Position paper of the ESC Working Group on Cellular Biology of the Heart:

Circadian rhythms in ischaemic heart disease. Key aspects for preclinical and translational research

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Abstract

Circadian rhythms are internal regulatory processes controlled by molecular clocks present in essentially every mammalian organ that temporally regulate major physiological functions. In the cardiovascular system, the circadian clock governs heart rate, blood pressure, cardiac metabolism, contractility and coagulation. Recent experimental and clinical studies highlight the possible importance of circadian rhythms in the pathophysiology, outcome, or treatment success of cardiovascular disease, including ischaemic heart disease. Disturbances in circadian rhythms are associated with increased cardiovascular risk and worsen outcome. Therefore, it is important to consider circadian rhythms as a key research parameter to better understand cardiac physiology/pathology, and to improve the chances of translation and efficacy of cardiac therapies, including those for ischaemic heart disease. The aim of this Position Paper by the European Society of Cardiology Working Group Cellular Biology of the Heart is to highlight key aspects of circadian rhythms to consider for improvement of preclinical and translational studies related to ischaemic heart disease and cardioprotection. Applying these considerations to future studies may increase the potential for better translation of new treatments into successful clinical outcomes.

1   **Introduction**

2

3   Circadian rhythms are endogenous (intrinsic) biorhythms that repeat approximately every 24

4   hours. They allow the body to continuously anticipate day-to-night environmental variations

5   consequent to the earth's rotation. Circadian rhythms are present in all organisms. In humans,

6   they play a central role in physiology and disease.

7

8   Many cardiovascular functions, such as blood pressure[1,2], cardiac contractility, heart rate[3], and

9   vascular resistance show 24-hour, diurnal variations. These rhythms are the product of

10   external (environmental, behavioural) factors and intrinsic (endogenous) circadian rhythms.

11   Circadian rhythms are driven by circadian clocks. Humans possess two clock types: 1) a central

12   biological clock in the suprachiasmatic nucleus (SCN) of the hypothalamus, that controls

13   circadian rhythms via the autonomic nervous system and humoral mediators (e.g. cortisol,

14   melatonin) and 2) peripheral clocks that locally enforce temporal governance in cells such as

15   the cardiomyocytes[4], vascular endothelial cells[5], smooth muscle cells[6], and cardiac progenitor-

16   like cells[7]. Both central and peripheral clocks are self-sustainable but can be altered and

17   entrained by environmental factors (called Zeitgebers) such as light, physical activity, and food

18   intake.

19

20   The circadian clock is a molecular mechanism that consists of clock proteins such as CLOCK,

21   BMAL1, PER1/2/3 and CRY1/2[8]. In brief, CLOCK and BMAL form a heterodimer and induce

22   transcription of PER and CRY proteins. The latter proteins subsequently form a complex and

23   inhibit the transcription of CLOCK and BMAL1, thereby generating a negative feedback loop.

24   This feedback loop is complemented by several other feedback loops, most notably REV-

25   ERBα/β, a member of nuclear receptor family (and thus a pharmacological target of the

26   circadian clock), and further regulated at different levels, including the post-translational and

27   epigenetic level. The circadian clock regulates transcription of approximately 10-15% of all

28   genes and proteins in the heart[9]. Oscillation of these genes and proteins causes 24-hour

29   fluctuation in processes like cardiac cellular growth, cell adhesion, metabolism, apoptosis,

30   fibrosis, electrophysiology[10], and contractile function.

31

1   An increasing number of studies support the idea that circadian rhythms affect almost all

2   functions of the cardiovascular system and play central roles in cardiovascular disease and

3   recovery. As a result, 24-hour rhythms have evolved from a niche topic to one that is

4   important in almost all pre-clinical and clinical research. It is a factor that, like age or sex[11],

5   may significantly impact the translation potential of cardiovascular research. In the current

6   position paper of the European Society of Cardiology Working Group Cellular Biology of the

7   Heart, we aim to provide key aspects on how circadian rhythms can be taken into account to

8   improve clinical and preclinical studies in cardiovascular disease, with a focus on ischaemic

9   heart disease.

10

11   **1. Circadian rhythms and physiological regulation of the cardiovascular system**

12   The cardiovascular system consists of various physiological parameters that exhibit 24-hour

13   (diurnal / time-of-day / day-night) variation. Many of these parameters are orchestrated by

14   the circadian clock (see figure1) and named circadian rhythms, although these terms are often

15   mixed. Blood pressure is one of the factors longest known to fluctuate throughout the day. It

16   is lowest around 3AM, rises just before awakening and peaks mid-morning, after which it

17   decreases again toward the night[2]. Blood pressure is regulated on a central level via

18   sympathetic, parasympathetic as well as hormonal influences such as the renin-angiotensin

19   system and the endothelin system[12]. On a local level, circadian clocks in cardiomyocytes and

20   vascular cells regulate heart rate, muscular contractile function, and endothelial function[13,14].

21   The 24-hour fluctuation in blood pressure is likely the result of all these factors combined,

22   although the role of the circadian clock is complex. Several animal models have been

23   instrumental to explore the role of each factor, for example by genetically disrupting the genes

24   coding for core clock proteins including CLOCK[15], BMAL[16], and PER[17] on whole body and organ

25   / tissue levels. These models demonstrate that genetic whole body disruption of the molecular

26   circadian clock leads to blunted diurnal variation of blood pressure[18]. Cell-specific disruption

27   of the circadian clock through *Bmal1* disruption in endothelial cells and vascular smooth

28   muscle cells shows similar results[19], but disruption of the circadian clock in cardiomyocytes

29   does not blunt blood pressure rhythmicity[20]. Studies in humans suggest that the normal rise

30   in blood pressure in the early morning is primarily driven by waking at this time, whereas the

31   secondary evening rise in blood pressure is driven by endogenous circadian clocks[21].

1   Heart rate[1] and many electrophysiological parameters such as PR-[22], QRS-[23], and QTc-interval

2   in the ECG[24], as well as heart rate variability[25] vary throughout the day in healthy humans.

3   Variation in electrophysiological parameters is regulated by both the (central) autonomic

4   nervous system and peripheral circadian clocks. Peripheral circadian clocks regulate the

5   expression and function of sodium-[26], potassium-[10], and calcium-channels[27]. As a result,

6   isolated cultured cardiomyocytes show 24-hour variation in spontaneous beating[28] and in

7   animal models where the molecular circadian clock is disrupted in cardiomyocytes specifically,

8   24-hour variation in heart rate is diminished[15].

9

10   Cardiac contractility also varies throughout the day. Daily variation is caused by circadian

11   rhythms in the previously described electrophysiology as well as cardiac metabolism and cell

12   signaling[4,16]. These rhythms are regulated by molecular circadian clocks within the heart, since

13   variation in contractility persists outside the body in explanted hearts[15]. In humans, variation

14   in cardiac contractility can be observed in 24-hour variation of cardiac echocardiographic

15   parameters describing cardiac relaxation / diastolic function[29].

