UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Human Development and Health

Biological rhythms in female reproduction

A LINK WITH CLINICAL DATA, UTERINE RECEPTIVITY AND IMPLANTATION

Linden Jane Stocker

Thesis for the degree of Doctor of Philosophy

February 2018

Abstract

Many aspects of physiological function are strongly circadian. Disturbance of these intrinsic modulators is implicated in disease states, but the role of biological rhythmic control in the context of reproduction is still largely unknown. The circadian network is apparent in all aspects of reproductive functioning; from menstruation to implantation and pregnancy. Endometrial dysfunction may occur if regulatory processes do not happen, with a disruptive effect on the synchronisation of implantation. Whether this dysregulation happens at the level of the endometrium, at the embryo-endometrial interface or at the level of clock genes is not known.

This work has investigated the role these biological rhythms, in particular those pertaining to that of uterine receptivity and implantation. By a systematic review and a meta-analysis of shift workers, a population being at risk of adverse early reproductive outcomes was identified. The link between experiencing poor early reproductive outcomes and sleep and activity was further investigated and it was shown that sleeping and activity patterns are different in reproductive pathology as compared with fertile healthy women.

The molecular basis of observed relationships between sleep and reproductive difficulty was investigated by examination of the uterine environment. Human samples were compared *in-vivo*, and with *in-vitro* culture models in unstimulated, normal menstrual cycles. This was to examine whether the difference in the circadian rhythm, which leads to deleterious effects in other pro-inflammatory disease, could be linked to the uterine environment of women with reproductive pathology. The immunomodulatory uterine secretome profile in women suffering from recurrent implantation failure (RIF) was shown to be different from fertile women.

The expression of core clock genes within the uterus was shown to be cyclical in a circadian manner. The effect of decidualisation appeared to effect the phase, but not the period of this expression. The distinct pattern of endometrial secretions in each group of women (RIF and fertile controls) was compared with the reciprocal core clock gene expression, and was shown to be correlated with a four-hour time lag. The gene-immunomodulator association was effected by decidualisation, more so in the women suffering from RIF than the controls. The addition of melatonin to the cell culture model made the RIF endometrium respond more like the control endometrium. After treatment with melatonin, cells from women with RIF had a more similar gene-immunomodulator profile to the control women. This effect was more noticeable after

decidualisation. Whether or not this can be considered a beneficial alteration has not been ascertained.

Table of Contents

List of Tables

List of Figures

List of Figures

List of publications and proceedings

Publications

1. **Stocker LJ, Cagampang F, Cheong Y** (2017) Identifying stably expressed housekeeping genes in the endometrium of fertile women, women with recurrent implantation failure and recurrent miscarriages. Sci Rep. 1;7(1):14857.
2. **Stocker LJ, , Bewley SJ, Cheong YC** (2016) Health consequences of shift work and insufficient sleep, BMJ, 355:i5210.
3. **Stocker LJ, Macklon NS, Bewley SJ, Cheong YC** (2014) Influence of shift work on early reproductive outcomes: a systematic review and meta-analysis. Obstet Gynecol. 124(1):99-110.

Proceedings

1. **Stocker LJ, , Cagampang F, Lee YH, Cheong YC** (2017) Can we turn back the clock? The effect of melatonin on the expression of clock genes in the endometrium of women with recurrent implantation failure (RIF). Human Reprod. 32(1):306.
2. **Stocker LJ, Cagampang F, Niranjan N, Cheong YC** (2016) Identifying Clock genes and comparing their circadian expression in the endometrium of healthy fertile women and those with recurrent implantation failure (RIF). Human Reprod. 31(1):84.
3. **Stocker LJ, Chan JCK, Lee YH, Cheong YCC** (2015) Does the endometrial secretory immunomodulatory profile differ between women with recurrent implantationn failure (RIF) and fertile controls? A case control study. Human Reprod. 30(1):301.

DECLARATION OF AUTHORSHIP

I, Linden Jane Stocker, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

BIOLOGICAL RHYTHMS IN FEMALE REPRODUCTION
I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as declared in 'publications and proceedings'.

Signed:.....

Date:.....

Acknowledgements

I thank the following people for sharing their protocols:

Dr Charlotte Weimar: tissue culture protocols from the University of Utrecht Laboratory, Netherlands.

Jon Ward: immunocytochemistry protocols of the Histochemistry Research Unit, Sir Henry Wellcome Laboratories, Southampton General Hospital.

Karl Staples: fluorescence-activated cell sorting (FACS) protocols (and collecting and interpreting the data with me).

Jianzhang 'Derek' Wang: Immunofluorescence protocols from Prince of Wales Hospital, Hong Kong (and teaching me about the ethos of Steve Jobs and Hong Kong cuisine).

I thank the following people for their technical assistance:

Yie Hou Lee: For performing the Luminex analysis of the uterine fluid samples, serum, culture media and the logistic modelling statistics.

Tom Ladyman and Shilong Lu of Fidelity Technologies Ltd, University of Southampton, for their modelling of the Actiwatch data, including frequency analysis and probabilistic modelling.

Gene Chi Wai Man: For performing immunohistochemistry on samples of endometrial tissue.

Sara Williams: For performing a literature search of temperature in the female reproductive system.

Dr Joanne Bailey: For practical advice on processing samples, laboratory demonstrations and as a point of reference on any laboratory techniques (or how to find out). Also her support, friendship and coffee-drinking ability.

