en vaatziekten is. Ieder mens heeft een levenslang risico van 20% om hartfalen te ontwikkelen en dit treedt met name op bij mensen van 65 jaar en ouder. Ondanks recente verbeteringen in behandelmethodes blijft hartfalen een dodelijke ziekte zonder een eenvoudig beschikbare remedie. Een harttransplantatie is vooralsnog de enige behandeling voor eindstadium hartfalen, maar er is een groot tekort aan donororganen. In Nederland worden jaarlijks minder dan 40 harttransplantaties uitgevoerd, wat te weinig is gezien de continue toename van het aantal personen met ernstig hartfalen. Met een sterftekans van 52.6% binnen vijf jaar kan hartfalen in feite beschouwd worden als "kwaadaardiger" dan vele vormen van kanker. Dit maakt het vinden van nieuwe benaderingen om het verloop van deze ziekte te begrijpen en beter te behandelen noodzakelijk.

Gebaseerd op de ejectiefractie (EF) kan hartfalen worden geclassificeerd als hartfalen met een gereduceerde EF (HFrEF; EF < 40%), hartfalen met een *mid-range* of enigszins gereduceerde EF (HFmrEF; EF tussen de 40% en 49%) en hartfalen met behoud van EF (preserved EF, HFpEF; EF > 50%). HFrEF – de focus van dit proefschrift – wordt gekenmerkt door een verminderde knijpkracht van de linkerhartkamer (systolisch hartfalen), terwijl bij HFpEF de relaxatie van de linkerhartkamer verstoord is (diastolisch hartfalen). Fibrotische remodelering is een fundamenteel proces bij het ontstaan en progressie van hartfalen, met verschillende types fibrose bij HFrEF en HFpEF.

Reparatieve of vervangende fibrose wordt gekarakteriseerd door overmatige afzetting van extracellulaire matrix (ECM) eiwitten als gevolg van schade aan het myocard (bijvoorbeeld een myocardinfarct). In eerste instantie is dit een nuttig respons om de structurele integriteit van het hart te waarborgen. Op de langere termijn zal dit proces zichzelf echter in stand houden en resulteren in excessieve ECM-deposities en daarmee leiden tot nadelige remodelering van het hartspierweefsel en een ongelijkmatige verdeling van de spiervezels met, uiteindelijk, hartfalen tot gevolg. Het gevormde litteken vervangt hier dus de verloren gegane cardiomyocyten. Dit type hartfibrose komt met name voor bij HFrEF en draagt via verschillende mechanismes bij aan systolische disfunctie. Eén daarvan is het verlies aan fibrillair collageen, wat leidt tot een verminderde overdracht van contractiekracht van cardiomyocyt naar

ECM, met een verlies van cardiale contractiekracht tot gevolg.

Reactieve interstitiële fibrose, daarentegen, is het gevolg van chronische maladaptieve signalen (bijvoorbeeld een ontstekingsreactie) gedurende de pathologische remodelering van het hart. Het wordt gekenmerkt door een expansie van de ECM die de cardiomyocyten omringt, zonder dat hier noodzakelijkerwijs een verlies aan cardiomyocyten bij plaatsvindt. HFpEF kenmerkt zich met name door dit type fibrose, waarbij diastolische disfunctie en een toegenomen stijfheid van het hart worden veroorzaakt door overmatige depositie van fibrillair collageen en juist een afname van het meer flexibele collageen type III.

Op dit moment zijn er geen effectieve therapieën beschikbaar voor de behandeling of het omkeren van het proces van beide vormen van hartfalen. De snelle wereldwijde toename in het aantal nieuwe gevallen van hartfalen noopt tot een verbetering van de beschikbare behandelmethodes. In dit proefschrift bieden we een uniek multidimensionaal perspectief op hartfalen.