16

17   Coagulation is another well-studied example that has 24-hour variation in function. Evidence

18   for circadian rhythm in platelet function and aggregation in healthy adult males was already

19   obtained in 1987[30]. Platelet aggregation activity is highest in the morning and, similar to blood

20   pressure, is regulated by both central and peripheral clocks. Central clocks regulate

21   catecholamine levels, platelet count (via thrombopoietin) and haemoconcentration that peak

22   upon arousal[31,32]. On a peripheral level, circadian clocks in platelets control platelet activation

23   independent of these central clocks: although platelets lack a nucleus and therefore the

24   canonical transcriptional-translational feedback loop, an alternative non-transcriptional clock

25   has been demonstrated in anucleate cells[33]. In addition, circadian clocks in endothelial cells

26   regulate expression of pro-thrombotic factors such as plasminogen activator inhibitor-1[34]. All

27   combined, this leads to increased coagulability in the morning, beneficial in ages when

28   humans were most likely to injure themselves in the morning, but nowadays more infamous

29   for increased morning incidences of thrombotic events such as cerebrovascular accidents[35]

30   and acute myocardial infarction (AMI)[36].

31

1   Traditionally, 24-hour variation in cardiovascular parameters has been attributed to the

2   autonomic nervous system. Increasing evidence, however, shows that the role of this

3   neuronal influence may be limited. Studies in animal circadian clock knock-out models for

4   example, demonstrate that disruption of the molecular circadian clock completely abolishes

5   24-hour variation in physiological parameters, whereas blockade of the autonomic nervous

6   system only diminished rhythmicity[37,38]. In humans, this is further supported by heart

7   transplantation studies, where 24-hour rhythmicity in heart rate and other parameters

8   continues after autonomic denervation[39]. Instead of the main driving force of 24-hour

9   variation, the autonomic nervous system is more likely one of the links between the central

10   and peripheral circadian clocks. A similar situation might be true for hormones such as

11   melatonin, cortisol, adrenalin, and insulin[40].

12

13   **2.   Circadian rhythms and ischaemic heart disease**

14   Circadian rhythms play a major role in cardiovascular disease at the level of incidence,

15   pathophysiology, and outcome[41–43]. Reviews have been written about this topic in specific

16   cardiovascular diseases such as stroke[44] and arrhythmias[38,45]. Here, we focus on ischaemic

17   heart disease in both its chronic and acute manifestations.

18

19   For decades, 24-hour rhythms have been studied in the context of disease onset. Acute

20   ischaemic heart disease (AMI) occurs more frequently in the early morning[46–49]. This may be

21   explained by a combination of factors, including the previously described morning increase in

22   hemodynamic stress (surge in heart rate and blood pressure), platelet aggregability, circadian

23   leukocyte oscillations, and recruitment of inflammatory leukocytes from blood to plaque

24   during this time of day[30,50–53].

25

26   In 2010, Durgan et al. published a break-through study investigating circadian rhythms in

27   tolerance of the heart to ischaemic insults. When myocardial ischaemia is induced at the sleep

28   to wake transition (subjective morning) in an animal model, infarct size, fibrosis and adverse

29   remodelling were significantly worse compared to ischaemia at the wake-to-sleep transition

30   (subjective evening)[41]. This illustrates that circadian rhythms are not only important in the

1   incidence and development of ischaemic heart disease, but also play a major role in outcome

2   of disease[41,42].

3

4   Several clinical studies observed differences in plasma levels of creatine kinase after AMI that

5   were similarly dependent on time-of-ischaemia onset[54–56]. Some clinical data further suggest

6   that morning onset of AMI is associated with increased risk of recurrent acute coronary

7   syndromes and coronary atherosclerosis progression[57]. However, other investigations failed

8   to confirm an association between time-of-day at symptom onset and infarct size or long-term

9   mortality in patients with ST-segment elevation myocardial infarction (STEMI) undergoing

10   primary percutaneous coronary intervention[58]. The variable outcome between clinical studies

11   has been discussed extensively[59,60]. One of the limitations in humans is certainly the relatively

12   high variability in patient characteristics (ethnic background, medication use, comorbidities

13   (such as diabetes), chronotype, culprit artery, time of ischaemia) as well as other study

14   parameters (statistical methodology, study size, and outcome measure), which might render

15   it difficult to validate an association between time of day of ischaemia onset and outcome.

16

17   To better assess direct causal relationships between circadian rhythmicity and infarct size,

18   mouse models of AMI have been instrumental[61]. Animal and *in vitro* models also help

19   understand the processes involved in diurnal variation of AMI outcome. In support of a

20   possible role for circadian clocks in ischaemic damage, genetic disruption of clock genes leads

21   to an altered infarct size in mice. Disruption of positive components of the molecular

22   clockwork such as *Bmal* and *Clock* caused an increased infarct size, whereas disruption of the

23   negative component *Per2* and *Rev-Erbα* reduced infarct size[41,42,62]. This further supported the

24   study of Durgan et al., which used a cardiomyocyte-specific *Clock* mutant mouse model to

25   demonstrate that the diurnal variation in AMI outcome is orchestrated by the cardiomyocyte

26   clock, possibly via a diurnal rhythm in ischaemia tolerance[28,41]. Clock disruption in other cell

27   types, such as the immune system and fibroblasts, may also contribute to the diurnal variation

28   of AMI. Studies in wound healing for example, demonstrate that 24-hour variation in wound

29   healing is caused by a rhythm in fibroblast activity, a process also important in post-AMI

30   cardiovascular remodelling[63].

31

1   Animal models also showed other important relations between outcome of ischaemic heart

2   disease and circadian rhythms, for example the effect of circadian disruption. In an

3   experimental mouse model of permanent left coronary ligation, disruption of light/dark cycles

4   promotes an unfavourable healing response after AMI[64]. More specifically, infarcted mice

5   were subjected to 10h light/10h dark cycles over 5 days, resulting in cardiac dysfunction and

6   poorer AMI tolerance. Circadian disruption had significantly greater adverse remodelling with

7   increased left ventricular internal systolic and diastolic dimensions, accompanied by

8   decreased fractional shortening and ejection fraction. Other studies investigated time-of-day

9   differences after myocardial infarction in depth, and found that in mice, AMI in the awake

10   period triggers genes associated with metabolic pathways, whereas an AMI in the inactive

11   period leads to upregulation of genes associated with inflammation[65]. This time-of day effect

12   of AMI on cardiac remodelling is regulated by the circadian clock[65]. Vice versa, there is

13   evidence that AMI may lead to circadian disruption, for example in the beta-adrenergic

14   receptor expression, thereby contributing to adverse cardiac remodelling[66,67].

15

16   The first studies investigating time-of-day and ischaemic heart disease focussed on melatonin,

17   an hormone produced by the pineal gland, under the influence of light, and one of the input

18   signals of the circadian clock[68]. Both animal and patient studies suggest that endogenous

19   melatonin levels correlate with lower ischemia-reperfusion injury [69–72]. Vice versa, AMI may

20   lead to decreased melatonin levels.[73,74] The relationship between melatonin and

21   cardioprotection in ischaemic heart disease is complex and involves multiple processes

22   including the regulation of the molecular circadian clock or direct effects of melatonin as a

23   regulator of multiple prosurvival signaling cascades within the heart, an antioxidant and an

24   anti-inflammatory molecule[75–77].