I would also like to thank all of those who have supported me in my work towards this project:

The long-suffering research team at Princess Anne; Dr Alexandra Kermack, Dr Bas Van Rijn and Dr Mukhri Hamden for their shared pursuit of the next, 'scientific breakthrough', and maintaining the pretence that they too, enjoy running. All the medical students who have helped along the way: Karuna Chhugani, Kasey Redler, Sarah Johnson and Jessica Evans. Professor Macklon for his encouragement and ability

to make anything seem possible, even Radio 4. Dr Felino Cagampang for being calm when I despair over dying cells and spread-sheets.

To my parents and Euan for their unwavering support and faith in my abilities. To Alistair and Olivia, for always making me laugh and their tolerance of another weekend dedicated to 'the PhD'.

I especially thank Professor Ying Cheong for her contagious enthusiasm, imagination and ideas, her endless support and sage advice (which I always take, ultimately).

Most of all I thank the women who, during some of their most harrowing life experiences were willing to partake in my project. Without them, this work would not have been possible.

Abbreviations

ACTH Adrenocorticotrophic hormone

ART Assisted reproductive techniques

bHLH Basic helix-loop-helix

CK1Casein kinase 1

CD8+ Cluster of differentiation T-cell

CK Cytokeratin

COX cyclooxygenase

CRH Corticosteroid releasing hormone

CU Circadian Units

DAPI 4,6-Diamidino-2-phenylindole, dihydrochloride

DEC Decidualisation/decidualised

DNA Deoxyribonucleic acid

E_2 Oestradiol

ECM Extracellular matrix

ESC Endometrial stromal cell

ESPL Early Spontaneous pregnancy loss

FSH Follicle stimulating hormone

GHRH Growth hormone-releasing hormone

HESC Human endometrial stromal cell

HKG Housekeeping gene

HPG Hypothalamic-pituitary-gonadal axis

HPO Hypothalamic-pituitary-ovarian axis

HPA Hypothalamic-pituitary-adrenal axis

IL Interleukin

LH Luteinising hormone

MMP Matrix metalloproteinase

Non Not decidualised

OSA Obstructive sleep apnoea

OR Odds ratio

PCOS Polycystic ovarian syndrome

PRL Prolactin

RHT retino hypothalamic tract

RIF Recurrent implantation failure

RM Recurrent miscarriage

RNA Ribonucleic acid

RR relative risk

RT-PCR Real-time polymerase chain reaction

SD Standard deviation

SVM Support Vector Machine

TNF-α Tissue necrosis factor alpha

USC uterine stromal cell

VIP Vasoactive intestinal polypeptide

VM Vimentin

ZT Zeitgeber time

Genes

clock: circadian locomotor out-put cycles kaput

bmal1: brain and muscle ARNT (arylhydrocarbon receptor nuclear translocator)-like protein)

cry: cryptochrome

f*rq:* frequency

npas: neuronal pas domain protein

per: period

tim: timeless

Abbreviations

Circadian glossary

Acrophase The time at which the peak of a rhythm occurs. Unit of measurement: hours (h) in relation to an absolute or arbitrary reference.

Actogram The graphical display of a time series along two time axes (actual duration vs duration of a cycle). Successive cycles are plotted on successive lines.

Amplitude The difference between the peak (or trough) and the mean value of a wave. Note: For symmetrical waves, the amplitude is half the value of the range of oscillation.

Bathyphase The time at which the trough of a rhythm occurs. Unit of measurement: hours (h) or degrees of circumference (°) in relation to an absolute or arbitrary reference.

Chronobiology The scientific study of biological rhythms.

Circadian Endogenous generation of cycles of approximately 24 hours and entrainable by a zeitgeber with a period in the circadian range (approximately 19 to 28 hours).

Circalunar Occurring or functioning in cycles of approximately one lunar cycle.

Circannual Endogenous generation of cycles of one year and entrainable by a zeitgeber with a period in the circannual range (approximately 8 to 16 months).

Clock A functional entity that indicates or records the time of day, usually by dividing the Earth's period of rotation into equal time intervals.

Cosinor A procedure for the analysis of biological rhythms based on the fitting of a cosine wave to the raw data. Note: The single-cosinor procedure fits a single cosine function with a presumed period (anticipated on the basis of prior experience) to the time series and provides estimates of mesor, amplitude, and acrophase. More complex cosinor procedures utilise a fundamental function and one or more harmonics.

Cycle (1) A single occurrence of a periodically repeated phenomenon. (2) A periodically repeated sequence of events (synonymous with rhythm, although by tradition the reproductive rhythm is called a cycle i.e. menstrual cycle).

Daily Having the duration of a day (24 hours). Daily may also mean ‘happening every day/once a day’, in other contexts.

Day (1) The 24-hour interval between two successive sunrises on Earth (solar day). (2) The interval of time between dawn and dusk.

Desynchronisation Loss of synchrony between a rhythm and its zeitgeber (external desynchronisation) or between two rhythms within an organism (internal desynchronisation).

Diurnal Occurring or active during the daytime

Enright periodogram A mathematical procedure for the determination of periodicity in time series with equally-spaced data points. The Enright periodogram is based on the variances of different segments of the time series sequentially aligned by period.

Endogenous Originating within an organism

Endogenous rhythm The ability of a cycle or rhythm to freerun in constant conditions (i.e. without environmental cues).

Entrain To synchronise a self-sustaining oscillation

Exogenous Originating outside an organism.

Fourier analysis A mathematical procedure for the determination of periodicity in time series with equally-spaced data points. Fourier analysis is based on the decomposition of the time series into

periodic components described by sine and cosine functions.