Ten eerste postuleerden we dat het toevoegen van de tijdsdimensie (4D) – wat betreft het circadiane ritme en de variatie daarin gedurende de dag – zou moeten worden overwogen bij onderzoek naar hartfalen. Het circadiane ritme is gedefinieerd als het biologische ritme waarvan de cyclus één dag (24 uur) duurt en dat alle organismes in staat stelt om zich voor te bereiden op veranderingen tussen dag en nacht door hun fysiologie en gedrag daarop af te stemmen. Omdat er overtuigend bewijs is voor het belang van het circadiane ritme in het algemeen en bij hart- en vaatziekten in het bijzonder, hebben we geprobeerd de relatie tussen circadiane ritmes en hartfalen bloot te leggen. Hartfalen zelf heeft al een tipje van de sluier opgelicht: neurohormonen die relevant zijn bij hartfalen hebben een circadiaan ritme, patiënten met hartfalen leiden vaak aan slapeloosheid en er is, vice versa, vastgesteld dat slapeloosheid de kans op hartfalen vergroot. Er is een groeiend besef dat het circadiane ritme een belangrijke rol speelt bij hartfalen. Het is echter nog onduidelijk hoe het precies bijdraagt aan het ontstaan van de ziekte, de progressie en de behandeling ervan.

Ten tweede hebben we, gezien het belang van fibrose bij de pathologie van hartfalen en een gebrek aan adequate humane modellen, een 3D *in vitro* model van humane hartfibrose voorgesteld en ontwikkeld. Traditionele monolaag (2D) celkweeksystemen en dierproeven hebben ons al waardevolle inzichten verschaft wat betreft cardiofysiologie en -pathologie. Maar het vertalen van effectieve anti-fibrotische interventies naar de kliniek verloopt zeer traag. Dit komt door de grote complexiteit en heterogeniteit van hartfibrose en de onderliggende mechanismes. Er is bovendien een gebrek aan onderzoeksplatformen die een getrouwe weergave zijn van *menselijk*

hartweefsel, wat nodig is om tot een beter begrip te komen van de mechanistische aspecten van fibrotische remodelering. Daarom hebben wij voorgesteld dat geavanceerde 3D *in vitro* modellen aan het oplossen hiervan kunnen bijdragen door het humane myocard adequaat na te bootsen, inclusief de pathologische veranderingen ervan.

Na een algemene introductie in **Hoofdstuk 2**, waarbij we het belang van het circadiane ritme in de context van hart- en vaatziekten plaatsen, vervolgen we met een onderzoek naar de factor tijd als de vierde dimensie bij hartfalen. In **Hoofdstuk 3** toonden we aan dat minimaal-invasief verkregen menselijke perifere weefsels – bloed, wangslijmvlies, baard- en haarzakjes – geschikt zijn voor het bestuderen van fysiologische processen die door het circadiane ritme worden beïnvloed. Het in kaart brengen van perifere klokgenen is van cruciaal belang voor zowel het bepalen van functionele circadiane ritmes als voor het herkennen van de oorzaken en gevolgen van afwijkende ritmes. Vervolgens gebruikten we in **Hoofdstuk 4** perifeer bloed voor het karakteriseren van circadiane ritmes bij patiënten met hartfalen, zoals deze tot uiting komen in de ritmische expressie van de belangrijkste endocriene producten van de centrale klok: melatonine en cortisol. Omdat het circadiane ritme veel belangrijke processen beïnvloedt die bij hartfalen worden waargenomen, hebben we geprobeerd te achterhalen of de functionaliteit van het circadiane ritme intact blijft bij hartfalen. We ontdekten dat de output van de centrale klok bij hartfalen gedempt is. Wat betreft de concentraties volgden beide hormonen het verwachte diametraal tegenovergestelde 24-uurspatroon waarbij melatonine gedurende de nacht piekte en cortisol gedurende de dag. De variatie gedurende de dag was echter significant lager in vergelijking met de controles zonder hartfalen. Bovendien kwam deze demping overeen met de hoge prevalentie van een nachtelijk niet-dalende bloeddruk en hartfrequentie. Dit terwijl de moleculaire perifere klok intact bleef, zoals op directe wijze geobserveerd in twee onafhankelijke diermodellen voor hartfalen: de zebravis (dagdier) en muis (nachtdier). Deze studie gaf een verdere bevestiging van de relatie tussen circadiane ritmes en hartfalen en benadrukte hiermee hoe belangrijk het is om circadiane ritmes mee te nemen bij onderzoek naar en de behandeling van hartfalen. Omdat de afgifte van melatonine en cortisol direct wordt gereguleerd door de centrale klok, weerspiegelt elke verandering in hun ritmiek ook een verandering in de functionaliteit van de klok zelf. Dit opent legio mogelijkheden om hartfalen aan te pakken en de kwaliteit van leven en de overleving van patiënten met deze ziekte te verhogen. Dit varieert van het ontwikkelen van nieuwe slaap-waakinterventies, zoals hormonale suppletie, tot het implementeren van de mate van 24-uurs variatie in bloeddruk en hartfrequentie bij het ontwikkelen van nieuwe prognostische en preventieve