25

26   In humans, disturbance of circadian rhythms is also associated with ischaemic heart disease[78].

27   Circadian rhythm disturbance (e.g. by sleep deprivation or shift work) induces a misalignment

28   between physical activity and intrinsic clocks, with adverse effects on cardiovascular

29   parameters, healing responses, and remodelling. Insufficient sleep, for example, affects the

30   blood transcriptome and disrupts its circadian regulation[79]. The identified genes, pathways

1   and biological processes affected by insufficient sleep include circadian clock genes as well as

2   inflammatory, immune, and stress response pathways.

3

4   The immune system appears to be a major contributor to the variation in AMI outcome.

5   Humans have diurnal fluctuations in immune cell numbers[41,80]. In particular, the innate

6   immune system including the inflammasome, the first immune response following an AMI and

7   involved in recruitment and activation of pro-inflammatory monocytes, is circadian

8   regulated[81,82]. Production and retention of neutrophils in the bone marrow is time-of-day

9   dependent[83,84]. Moreover, circulating neutrophils at the beginning of the active phase have

10 higher capacity to migrate into the myocardium due to upregulated CXCR2 (C-X-C Motif

11 Chemokine Receptor 2) expression[80]. Other immune cells such as classical monocytes are

12 regulated by the circadian clock and involved in AMI outcome[85,86]. Disruption of the molecular

13 clock in these monocytes worsens inflammation[81,85]. Recently, a study showed that the

14 inflammatory role of the gut microbiome in AMI and heart failure is influenced by the circadian

15 clock[87].

16

17 The molecular circadian clock is not only important in AMI, but also plays a major role in

18 chronic ischaemic heart disease. Disruption of the molecular clock can dampen blood pressure

19 rhythmicity, reduce the production of vasoactive hormones and cause endothelial

20 dysfunction[88], thereby increasing the development of atherosclerosis[89]. For example, the

21 aortae of clock-mutant mice exhibit impaired cholesterol metabolism and enhanced

22 atherosclerosis[90]. Interestingly, the mechanism appears to be cell intrinsic as significant

23 atherosclerosis develops when the aortae from clock mutant mice are transplanted into wild

24 type mice[88]. Pharmacological targeting of clock components decreased atherosclerosis in

25 mouse models, likely secondary to effects on inflammation[53].

26

27 Finally, to illustrate some relevant links beyond our focus on ischaemic heart disease , global

28 and cardiomyocyte-specific clock-mutant mice develop dilated cardiomyopathy[16,91].

29 Moreover, cardiomyocyte-specific downregulation of BMAL1 results in reduced heart rate,

30 prolonged RR and QRS intervals, and increased episodes of arrhythmia. The phenotype is

31 linked with reduced circadian expression of the sodium and potassium channels, which may

1   contribute to the sudden cardiac death observed in cardiomyocyte-specific *Bmal* knockout

2   mice[26,92].

3

4   **3. Circadian rhythms and interacting factors**

5   Multiple factors are associated with circadian rhythms and cardiovascular disease. This

6   relation is often bi-directional: disruption of circadian rhythms is associated with an

7   increased severity of many cardiovascular risk factors and, vice versa, the presence of most

8   cardiovascular risk factors is associated with a disturbance in circadian rhythms. The

9   circadian rhythms may be disturbed on various levels. Often, clock input signals

10   (Zeitgebers) are misaligned with intrinsic molecular clocks. This occurs, for example, with

11   travel to a different time-zone (jetlag) or in case of shift-work. Intrinsic factors may also

12   disrupt the molecular clock directly.

13   In table 1, we summarize the relationship between circadian rhythms and various

14   traditional and environmental interacting factors known to affect cardiovascular risk, from

15   age, sex to environmental factors including light, temperature and noise. It is important to

16   note that an association between an alteration of circadian rhythms and different stimuli

17   is often observed in both preclinical and clinical settings but a causal relationship is yet to

18   be proved with most factors.

19   **Table 1**

Interacting factors		Relationship between interacting factor and circadian rhythm	Relevant references
Physiological factors	Sex	Sex-specific differences in the mechanisms that establish circadian rhythms. In healthy adults, circadian misalignment is associated with sex-specific changes in energy homeostasis independent of behavioural/environmental factors. In female rodents, oestrous cycle stage has tissue-dependent effects on the expression of clock genes.	11,93–96
	Aging	Aging has been associated with changes in the period and amplitude of circadian rhythms in	97,98

		rodents. Advanced age associated with a modulation to the light-dark cycle and a loss of responsiveness to the phase shifting. In both humans and rodents, melatonin levels decline with aging.	
	Pregnancy/ lactation	The central circadian clock undergoes marked adaptations with the onset and progression of pregnancy. Circadian rhythms modulate metabolic and hormonal adaptations necessary to initiate and sustain lactation, and several components of breast milk show circadian variations. Lactation is associated with improved nocturnal sleep in breast-fed infants.	99–102
Co-morbidities	Hypertension	Molecular clocks regulate the circadian regulation of blood pressure). Spontaneously hypertensive rats display alterations in the circadian genes expression in several organs including the heart and the aorta. In humans, antihypertensive treatment given at bedtime reduces cardiovascular risk Epidemiological studies suggest a correlation between endogenous melatonin levels and incident hypertension.	18,103–105
	Metabolic diseases	Genetic alterations of the molecular clock have pronounced effects on both peripheral and central metabolic regulatory signals. Disruption of the circadian rhythm is associated with increased risk in metabolic diseases. Alterations in energy balance are associated with disruptions of the circadian clock function, of the blood pressure circadian rhythm and changes of clock genes expression in the vasculature.	106–108
Psychological factors	Depression	Major depressive disorders are frequently associated with a disruption of the expression of the	109,110

		clock genes. In rodents, models of circadian disruption are characterized by depressive-prone features.	
	Mental stress	A variety of mental stressors are associated with alteration of peripheral clocks in animals. It is suggested that mental stress causes the dysregulation of circadian rhythm by inducing oxidative stress which disrupts circadian clock proteins.	[111–113]
Behavioural factors	Physical activity	Physical performance is partly dependent on circadian clock proteins and, vice versa, physical inactivity or exercise can influence the circadian system in mammals. Exercise, if performed at the appropriate time of day, shifts the internal circadian rhythm phase and thus improves circadian alignment. Late chronotypes ('evening people'), who experience circadian misalignment may benefit from phase advances induced by morning exercise, whereas evening exercise may exacerbate circadian misalignment in the early chronotypes. Thus, the personalized prescription of exercise times based on the chronotype could alleviate circadian misalignment in young adults.	[114–119]
	Food intake	Food intake is a Zeitgeber for the circadian clock. The timing of food intake influences the effect of nutrients on the cardiovascular system. Time-restricted feeding prevents metabolic disease and cardiac ageing in animal models, and is currently investigated in clinical trials.	[120–122]
	Alcohol consumption	Circadian clock disruption may favour alcohol addiction and chronic alcohol consumption in rodents disrupts molecular clocks. Alcohol	[123–126]