Freerun The state of a self-sustaining oscillation (rhythm) in the absence of effective zeitgebers or other environmental agents that may affect the period of the oscillation.

Frequency (f) The number of times a specified phenomenon occurs within a specified time interval ($f = 1 / \tau$).

Infradian Occurring with a frequency lower than circadian (i.e., with a period longer than circadian).

Illuminance The total luminance flux (measured in candela, cd) incident on a surface, per unit area (cd/m^2, or lux). It is a measure of how much light illuminates the surface, wavelength-weighted by the luminosity function to correlate with human brightness perception.

Masking Disruption in the expression of overt rhythms caused by an external agent without a direct effect on the period or phase of a pacemaker.

Mesor An estimate of central tendency of the distribution of values of an oscillating variable (the average value around which the variable oscillates). The mesor is a circadian rhythm-adjusted mean based on the parameters of a cosine function fitted to the raw data. When a process is known to be rhythmic, and the data points are not equidistant or the sample size is small, the Mesor often provides a more appropriate unbiased estimator of central tendency than does the arithmetic mean of the raw data.

Nadir The lowest value of an oscillatory function.

Nocturnal Occurring or active during the night time.

Nycthemeral rhythms Having the duration of a day (24 hours) whose endogenous nature has not been ascertained

Oscillator An entity capable of generating a periodic variation in the value of a quantity.

Peak The point of culmination of an oscillatory function.

Period (τ)The time elapsed for one complete oscillation or cycle (the distance in time between two consecutive points of a recurring wave). ($\tau = 1 / f$).

Periodogram A function relating periodic components of a time series to their spectral power. There are many types of periodograms (e.g., Fourier, Enright, Lomb-Scargle, etc.).

Phase (1)The relative angular displacement between a periodic quantity and a reference angle (2). A distinct stage of a process (such as the luteal phase of the menstrual cycle).

Phaseshift A discrete displacement of an oscillation along the time axis.

Rhythm A periodically repeated sequence of events.

Shift work A work schedule involving non-traditional working hours, usually during the evening and night.

Sidereal day. The 23.9-hour interval during which the Earth completes one rotation on its axis

Slave oscillator An oscillator that is driven or entrained by another oscillator.

Synchronisation (1) The action of causing two or more processes to happen at the same rate (entrainment) (2) The action of causing two or more processes to start at the same time

Ultradian Occurring or functioning with a frequency higher than circadian (i.e., with a period shorter than circadian)

Abbreviations

Wavelength. The distance between two successive points of a regular wave (a metric measure of period)

Zeitgeber A synchronizing agent (a stimulus capable of resetting a pacemaker or synchronising a self-sustaining oscillation). The zeitgeber dictates the local time, not the ability to keep time

Zeitgeber hour The unit of time corresponding to 1/24 of the period of a zeitgebers.

Zeitgeber time A standard of time based on the period of a zeitgeber. Under standard light-dark cycles, the time of lights on usually defines zeitgeber time zero (ZT 0).

Summary

Human physiology is governed by tightly controlled sequences of events. In humans, one of the most obvious examples of regulation is demonstrated by the sleep-wake cycle of circadian rhythms, but many biological regulators are important for optimal functioning. Disruption to these intrinsic modulators is implicated in disease states, but the role of biological rhythmic control in the context of reproduction is still largely unknown, although the circadian network of biological systems is apparent in all aspects of reproductive functioning; from menstruation to fertility, pregnancy and beyond. This work aims to investigate the role these biological rhythms, in particular, that pertaining to that of uterine receptivity and implantation.

Chapter one outlines the importance of biological rhythms in reproductive functioning. In more detail, it presents several rhythms and cycles, thought to be important in regulating female reproduction and hypothesises as to why derangement to these might cause adverse reproductive effects.

Chapter two presents a systematic review and a meta-analysis of shift workers and identifies this population as being at risk of adverse early reproductive outcomes. An association, not causal link is demonstrated. The following chapters aim to explore the link between sleep/activity in a group of women experiencing poor early reproductive outcomes and compare this data with healthy fertile controls.

Chapter three describes the findings of sleep-wake cycle information from women with reproductive pathology and controls. This includes monitoring of their sleep patterns with actigraphy watches, sleep diaries and questionnaires.

Chapter four presents a series of experiments, designed with the aim to investigate the molecular basis of observed relationships between sleep and reproductive difficulty. Human samples are compared *in-vivo*, and with *in-vitro* culture models. This chapter examines whether the difference in the circadian rhythm, which leads to deleterious effects in other pro-inflammatory diseases, could be linked to inflammatory markers in the uterine environment of affected women and if and how this could be modulated by melatonin.

Chapter five is a general discussion of the amalgamation of all the results presented within this thesis, and the conclusion.

Chapter six proposes a summary of future work.

Chapter 1 Introduction

1.1 Chronobiological rhythms

From the ancient Greek *chromos* time + *biological* relating to living organisms

1.1.1 Biological rhythms

Living organisms have been developing for millions of years with few elements conserved but nature's intricate clockwork of biological rhythms. The first documented evidence for the human appreciation of biological rhythms was made in fourth century BC as Androsthenes noted diurnal movements of the tamarind tree, *Tamarindus indicus* (1). These optimally timed biological rhythms where physiological processes such as feeding and reproduction coincide with environmental timings of the Earth's rotation have evolutionary advantages and hence are highly conserved.