strategieën.

In **Hoofdstuk 5** onderzochten we of bepaalde biomarkers ook belangrijke biologische kenmerken zijn die worden aangestuurd door circadiane ritmes. We toonden aan dat oplosbaar ST2, een sterke prognostische biomarker voor hartfalen, een variatie gedurende de dag laat zien, met een piek in de middag en de laagste concentraties in de nacht. Fysiologische variatie gedurende de dag van de concentratie van oplosbaar ST2 kan leiden tot misinterpretatie van de gevonden waarden, afhankelijk van op welk moment het bloed van de patiënt is afgenomen. Deze kennis kan belangrijke gevolgen hebben voor het diagnostisch en prognostisch gebruik van ST2 in de kliniek.

In **Hoofdstuk 6** kwamen we nog dichter bij de klinische setting door te illustreren hoe het circadiane ritme het klinisch beslisproces kan beïnvloeden. We onderzochten de bloeddruk in longslagaders, dagelijks verkregen door gebruik van CardioMEMS sensoren, om zo het optimale moment op de dag te bepalen waarop deze druk bij patiënten met hartfalen gemeten dient te worden. In de kliniek leiden deze implanteerbare draadloze drukmonitors in de longslagader tot een belangrijke verbetering van de zorg bij hartfalen, waardoor minder patiënten met hartfalen in het ziekenhuis hoeven te worden opgenomen. We bevestigden dat de bloeddruk in longslagaders gedurende de dag varieert, waarbij de waarden 's avonds stijgen. Hieruit concluderen we dat deze bloeddruk vanwege de stabiliteit het beste in de ochtend gemeten kan worden. De uitkomsten van deze studies wijzen erop dat circadiane ritmes verstrekkende gevolgen hebben in de klinische praktijk. Clinici dienen zich hier dan ook van bewust te zijn bij het interpreteren van patiëntdata, met name in het geval van nieuwe biomarkers of uitlezingen waarvoor een mogelijke variatie gedurende de dag ten gevolge van het circadiane ritme nog niet is onderzocht. Om het effect van een behandeling niet verkeerd te interpreteren, de prognostische waarschuwingssignalen niet te over- of onderschatten, dan wel om tot zuivere uitkomsten van een klinische trial te komen, is het cruciaal dat men zich bewust is van het tijdstip van metingen en dat vervolgmetingen op hetzelfde tijdstip plaatsvinden.