		upregulates the expression of *Clock* and *Per2* circadian clock genes.	
	Smoking	Cigarette smoking alters gene expression of the central (brain) and peripheral (lung) clock genes.	[127,128]
Environmental factors	Temperature	Temperature oscillations as small as 1°C alter expression of circadian genes and thereby affects circadian amplitude and phase. The circadian period length (tau) on the other hand remains approximately constant through the homeostatic mechanism of temperature compensation.	[129–132]
	Noise	Auditory function is regulated by biological clocks, and, vice versa, sound stimuli can influence circadian rhythms. As an example, sleep deprivation induced by aircraft noise will increase vascular and cerebral oxidative stress, an effect associated with the modulation of circadian clock genes in murine aorta, heart and kidney.	[133–140]
	Light/ season	Light is the most powerful environmental signal for phase-shifting circadian rhythms. The time and amount of solar irradiation vary dynamically with the season, especially with increasing distance from the equator. Chronotype is influenced by seasonal change, most likely due to light differences. Inter-individual differences in photoperiod responsiveness indicate that some people are more affected than others, possibly due to variation in molecular clock and/or previous light history.	[127]
	Sleep disorders	Shift work and sleep disorders (short duration and/or poor quality sleep) chronically disrupt the circadian clock system. In shift workers, circadian blood pressure rhythm changes from a "dipper" pattern to a "non-dipper" pattern during night shifts	[141–149]

		and reverses back to a dipper pattern within a few days after the end of the shiftwork.	
	Air pollution/ toxins	Chemical pollutants can have a significant impact on circadian rhythms, altering sleep/wake pattern and increasing the risk for cardiovascular disease by altering rhythmic cardiovascular functions. Air pollution alters redox regulation of the circadian molecular clock. Heavy metals or pesticides induce oxidative stress which mediates redox modifications of circadian clock proteins.	139,150–154

1

2 **4. Current pitfalls in clinical and preclinical studies**

3 Circadian rhythms are currently only considered in a minority of clinical and preclinical studies.

4 This omission leads to several potential pitfalls (figure 2) and the most common ones are

5 discussed below:

6

7 4.1 Clinical studies

8 A. Collection of clinical (time-of-day) parameters

9 In many studies, no data about circadian parameters are collected or reported, resulting to

10 the risk of both type I and type II errors. To illustrate this, take a hypothetical trial in which the

11 ability of a new drug to reduce infarct size long-term, is compared to placebo. In the accidental

12 case that most patients (or experimental animals) in the drug arm of the study experience an

13 AMI in the afternoon (smaller infarct), whereas the placebo subjects more often have their

14 AMI in the morning (larger infarcts), one could falsely conclude that the new drug decreases

15 infarct size (Type I error). Alternatively, when subjects in the drug arm of the study more often

16 suffer from AMI in the morning compared to placebo subjects, a beneficial effect of the drug

17 will remain unnoticed (Type II error).

18

19 Furthermore, as a disrupted circadian rhythm (as explained in chapter 3) is related to disease

20 incidence, outcome, and effect of an intervention, this pre-existing "comorbidity" may act as

1   a confounder in clinical studies that thus far goes unnoticed: unlike other comorbidities and

2   demographic factors, it is hardly ever analysed or corrected for in the data analysis.

3

4   Finally, an intervention may directly disrupt (or restore) the circadian rhythm of subjects,

5   which may affect the outcome of a clinical study or cause unexplained variation if

6   preferentially incident in a subgroup of patients or controls. Unless questionnaires or

7   measurements are taken to establish intactness of the circadian clock, this type of

8   confounding factor will not be registered.

9

10 Thus, recording of parameters related to both collecting time and circadian rhythm, before

11 and during the study will improve the accuracy of the study results and may reduce type I and

12 II errors.

13

14 B. Sampling

15 Circulating factors in clinical laboratory measurements such as troponin[155] and soluble ST2[156],

16 as well as functional (e.g. coagulation-related[34]) tests display physiological diurnal variation.

17 In almost all nucleated cells and tissues, circadian clocks are active and will rhythmically

18 regulate approximately 10-15% of the transcriptome and proteome. Therefore, blood and

19 tissue sampling at random times may cause undue variation, whereas structurally different

20 sampling times between groups may cause bias and false negative or false positive results.

21

22 C. Physiological parameters

23 Blood pressure is a well-known example of a physiological parameter that can only be

24 compared when measured around the same time of day. In addition, its diurnal/nocturnal

25 pattern appears more clinically relevant than a single measurement. Performance at exercise

26 tests, vascular reactivity, and ECG parameters such as QTc time[24] are other examples of

27 measurements that are affected by diurnal variation, yet often performed at random times.

28

29 Consciously choosing and reporting a specific time-of day to do a measurement may be

30 beneficial. Measuring a cardiovascular parameter always at a specific time limits the variation

1   of the measurement. This reduces the number of subjects needed to find significant outcomes

2   (increased statistical power) and increases chances of replication by other research groups.

3

4   D. Drug therapy

5   Clinical studies that investigate circadian aspects of intervention, previously showed that both

6   the efficacy and side effects of therapy may depend on the timing of treatment

7   administration.[157] In some cases, a treatment that is not effective or has unacceptable side

8   effect at a certain time of the day, may display a better risk-benefit ratio at a different time.

9   When circadian parameters are not collected, optimal timing of therapy will not be

10   determined, and a good treatment tested at the wrong time of the day may not make it into

11   the clinic setting. This variation may be caused by pharmacokinetics and pharmacodynamics

12   that are oscillating over 24 hours, and/or by target responsiveness – for example if the target

13   receptor is variably available due to regulation by the circadian clock. Furthermore, drugs may

14   disturb or enhance the circadian system as an off-target effect; melatonin and corticosteroids

15   are classic examples, but newer drugs may also interfere with clock function.

16

17   Beta blockers abolish the circadian patterns of ischaemic events, therefore the use of

18   extended-release beta blockers in the evening might reduce vulnerability for cardiac events in

19   the morning[158]. In parallel, long-acting anti-hypertensive drugs (e.g. ACE-inhibitors) might

20   decrease the blood-pressure peak in the morning[159]. In a prospective, randomized trial low-

21   dose aspirin showed a time-dependent effect on the blood pressure of untreated

22   hypertensive patients. While before-bedtime aspirin intake reduced the blood-pressure, the

23   intake of aspirin in the morning even slightly elevated blood-pressure levels[160]. The MAPEC

24   study investigated prospectively the effect of administration time of anti-hypertensive

25   medication and randomized over 2000 patients into morning dosing all BP medications or

26   dosing ≥1 BP medications at bedtime. After a mean follow-up of 5.6 years, the patients in the

27   evening dosing group showed lower mean asleep blood pressures, a lower prevalence of non-

28   dipping pattern and improved ambulatory BP. In addition, bedtime intake reduced major and

29   total cardiovascular events including deaths[161]. Recently, a follow-up study showed similar

30   results in prospective study of more than 19000 patients[105]. Both studies elegantly show the

31   potential impact of chronotherapy on patient outcome. Meticulous planning of the timing of

1   pharmacotherapeutical administrations is necessary to achieve reliable and reproducible

2   outcome data.