However, some biological rhythms are conserved in extreme circumstances, where survival and procreation depends on very few external cues. For example the entrenched biological rhythms of the subterranean and blind naked mole rat, *Heterocephalus glaber* (2). The deep sea squid *Euprymna scolopes* has a drive great enough to maintain biological cycling that it acquires circadian transcriptions from its ingested symbiotic luminous prey, the *Vibrio fischeri* (3). The perseverance of human sleep patterns without external clues was first demonstrated by Kleitman in the early 1900s in underground Mammonth Cave.

Whilst some organisms such as subterranean mammals and deep-sea fish may experience minimal day and night variation in environmental conditions, virtually all species are subjected to important cyclical changes. Light and dark is only one essential component of the photoperiodic system that measures day length. It uses changes in day length to help regulate seasonal adaptations (4). The use of other cues to time seasonal adjustments demonstrates other adaptive rationales for the retention of biological rhythms. Animals living above the Arctic Circle experience extensive periods of permanent light and then complete darkness over the course of a year. This extreme environment poses unique challenges. One adaptive response in the Arctic reindeer, *Rangifer tarandus*, is the periodic seasonal shifts in eye colour to adapt to the seasonal changes in which the deer must survive (5, 6). Whilst the importance of biological rhythms is consistently demonstrated across species, the methods of regulation behind these clocks vary enormously.

It seems unlikely that the capacity of such animals to display rhythms is merely because the species has intact redundant ancestral systems. These cycling capacities may be retained because they are necessary for specific functions. For example, it might be these rhythms could be valuable for providing internal temporal order which provides advantages, even in organisms that inhabit relatively constant environments (7). Whilst external cues are crucial to the tuning of the 'body clock', the inherent endogenous clock may be robust, yet flexible, as seen in species resident at high latitudes (such as the artic reindeer). Whilst exposed to a year-long dark or light cycle, they achieve precisely timed annual reproductive rhythms despite the absence of daily photoperiodic information (8). Their biological rhythm is acutely responsive to the light and dark cycles, but not to circadian (24 hour-long) phase. The molecular clockwork that normally drives internal circadian rhythms is weak, but this does not mean complete arrhythmia. Seasonal timing may be driven directly by light information received at a limited time of year, involving a distinct 'circannual' clock.

Whilst the nature of these biological rhythms varies widely across species they appear to occur in all organisms corresponding to, and in response to, periodic environmental change. Examples of such change include cyclical variations in the relative position of the Earth to the sun and the immediate effects of such variations. For example, day alternating with night, high tide alternates with low tide. Thus biological rhythms are a direct response to various changes in exogenous stimuli. They are also an endogenous rhythm that ensures behaviour is in synchrony with the exogenous 'period'. Or, more likely, they utilise a mechanism which is a combination of both. Regardless of how these rhythms are maintained, the importance of chronobiology cannot be understated. It is a method of seeking to discover more about the method and rationale of the cyclic phenomena of living organisms as an adaptation to external rhythms. Chronobiology and an understanding of how and why certain intrinsic functions are preserved are very much in their infancy. Studies including comparative anatomy, physiology, genetics, molecular biology and behaviour of organisms within biological rhythm-mathematics provide basic information. In development and reproduction, in order to proceed with this knowledge and fully understand what, how and why biological rhythms exist and are maintained, requires further investigation. Perhaps as importantly is whether or not chronobiology can be manipulated and its relationship into broader aspects of cyclicity and related biological outcomes.

1.1.2 Types of biological rhythms

Zeitgeber from German, Zeit time + Geber giver

By definition, biological rhythms are maintained with and without external governance, but are also able to synchronize with the environment via 'Zeitgebers'(9). These rhythms regulate a variety of processes from a cellular level to systemic effects that mediate functioning. Expression of endogenous biological rhythms when subjects are isolated from external cues is described as free running. The types of rhythm are summarised in Table 1-1.

Table 1-1: Types of biological rhythm

	Duration of rhythm	Implication for human female reproduction
Circannual	12 months	Endocrine control (peak testosterone levels, LH, PRL) (10, 11) Birth rates (12)
Circa lunar	28 days	Menstrual cycle (13)
Circa septan	7 days	Spontaneous birth (14)
Circadian	24 hours	Temperature, sleep, hormones or endocrine control as above

1.1.2.1 Circannual

From Latin *circa* about + *annus* a year

Any biological rhythm involving processes that occur or fluctuate at intervals of approximately one year, even in controlled environments from which seasonal cues have been eliminated. The intrinsic timing is indicated by the manner the physiological rhythms 'free-run,' with a period significantly different from the Sidereal year (a measure of the position of the Earth in its rotation around its axis). These rhythms have been demonstrated to be unperturbed under constant laboratory conditions from which fluctuations in temperature, daylight, and other seasonal markers have been excluded (15). After time, temporal asynchrony will develop between individuals but even in animals born into constant conditions the intrinsic nature of circannual timers persists (16, 17). As a rhythm that is not so readily seen in human subjects, more obvious examples are the seasonal changes in behaviour of some migratory birds, the onset of hibernation of animals and the flowering time of plants.

The biological importance of such rhythms is to allow physiological changes to be complete, well in advance of seasonal environmental changes. Physiological and/or morphological adaptations that require time to develop must be initiated early enough to meet and survive the environmental challenges. For example, to guarantee offspring

are born at the optimal time of year, the process of gonadal development must be initiated by a forewarning environmental cue that reliably gives information about the season to come, well in advance.