Tot slot onderzochten we de derde dimensie bij hartfalen. Hiervoor verlegden we onze aandacht naar de moleculaire achtergrond van hartfalen door in te zoomen op fibrotische remodelering als een fundamenteel proces bij het ontstaan en de progressie van deze ziekte. In **Hoofdstuk 7** ontwierpen we een 3D *in vitro* model van humane hartfibrose dat geschikt is om de pathofysiologische mechanismen van fibrose te onderzoeken en dat ook dienst kan doen als weefselspecifiek platform voor het testen van medicijnen. Met het gebruik van humane geïnduceerde pluripotente stamcellen hebben we een alternatief ontwikkeld voor conventionele 2D celkweekmodellen en daarmee

een deur geopend naar een meer gepersonaliseerde en gehumaniseerde *in vitro* benadering voor het behandelen van hartfalen.

Dit proefschrift benadrukte het belang van een breder perspectief bij wetenschappelijk onderzoek: door de tijdsdimensie (4D) toe te voegen – wat betreft het circadiane ritme en de variatie daarin gedurende de dag – en door gebruik te maken van geavanceerde 3D *in vitro* modellen die het humane myocard adequaat nabootsen, hebben we nieuwe inzichten in deze dodelijk ziekte opgedaan. In grote lijnen is het zo dat klinische observaties tot hypotheses leiden die vervolgens in het laboratorium getest en gevalideerd worden om uiteindelijk hun weg te vinden naar het bed van de patiënt in de vorm van nieuwe therapieën of aanbevelingen voor patiëntenzorg. Deze weg zou onder andere relevante 3D *in vitro* modellen moeten bevatten, die een betrouwbaardere representatie van menselijke weefsels zijn. Tevens is het van belang om de factor tijd in ogenschouw te nemen, gezien de belangrijke invloed van het circadiane ritme, waarbij kennis van wetenschappers en artsen elkaar aan kan vullen. Samenvattend hebben we met het werk in dit proefschrift de deur geopend naar nieuwe dimensies in de diagnostische, prognostische en therapeutische aanpak van hartfalen.

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Love, Sandra

List of publications

Published

1. Voskamp, M. J., Li, S., van Daalen, K. R., **Crnko, S.**, Ten Broeke, T. & Bovenschen, N. Immunotherapy in medulloblastoma: Current state of research, challenges, and future perspectives. *Cancers (Basel)* **13**, 5387 (2021).
2. de Jong, L. C., **Crnko, S.**, Ten Broeke, T. & Bovenschen, N. Noncytotoxic functions of killer cell granzymes in viral infections. *PLoS Pathog* **17**, e1009818 (2021).
3. Bouwman, A. C., van Daalen, K. R., **Crnko, S.**, Ten Broeke, T. & Bovenschen, N. Intracellular and extracellular roles of granzyme K. *Front Immunol* **12**, 677707 (2021).
4. **Crnko, S.**, Brugts, J. J., Veenis, J. F., de Jonge, N., Sluijter, J. P. G., Oerlemans, M. I. F. & van Laake, L. W. Morning pulmonary artery pressure measurements by CardioMEMS are most stable and recommended for pressure trends monitoring. *Neth Heart J* **29**, 409–414 (2021).
5. **Crnko, S.**, Schutte, H., Doevendans, P. A., Sluijter, J. P. G. & van Laake, L. W. Minimally invasive ways of determining circadian rhythms in humans. *Physiology* **36**, 7-20 (2021).
6. **Crnko, S.**, Printezi, M. I., Jansen, T. P. J., Leiteris, L., van der Meer, M. G., Schutte, H., van Faassen, M., du Pré, B. C., de Jonge, N., Asselbergs, F. W., Gaillard, C. A. J. M., Kemperman, H., Doevendans, P. A., Sluijter, J. P. G. & van Laake, L. W. Prognostic biomarker soluble ST2 exhibits diurnal variation in chronic heart failure patients. *ESC Heart Fail* **7**, 1224–1233 (2020).
7. **Crnko, S.**, du Pré, B. C., Sluijter, J. P. G. & van Laake, L. W. Circadian rhythms and the molecular clock in cardiovascular biology and disease. *Nat Rev Cardiol* **16**, 437–447 (2019).
8. **Crnko, S.**, Ernens, I. & van Laake, L. W. New dimensions in circadian clock function: The role of biological sex. *Cardiovasc Res* **114**, 203–204 (2018).
9. **Crnko, S.**, Cour, M., van Laake, L. W. & Lecour, S. Vasculature on the clock: Circadian rhythm and vascular dysfunction. *Vascul Pharmacol* **108**, 1–7 (2018).
10. du Pré, B. C., Dierickx, P., **Crnko, S.**, Doevendans, P. A., Vos, M. A., Geijsen, N., Neutel, D., van Veen, T. A. B. & van Laake, L. W. Neonatal rat cardiomyocytes as an *in vitro* model for circadian rhythms in the heart. *J Mol Cell Cardiol* **112**, 58–63 (2017).
11. du Pré, B. C., Demkes, E. J., Feyen, D. A. M., Dierickx, P., **Crnko, S.**, Kok,