3

4   <u>E. Invasive therapeutic interventions</u>

5   *Cardiac surgery*

6   During cardiopulmonary bypass, cardioplegia and subsequent reperfusion inevitably result in

7   ischaemia/reperfusion injury. A small (n=88) prospective randomized single-centre study

8   found perioperative myocardial injury in surgical aortic valve replacement to depend on the

9   time of the day[42]. Patients operated in the afternoon showed decreased perioperative

10 troponin T release and reduction of major cardiac events, which was associated with

11 transcriptional regulation of *REV-ERBα*.

12

13 These data could not be confirmed in a large retrospective analysis of the Society of Thoracic

14 Surgeons (STS) Adult Cardiac Surgery Database (n=14078 patients) including 11 surgical

15 centres in the US[162]. However, further randomized data including larger numbers of patients

16 are necessary to substantiate the impact of surgical timing on outcome and thus whether

17 timing of surgery should be taken into account in the design and analysis of clinical studies.

18

19 *Catheter intervention*

20 Results of percutaneous coronary intervention (PCI) have variably been related to time-of-

21 day. An observational study from a Swiss registry of 2860 patients with STEMI found that

22 effectiveness of thrombus aspiration was dependent on time of symptom onset with greatest

23 myocardial salvage for patients with symptom onset between 6 a.m. and 6 p.m.[163]. In another

24 registry-based study a circadian STEMI pattern with a peak during morning hours without

25 impact on the clinical outcome was found in 8608 patients [164]. A retrospective analysis of 1021

26 patients showed a decreased rate of periprocedural (Type 4A) AMI in patients undergoing

27 elective PCI between 7 a.m. and noon, whereas treatment in the afternoon increased the risk

28 for type 4A AMI[165]. Clearly, there is an urgent need for prospective data regarding the time of

29 the day during PCI procedures in patients with ischaemic heart disease to substantiate the

30 role of circadian mechanisms in PCI-related outcomes. In addition, time of symptom onset and

1   timing of intervention should be taken into account in future PCI studies to exclude a bias of

2   circadian mechanisms.

3

4   *Heart transplantation*

5   Ischaemia during organ harvesting and reperfusion after termination of cross-clamping is the

6   prime example for ischaemia/reperfusion injury. Prolonged ischaemia time is associated with

7   rejection and impaired long-term outcome[166]. A retrospective cohort study including 16573

8   patients undergoing heart transplantation found no significant association between daytime

9   or night-time surgery and survival up to 1 year after organ transplant[167], but no specification

10   was made between time points within 12 hour blocks. A similar study in lung transplant

11   patients on the other hand, did find time-of-day effect on outcome. Transplants performed

12   between 4 am and 8 am, had a relatively high risk of primary graft dysfunction[168]. Again, it

13   would be desirable to include time of surgery as a parameter in prospective outcome analysis

14   to clarify possible circadian effects.

15

16   *Hospital operational rhythms and shift work*

17   Hospitals provide care 24 hours a day. Observational studies show that almost all care,

18   including diagnostics, treatments and referrals take place according to a fixed daily pattern

19   [169]. These patterns are generally not motivated by medical necessity but by practical

20   considerations, such as availability of staff, changing of shifts, or simply habits. In addition,

21   hospital care is delivered by staff who are also under the influence of circadian rhythms. Night

22   shifts disrupt day-night rhythms, leading to reduced alertness and a greater risk of making

23   mistakes. Observational studies show a worse outcome of operations performed at night

24   (although bias is hard to rule out properly in these studies)[170].

25

26   4.2 Preclinical studies

27   It is clear that, like humans, animals in laboratory conditions show time-dependent behaviour

28   in for example food intake and physical activity. Indeed, physiological and molecular 24-hour

29   variation is demonstrable in these animals and therefore relevant in the setup of

30   experiments[65]. Just as time of incidence in humans may affect the outcome of observational

31   and interventional studies, the time of an induced event can affect the outcome in preclinical

1   animal models. A clear example is the timing of coronary artery ligation to induce AMI in

2   rodents, given the ample evidence that timing affects infarct size in this model[41,59,171].

3

4   Besides this timing of "incidence", sampling, physiological parameters, drug therapy and

5   invasive interventions entail the same pitfalls in preclinical animal models as in clinical or

6   epidemiological studies, described in section 4a. In addition, several relevant considerations

7   are specific to preclinical experimentation.

8

9   <u>A. Choice of animal model</u>

10   In laboratory animal studies *in vivo*, the alternans of light and dark periods congruent with

11   outside day and night is used as standard day-night conditions, named photoperiod. In diurnal

12   species (pigs, dogs, sheep, monkeys and humans), the acrophase (physiological peak phase)

13   of body temperature and locomotor activity occurs during daytime. The acrophase of

14   nocturnal animals (mice, rats, hamsters) occurs at night. When noise is present or experiments

15   are performed in nocturnal animals during human working hours, this leads to several

16   problems. First, animals are disturbed in the inactive period, causing stress, non-physiological

17   light exposure leading to hormonal (e.g. melatonin) disruptions and thereby non-physiological

18   results of the experiments performed. Secondly, if the procedure is performed in the inactive

19   period, results do not translate well to a clinical situation when a human receives a treatment

20   during working hours. A recent study investigating cerebral ischaemia, suggests that the

21   difference between nocturnal animals and diurnal humans contributes to the translational

22   failure of novel, promising neuroprotective strategies[172]. In addition to being representative

23   with respect to size, pigs have similar dietary habits (omnivorous) and diurnal behaviours as

24   humans. Zebrafish and Drosophila, often used in transgenic and repair studies, are diurnal

25   animals as well.

26   Age and sex are other factors to consider when choosing the best animal model for an

27   experiment, since both may affect the circadian clock as described in section 3. Overall,

28   circadian amplitude may be expected to be lower in older than in younger animals, while the

29   differences between sexes in experimental animals require further investigation as data in

30   female animals are scarce[173].

Further, the translational power of a preclinical study may be reduced if the animal model does not reflect the degree of circadian disruption in the patient category that it aims to represent. Atherosclerotic comorbidities affect central and peripheral circadian rhythms, a factor rarely considered in animal experiments[64,174]. A therapy may work well in a young healthy animal with normal clock function, but not in an older patient with disturbed clock function due to external factors or age itself. Interventions unique to the preclinical situation may disrupt the circadian rhythm in animals specifically. General anaesthesia, for example, which is known to deregulate the circadian clock[175], is usually performed for echocardiography in animals but not humans. Ear-based instrumentation of pigs led to increased stress of the animals with consequent diminished circadian rhythm of temperature oscillations lasting up to 3 days[176]. Thus, as with other comorbidities such as diabetes or obesity, circadian clock function in this way may act as a contributor to the translational gap.

B. Housing

Light and darkness will obviously affect the circadian rhythm in experimental animals. Light /darkness (L:D) schedules of 12:12 L:D schedules and 14:10 schedules are standard, and occasionally seasonal variation is taken into account in the laboratory setting. Different L:D schedules between labs may account for variation in results. Importantly, lack of acclimatization after a transfer, but also unintended light disruption at night, are likely to cause profound disruption of the circadian rhythm and may thus affect study results without the researchers being aware[177].