However, these rhythms do exist in human populations with effects on physiological and psychological functioning. There has been demonstrable circannual variation at a molecular level with effects on DNA synthesis and protein function, to alterations in physiological parameters such as blood pressure to system-wide endocrine and metabolic changes(18-20). For example, endocrine control under the influence of annual fluctuations alters the peak testosterone levels, luteinising hormone (LH), testosterone and prolactin (PRL) levels in adult humans (10, 11). This can also be seen in the downstream effect on the annual cyclicity in human sperm concentration and total sperm count (21), conception rates and birth rates (12, 22).

It is inherently difficult to study annual rhythms in the absence of external modulators in humans and it could be argued that by definition these changes are in fact seasonal and light dependent, rather than truly circannual. For example, whether or not circannual rhythm plays a role in the higher prevalence of seasonal affective disorder (SAD) in winter, or whether it is purely photo dependent is difficult to ascertain (23). However other higher psychiatric functioning has also been linked to acircannual roots, with a higher prevalence of *anorexia nervosa* being seen in children born in later months of the year so a physiological circannual rationale is plausible (24).

1.1.2.2 Circa lunar

From Latin *circa* about + *lunar* pertaining to the moon

The biorhythm that corresponds with the lunar cycle (approximately 29.5 days) (25) affects the reproductive cycles of many organisms, especially marine organisms (26, 27). These are linked to changing levels of moonlight and the tidal rhythm, both of which are governed by the phases of the moon. Lunar rhythms have an influence upon human culture, with commonly held beliefs that our mental health and other behaviours are modulated by the phase of the moon (28). There is some evidence that human biology is regulated by the lunar rhythm, with the subjective and objective measures of sleep structure varying according to lunar phase and this may reflect the possibility of circa lunar rhythmicity in humans (29).

Whilst not strictly circa lunar, and should be correctly termed Circa trigintan (From Latin *circa* about + *triginta* thirty), the human female menstrual cycle is mean length of between 27.3 to 30.1 days between ages 20 and 40 years (13). The derivation of the word itself having come from the moon (Latin *mensis* month *cf* Greek *mene* moon). It

has been postulated that menstrual synchrony is related to the lunar cycle. There is no firm evidence that this is the case. It is however one of the most evident human biological rhythms that will persist without external modulation (30-32).

1.1.2.3 Circa septan

From Latin *circa* about + *septan* recurring on the seventh day

Circaseptan rhythms initially perhaps appear to present an artificially and socially imposed biological rhythm. However, early studies have demonstrated cellular differences in response and the susceptibility of tissues at different stages of their circaseptan cycle (33). Circaseptan hormonal biological rhythms have also been shown to be initiated by prolonged weekly drug administration which persist into the long-term following a period of entrainment (34). This has been reproduced with environmental entrainment (35) and may explain the altered response to behavioral patterns and the change in pharmacological actions seen in long term drug use.

Physiological processes such as spontaneous birth and various pathologies have also been noted to have weekly variation, for example, myocardial infarctions, and strokes (14). Whether or not truly inherent or partly entrained, the knowledge of such cycles is important for understanding the basis of synchronisation between the external environment and internal regulators.

1.1.2.4 Circadian

From Latin *circa* about + *diem* day

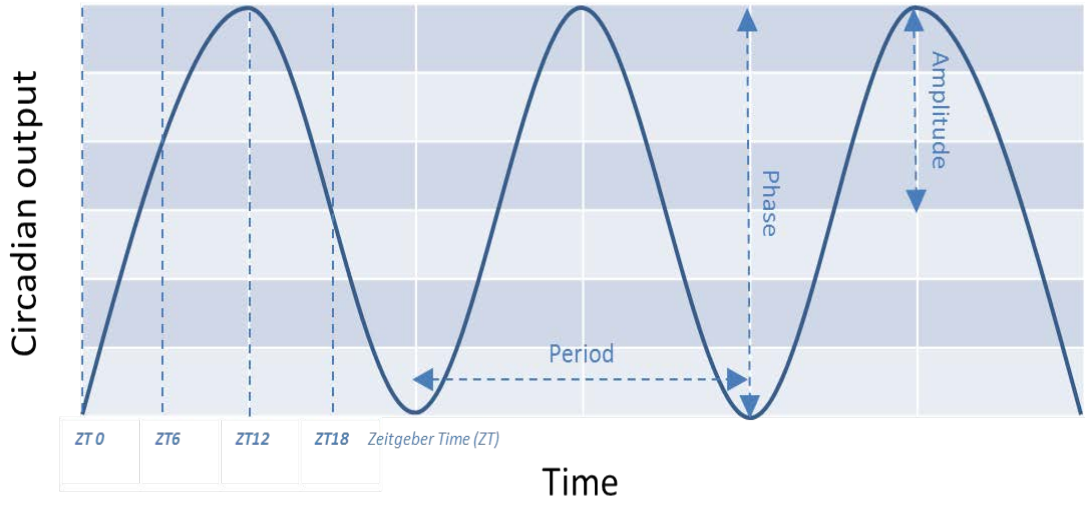
If rhythms persists under constant environmental conditions and have a period of around one day, the rhythm is termed circadian. Like all biological rhythms they are endogenously generated and self-sustaining, persisting under constant environmental conditions, typically constant light or dark. Circadian rhythms also display temperature compensation with the period remaining relatively constant over a range of ambient temperatures (36). This is thought to be one mechanism that buffers the clock against changes in cellular metabolism (37). Circadian rhythms have been demonstrated in plants, animals, fungi and cyanobacteria (38, 39).