B. J. M., Sluijter, J. P. G., Doevendans, P. A., Vos, M. A., van Veen, T. A. B. & van Laake, L. W. SCA1+ cells from the heart possess a molecular circadian clock and display circadian oscillations in cellular functions. *Stem Cell Reports* **9**, 762–769 (2017).

12. du Pré, B., van Veen, T., **Crnko, S.**, Vos, M., Deddens, J., Doevendans, P. & van Laake, L. Variation within variation: Comparison of 24-h rhythm in rodent infarct size between ischemia reperfusion and permanent ligation. *Int J Mol Sci* **18**, (2017).

In revision

13. Bracco Gartner, T. C. L.*, **Crnko, S.***, Leiteris, L., van Adrichem, I., van Laake, L. W., Bouten, C. V. C., Goumans, M. J., Suyker, W. J. L., Sluijter, J. P. G. & Hjortnaes, J. Pirfenidone has anti-fibrotic effects in a tissue-engineered model of human cardiac fibrosis.
14. **Crnko, S.***, Printezi, M. I.*, Leiteris, L., Lumley, A. I., Zhang, L., Ernens, I., Jansen, T. P. J., Homsma, L., Feyen, D., van Faassen, M., du Pré, B. C., Gaillard, C. A. J. M., Kemperman, H., Zwetsloot, P. M., Oerlemans, M. I. F. J., Doevendans, P. A. F. M., Sluijter, J. P. G., Devaux, Y. & van Laake, L. W. The circadian clock remains intact, but with dampened hormonal output in heart failure.

Curriculum vitae

Sandra Crnko was born on 12 August 1991 in Rijeka, Croatia. There, she completed her high school education at the First Croatian Gymnasium in Sušak, language track. Although the focus of her track was more on languages and social sciences, from the very start Sandra showed affinity to biology and chemistry. Therefore, in 2010 she enrolled in her Bachelor's at the University of Rijeka where she studied Biotechnology and Drug Research. In 2015, Sandra completed her Master's degree Biotechnology in Medicine *summa cum laude* at the same University. She did the Master's research internship at the University of Copenhagen, Center for Glycomics, at the lab of prof. dr. Henrik Clausen and under the supervision of prof. emerita Jasna Peter-Katalinić and assoc. prof. Sergey Vakhrushev. During the course of her studies, Sandra won several awards and scholarships: Rector's Award for academic excellence and Academic Excellence Scholarship from the University of Rijeka, as well as the Erasmus+ Scholarship for Study Exchange Program and Scholarship of city of Rijeka for gifted students.

In 2016, Sandra moved to the Netherlands to start her PhD training in the Experimental Cardiology group (Regenerative Medicine Centre Utrecht) under the supervision of assoc. prof. dr. Linda van Laake, prof. Joost Sluijter, and prof. dr. Pieter Doevendans. Her research mainly focused on circadian rhythms and heart failure, but also on stem cells, cardiac fibrosis, and development of the 3D *in vitro* cellular models. The results of her PhD trajectory are published in this thesis.