Circadian disruption can also be performed intentionally to mimic a clinical situation. Patients with AMI are treated in coronary care units or intensive care units (ICUs), with interrupted endogenous circadian rhythms and sleep periods due to noise of monitors and lights. Alibhai et al. demonstrated that rats with AMI subjected to disrupted diurnal rhythm had disturbed metabolism, innate immune response, and altered scar formations and overall worse prognosis[64]. Similar to rodents, continuous sedation, mechanical ventilation and medical maintenance of the circulation of pigs, simulating human intensive care unit conditions led to lost or desynchronized internal biological circadian rhythm[178].

1   Furthermore, any stress-inducing housing circumstance (e.g. limited nesting opportunities,

2   lack of socialization) will increase cortisol levels[179,180], which in turn may cause disruption of

3   the circadian clock since diurnal oscillations of cortisol are associated with varying numbers of

4   circulating immune cells relevant in cardiac repair[181].

5   <u>C. Feeding</u>

6   Feeding-fasting patterns are not only a direct inducer of cardiovascular changes but also a

7   known Zeitgeber for the circadian clock[182]. Furthermore, hormones such as insulin, glucagon,

8   cortisol and the microbiome regulate metabolism via circadian clocks.

9   Feeding-fasting pattern in animal experiments have been shown to influence results. Mice

10   that have ad libitum access to standard diet, eat mostly during their active period

11   ('darkness')[183]. Changes in diet composition, for example to a high fat diet in ad libitum

12   condition, lead to more food being consumed in the inactive phase ('light')[184]. When the

13   animals are forced to eat a high fat diet (with the same amount of calories) in metabolic

14   changes that are different and generally less severe than their ad libitum counterparts[185].

15   Effects of time-restricted feeding are not only present in high fat diet conditions, but also in

16   high fructose or normal diets and are important[186]: Time-restricted feeding vs ad libitum

17   feeding of a normal diet, leads to 40% difference in endurance after correction for body

18   weight[186].

19   Choices in diet composition and timing influence circadian rhythms and the outcome of animal

20   experiments. Although most studies standardize their feeding protocol, these influences are

21   often large but overlooked.

22   Of note, many of the points discussed for pre-clinical studies, apply to clinical studies and vice

23   versa. Similar to pre-clinical studies, light/darkness effects may confound clinical studies. A

24   group of ischaemic heart disease patients treated in winter for example, (when daylight hours

25   are short) may yield different results when treated in summer (when daylight hours are

26   longer). As a second example, meal timing influences various cardiometabolic parameters and

27   the circadian clock[187,188]. When these confounders remain unnoticed, this may influence

28   outcome and interpretation of clinical studies.

1   4.3 *Ex vivo* and *in vitro* studies

2   The dependency of cell responses on the circadian clock is cell autonomous and can be

3   considerable in cardiovascular cell types including cardiomyocytes, fibroblasts, and vascular

4   cells. For example, beating frequency[28], difference in the amplitude of calcium surges[4],

5   response to stimuli involved in contractility, and metabolism appear to be modulated in a

6   circadian-related fashion even *ex vivo* and *in vitro*[4,15,26,42,189]. It follows that *ex vivo* and *in vitro*

7   experiments are subject to confounding caused by circadian factors.

8

9   A. Primary cells

10   When cardiovascular cells are kept in primary cultures, they maintain a synchronized circadian

11   gene expression pattern and physiological activity for a several days. Therefore, the time of

12   isolation is an important parameter that is rarely noted.

13

14   B. Long term cultures

15   Upon prolonged culture, circadian rhythms may be desynchronized but can be restored or

16   experimentally modified with 'Zeitgebers', which in the *in vitro* situation are exogenously

17   administered stimuli (e.g. serum starvation followed by re-addition) that can re-synchronize

18   the circadian clock in culture (but may have other undesired effects on the *in vitro* situation).

19   Importantly, even standard procedures such as splitting cells or refreshing culture medium

20   may act as synchronizers. On the other hand, experimental interventions may in fact disrupt

21   the pre-existing circadian rhythm *in vitro*. Circadian rhythms can also develop during

22   differentiation and maturation in prolonged culture, as has been shown for human pluripotent

23   stem cell-derived cardiomyocytes[190].

24

25   Whether or not the circadian clock is active and therefore relevant to a specific *in vitro* system

26   is commonly unknown and needs attention. Since about 10-15% of the entire gene expression

27   program in e.g. cardiomyocytes is under control of core clock genes and thus susceptible to

28   time-dependent changes, this may profoundly affect the results up to the level of cellular

29   function - including response to drugs and resistance to mimicked ischaemia-reperfusion[28],

30   again causing type I or type II errors. Thus, careful standardization of timing in relation to

31   culture procedures (for example, related to the time after splitting and medium change) and

1   interventions is essential. Assessment of a treatment effect at multiple circadian times is rarely

2   performed, but it would increase the chance that such an effect is found.

3

4   **5. Considerations on inclusion of circadian rhythm aspects in clinical and preclinical**

5   **studies related to ischaemic heart disease**

6   Circadian rhythms play an important role in cardiovascular disease including ischaemic heart

7   disease[191]. Based on the considerations presented in this paper, the ESC WG on Cellular

8   Biology of the Heart and invited experts provide the following suggestions on circadian

9   rhythms in preclinical and translational research, and potentially also in clinical studies[192].

10   Before the start of the study, it is key to conduct a literature search investigating what is

11   already known about circadian rhythms in the specific topic as presented in figure 3. Several

12   clinical-focussed reviews can be a starting point[193,194]. Good summaries of current knowledge

13   from preclinical studies are also available[195] but here, evidence is rapidly accumulating so a

14   new search before each study design is strongly advised. When studying a specific gene or

15   protein, transcriptome[9,196] and proteome[197] studies that analysed the heart specifically may

16   be of help. It is important to realize that rhythmicity may be present in all aspects of research.

17   Many cardiac outcome measurements including troponin[155], blood pressure, and

18   repolarization duration[24] for example, are known to vary throughout the day and may

19   influence results. If no previous data is present, a pilot experiment can be helpful to determine

20   if rhythms are present and significant.

21   When literature or pilot experiments suggests circadian rhythms may be present in one or

22   more of the research factors, there are several options to incorporate this in a study. If the

23   researcher is interested in a potential circadian effect, measurements will have to be done at

24   several time-points throughout the day and statistics including sample-size will have to be

25   adjusted accordingly. If the researcher on the other hand, wishes to exclude circadian rhythms

26   as a potential confounder, a different option is to do all measurements at the same time-point

27   or equally distributed throughout the day. An additional advantage is that standardization of

28   time-points will reduce variation and therefore allow for a reduction of sample size in some

29   cases.

1   In preclinical research, the choice of the species/strain, sex, and gender will need to be

2   carefully weighed to mimic the clinical setting. The time of feeding, the housing conditions

3   (i.e. temperature, light exposure, noise), intervention (whether it is the type of the surgery, of

4   the experimental procedure, or the drug delivery), and the time of sample collections will

5   need to be chosen carefully and recorded to avoid bias of circadian rhythms into the analysis

6   of the outcomes (see figure 3), as well as to maximize potential (therapeutic) effects for later

7   translation.