To facilitate the study of circadian rhythms, universal terms have been described (see Figure 1-1). Period is defined as the time to complete one cycle of 24 hours. It is commonly measured from peak to peak but could equally be measured from trough to trough or from any specified phase marker. Phase is the time of day for any given event with the baseline being dawn. Conventionally measured in Zeitgeber Time (ZT).

The onset of light, as a powerful zeitgeber, means that sunrise (light onset) is defined as ZT0. The amplitude of the rhythm is defined as half the maximal difference in any given fluctuation of the circadian rhythms being measured.

Figure 1-1: Circadian terminology:

Period, time to complete one cycle (if circadian, circa 24 hours); Phase, time of day for any given event measured in Zeitgeber time (ZT); Amplitude, half the maximal difference in any given fluctuation of the circadian rhythm. Artwork by LJS.



Circadian rhythms control a variety of biological processes in living systems. Many living processes are regulated by circadian rhythms, including body temperature, feeding behaviour, blood pressure, hormone secretion, metabolism, glucose homeostasis and cell-cycle progression (40) and the human circadian rhythm manifests itself in many physiological and behavioural outputs (9, 41, 42). Perhaps the most obvious and well-studied circadian rhythms is the sleep and wake cycle in animals. In order to distinguish circadian rhythms from simple responses to daily external cues, the period of the rhythm in constant conditions is called the free-running period (τ). In diurnal animals (active during daylight hours), in general τ is slightly greater than 24 hours, whereas, in nocturnal animals (active at night), in general τ is shorter than 24 hours.

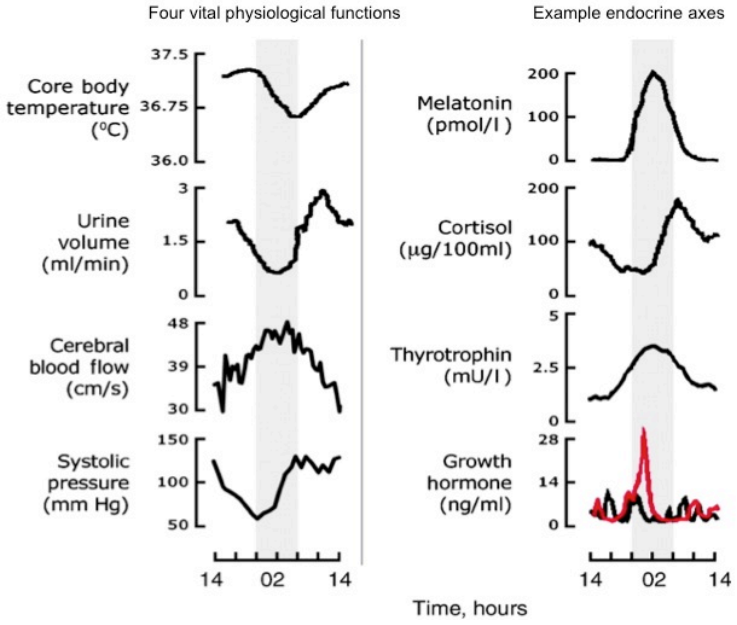
1.1.2.5 The clinical manifestation of human biological rhythms

Whilst all biological rhythms are demonstrated within human functioning, the most apparent and widely studied rhythm is circadian, and virtually all human physiology will demonstrate some level of circadian control. Commonly studied phase markers for measuring the timing of circadian rhythm are sleep, temperature and various hormonal markers, particularly melatonin levels (see Figure 1-2).

Figure 1-2: Representative physiological and endocrine circadian rhythms in humans held under constant routine conditions

From Hastings M, O'Neill JS, Maywood ES. Circadian clocks: regulators of endocrine and metabolic rhythms. J Endocrinol. 2007;195(2):187-98 (43)

Representative physiological and endocrine circadian cycles in humans held under constant routine conditions. Graphs on left show circadian rhythm in four key physiological functions. The right graph shows four endocrine axes. The shaded area depicts when the subjects would normally have been sleeping, but in the constant routine they remained awake. In the case of growth hormone, levels are not rhythmic in the absence of sleep (black line) but if sleep is permitted a clear circadian cycle emerges (red line).



1.1.2.5.1 Temperature

The circadian rhythm of core body temperature (CBT) is a well-documented physiological phenomenon which has been investigated since it was first reported in the 19th century (44). The body temperature of humans varies during the day by about 0.5 ˚C (0.9 ˚F), with lower temperatures in the morning and higher temperatures in the late afternoon and evening (45). The core body temperature of an individual tends to its lowest in the second half of the sleep cycle and its nadir, one of the primary markers for circadian rhythms, is reached approximately two hours before habitual wake time. Core temperature reaches its highest value approximately 12-14 hours after waking but absolute values vary amongst individuals depending on phenotype (see Circadian typology).

Temperature changes can be induced by external factors such as smoking, alcohol consumption and change in schedule, biological factors such as age and disease and physiological processes such as exercise and metabolism (46, 47). The underlying control of temperature change is governed by the paraventricular nucleus and

hypothalamus by altering activity of the autonomic nervous system. Core body temperature is variable within its circadian restraints and pro-inflammatory cytokines, again considered biological clock-controlled mediators (see section 1.2), for example Tumour Necrosis Factor (TNF), interleukin 1 (IL1) and interleukin 6 (IL6) in immune-mediated inflammatory reactions can activate the hypothalamus and reset temperature homeostasis (48, 49). Similarly environmental insult such as hyperthermia results in physiological actions such as higher rates of metabolism and an altered immune response. This in turn leads to enhancement of Cluster of Differentiation 8 (CD8+), (the transmembrane glycoprotein that serves as a co-receptor for the T cell) expression, as well as cytotoxic T-cell generation and differentiation, again considered clock-controlled mechanisms (50).

Skin temperature is the inverse of the core rhythm, and a decrease in core body temperature and an increase in peripheral skin temperature are associated with sleep onset with the reverse happening in the wake period. The circadian rhythm of core body temperature is determined both by changes in heat production and changes in heat loss. Heat production undergoes a circadian rhythm which is phase advanced by 1.2 hours compared with the circadian rhythm of heat loss (51).

1.1.2.5.2 Temperature in female cycles

As part of normal biological cycling, body temperature varies throughout the menstrual cycle in women of reproductive age. However, the underlying circadian rhythm of body temperature persists so the menstrual cycle mediates the underlying circadian rhythm. Normal cycling women maintain a similarly shaped circadian body temperature pattern in both the follicular and luteal phases but the overall temperature is shifted upward in the luteal phase compared with the follicular phase (see Table 1-2 and Figure 1-3). There is an increase in the waking and mean temperature but the difference between peak and trough values can decrease during the luteal phase. The decrease in amplitude occurs because the increase in trough value with ovulation is not accompanied by the same degree of increase in peak temperature value, resulting in a lessening of the difference between the two values (46).

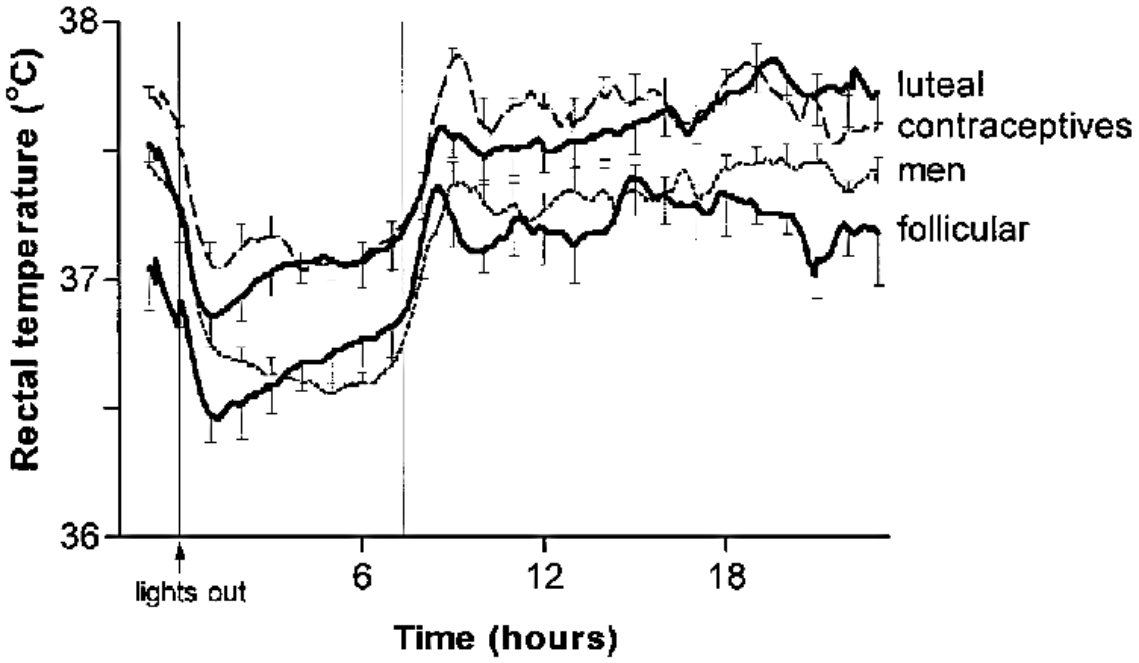
Table 1-2: Temperature variation in the normal menstrual cycle

Author	Follicular phase temperature daily mean (°C) ± SD	Luteal phase temperature daily mean (°C) ± SD	Mean increase in luteal phase (°C)
Baker 2001 (52)	37.0 (0.3)	37.4 (0.2)	0.4
Cagnacci 2002 (53)	37.0 (0.1)	37.3 (0.0)	0.3
Coyne 2002 (54)	37.1 (0.1)	37.4 (0.1)	0.3
Simic 2013 (55)	Lower in follicular phase		

Figure 1-3: 24 hour rectal temperatures from naturally cycling women

In the follicular phase, the and luteal phase, in women taking contraceptives, and in men.

From: Baker FC, Waner JI, Vieira EF, et al. Sleep and 24 hour body temperatures: a comparison in young men, naturally cycling women, and women taking hormonal contraceptives. J Physiol 2001;530:565-574(52).



It has been assumed that the biological rhythm in core body temperature is mirrored by the reproductive organs, also providing a rhythmic temperature cycle to the gametes and the embryo in the periconceptual environment. However evidence suggests that temperature actually varies within the reproductive organs as well as the circadian and circa lunar rhythm of mammals (see Table 1-3). This may be because temperature serves as an important regulator of reproductive functioning (56). It has been postulated that these observed effects are a result of endothermic processes within the maturing gonads (57) but deliberate cooling of follicles could occur pre-ovulation by

changes in mucification or blood flow (56, 58). Whether a cause or effect of timely modulators within the reproductive tract remains unknown (see section 1.6.1). This temperature variation is unlikely to be without biological rationale given that temperature change alters fertilisation rates (59-61).