8   Similarly, clinical studies may need to consider possible variations of the circadian rhythm due

9   to age, sex, ethnicity of the population, the presence of classic cardiovascular risk factors and

10   other risk factors such as the light exposure, sleeping conditions, noise, dietary conditions,

11   and time of the meals. Whenever possible, all these conditions should be clearly reported into

12   the publication. In addition, time of the surgical or clinical intervention, time of the drug

13   delivery, or sample /data collection can be recorded if the investigator wishes to ensure that

14   the variability of the outcomes is or is not consecutive to the circadian rhythm's influence (see

15   figure 3).

16   Recently, many convincing pre-clinical concepts in cardioprotection and ischaemic heart

17   disease, including the use of cyclosporin[198] , remote ischaemic preconditioning[199], and a study

18   investigating circadian rhythms in cardiac surgery[162], failed to show clinical benefit in trials.

19   Multiple factors may contribute to this translation failure, and these include lack of

20   standardized research protocols, randomized study designs, blinding of investigators and the

21   use of inadequate animal models (discussed previously[200,201]). Incorporation of circadian

22   rhythms in pre-clinical and translational research may also contribute to reduce the gap

23   between bench and bedside and thus improve translation of preclinical concepts to the clinic.

24   **6. Conclusion**

25   It must be acknowledged that clinical data showing circadian variation in cardiovascular

26   outcome in myocardial injury are still sparse and there is a lack of randomized clinical trial

27   demonstrating the circadian rhythm impact on a myocardial ischaemia/reperfusion injury

28   endpoint. Nevertheless, as shown mostly in preclinical studies, circadian rhythms may play an

29   important role in the incidence, development, outcome, and treatment of ischaemic heart

30   disease. So far, however, many studies have not adequately incorporated circadian rhythms into

1   the design, methodology, and analysis of preclinical and clinical data, potentially leading to

2   suboptimal research results. We believe that including circadian rhythms in the design and the

3   analysis of research may benefit translation of cardioprotective studies related to ischaemic

4   heart disease and may expand to other cardiac and non-cardiac diseases. Similar to age and

5   gender, circadian rhythms may be an important physiological parameter that, when

6   incorporated, may improve reliability of research, thereby helping to better understand and

7   cure ischaemic heart disease.

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Funding:

Table Legend

Table 1: Relationship between physiological, co-morbidities, psychological, behavioural, environmental factors and circadian rhythms

Figure Legends

Figure 1: The role of circadian rhythms in cardiovascular physiology. A) The circadian clock sustains a 24-h rhythm that regulates the cardiovascular system, including electrophysiological parameters, blood pressure, cardiac contractility, coagulation, vascular function, and the cardiovascular involvement of the immune system. B) The circadian clock is a molecular mechanism consisting of positive and negative feedback loops C) The circadian clock causes time-of-day variation of clock components as well as physiological parameters

Figure 2: Aspects of preclinical and clinical cardioprotective research influenced by circadian rhythms.

Figure 3: Practical flow chart to include circadian rhythm aspects in the design of preclinical, translational and potentially clinical cardioprotective studies.
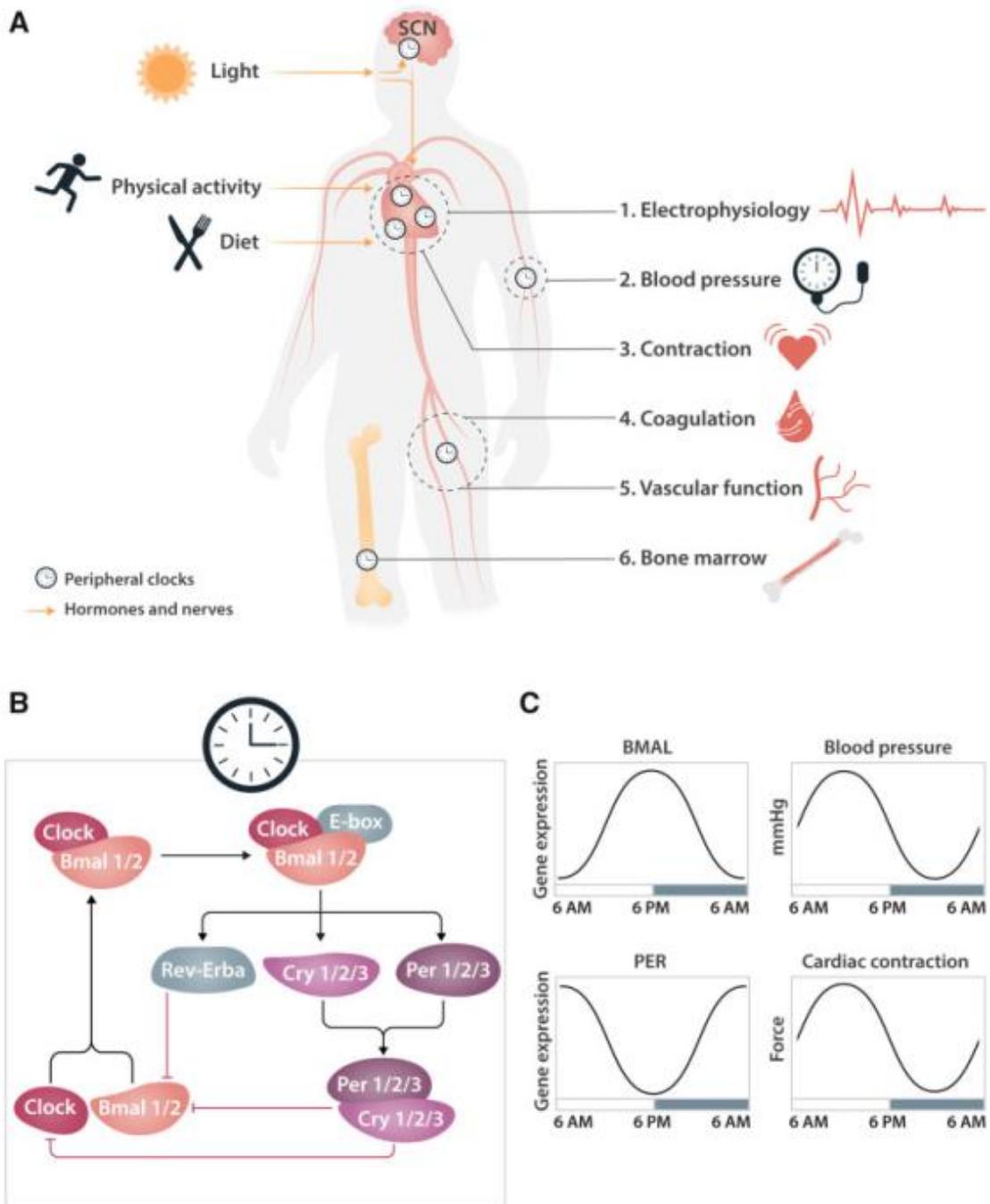
Figure 1

Key points related to circadian rhythm that may interfere with the outcomes of research studies on cardioprotective strategies
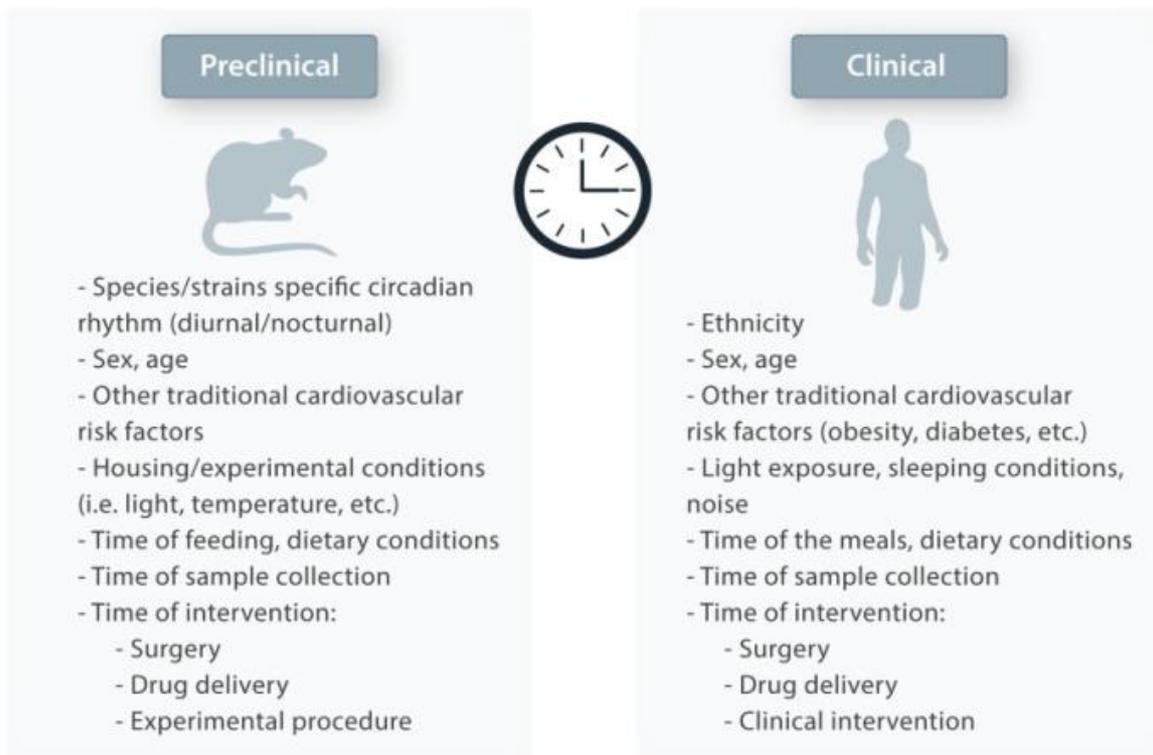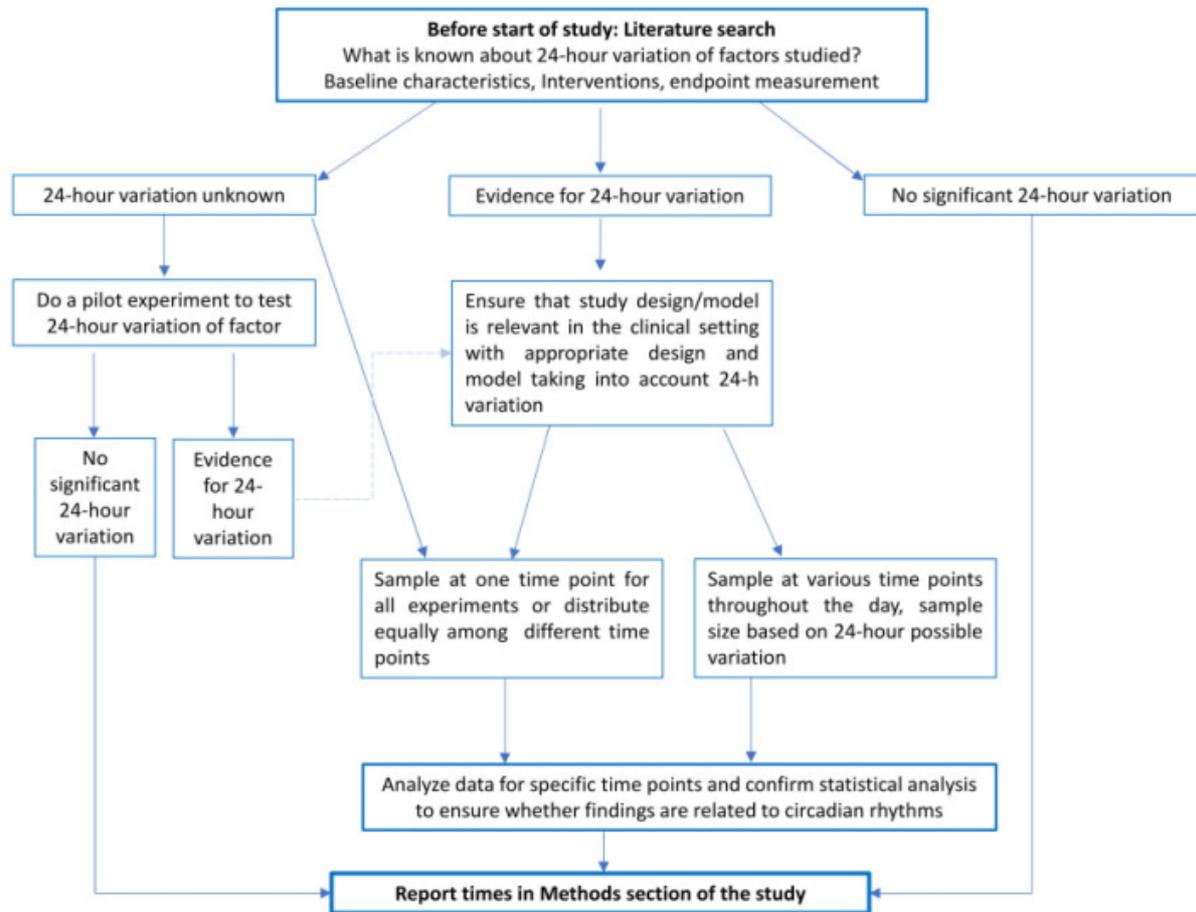

Preclinical

- Species/strains specific circadian rhythm (diurnal/nocturnal)
- Sex, age
- Other traditional cardiovascular risk factors
- Housing/experimental conditions (i.e. light, temperature, etc.)
- Time of feeding, dietary conditions
- Time of sample collection
- Time of intervention:
    - Surgery
    - Drug delivery
    - Experimental procedure

Clinical

- Ethnicity
- Sex, age
- Other traditional cardiovascular risk factors (obesity, diabetes, etc.)
- Light exposure, sleeping conditions, noise
- Time of the meals, dietary conditions
- Time of sample collection
- Time of intervention:
    - Surgery
    - Drug delivery
    - Clinical intervention

Figure 2

Figure 3