Table 1-3: Temperature across the normal menstrual cycle in mammals

Author	Model	Organ	Timing	Temperature difference (absolute) (˚C)	Temperature difference
Grinsted 1980 (62)	Rabbit	Pre ovulatory Follicles/ ovarian stroma	Pre-follicular simulated and unstimulated	1.4	Ovarian stroma > follicles
Grinsted 1985 (63)	Human	Pre ovulatory follicles/ ovarian stroma	Pre-ovulatory	No value	Follicular fluid < ovarian stroma
Hunter 1986 (64)	Rabbit	Isthmus and ampulla	Pre ovulation	0.8	Isthmus < ampulla
			Post ovulation	1.6	
Bahat 2005 (59)	Rabbit	Isthmus	Non-mated	(35.1)	Isthmus < ampulla < rectum
			Pre ovulation	0.7 (35.8)	
			Peri ovulation	-0.9 (34.9)	
			Post ovulation	-1.1 (34.7)	
		Isthmal-ampullary junction	Non-mated	35.9	
			Pre ovulation	0.7 (36.6)	
			Peri ovulation	0.3 (36.2)	
			Post ovulation	0.4 (36.3)	
El-Sheik Ali 2013 (65)	Cow	Vagina	Average over menstrual cycle	(37.8)	Temperature gradient increases towards uterine horns
		Cervix		0.2 (37.9)	
		Uterine body		0.1 (37.9)	
		Uterine horn		0.2 (38.0)	

1.1.2.5.3 Melatonin

Pleitropy, from Greek *pleion* more + *tropos* turn

Melatonin, a neuro hormone of the pineal gland, is involved in the phasing of circadian rhythms and sleep promotion, it regulates the sleep/wake cycle, other circadian and circannual and circa lunar rhythms and acts as an immunostimulator and cytoprotective agent (66). Its cyclical release is a universal feature of all vertebrates (67).

As a circulating hormone, the circadian rhythm of melatonin can reach every cell via the bloodstream. The importance of this regulation can be demonstrated by the wide distribution of melatonin receptors (68, 69) and it is likely that these receptors exist on the membranes of all cells whose activity must be synchronised by the melatonin rhythm (70).

Melatonin acts via G protein-coupled receptors expressed in various areas of the central nervous system and in peripheral tissues, as well as via binding sites in the nucleus (66, 71). The biding sites in the nucleus are ROR orphan receptors (Retinoic acid-related Orphan) that include three subtypes (α, β, γ) and four splicing variants of the α-subtype. Only the splice variant c of the RORα subtype and the RORγ subtype are related to melatonin actions in the nucleus (72). Some of the genomic effects of melatonin are related to its interaction with these type of receptors. However, its actions are pleiotropic, that is it influences multiple, seemingly unrelated phenotypic traits. These effects are also mediated via nuclear receptors and other binding sites or chemical interactions. The cellular processes that controlled by melatonin independently from receptors may mean that the membrane mediators may not be a requirement for melatonin to influence circadian gene expression in peripheral cells (70).

All species that secrete melatonin do so at night because light immediately suppresses its synthesis. It has been established that if samples (saliva or blood or urine) are collected under dim light and controlled posture conditions, the melatonin rhythm provides an optimal marker of circadian phase in humans (73). Dim light melatonin onset (DLMO) is the easiest marker of circadian rhythm, because it can be feasibly measured in saliva before a person goes to sleep. However, melatonin assays are not a rapid or an easily available method for everyday diagnostic use.

The role, if any, of melatonin during human pregnancy is unclear. It has been suggested that the diurnal maternal rhythm serves as a signal for the fetus to entrain the circadian rhythms in newborns after delivery as serum melatonin levels during human pregnancy are higher than in a non-pregnant state (74). Only one meta-analysis has been performed specifically assessing the use of melatonin in IVF pregnancies and found no difference in clinical pregnancy rates when supplemented with melatonin or not (75). However, there was much heterogeneity and imprecision encountered in all studies considered. Melatonin is also known to be safe, with the Cochrane systematic

review and meta-analysis finding no association between melatonin supplementation and adverse effects for women involved in treatment (76).

The earliest identified manifestation of the circadian melatonin rhythm being influential in reproductive physiology was in 1963, when Wurtman et al reported that administration of melatonin reduces the weight of the ovaries of female rats (77, 78). There is evidence that melatonin affects reproductive ability in a wide variety of species and that the pattern of melatonin secretion, mediated by light exposure, directly influences reproductive function (78). Melatonin production has been demonstrated in the human ovary (79, 80) and placenta (81). Melatonin may be responsible for the circannual trends in human reproduction (see 1.1.2.1). Among people living in the Arctic, pituitary-gonadal function and conception rates are lower in the dark winter months than in the summer (22), which correlates with increases in serum melatonin concentration (82).

There has been some work done on the relevance of melatonin in oocyte and sperm quality (83-87). It has also been considered as an additive in embryo culture media (88) and its effects on luteal function have demonstrated increased levels of progesterone after melatonin supplementation (89, 90). The positive implications of higher melatonin levels on the human menstrual cycle, fertility and pregnancy are therefore well documented but with varying levels of evidence (91-93). Nevertheless, it appears that melatonin serves a purpose in the human reproductive system, with many of its observed effects not yet being linked to circadian functioning.

1.1.2.5.4 Activity

The most obvious circadian rhythm is activity. Knowledge of the properties of the circadian system, the role of zeitgebers for adequate synchronisation to the 24-hour day, and how sleep is regulated, has led to the development of stringent protocols to investigate the characteristics of circadian rhythms and activity. These studies have provided gold standards for estimating circadian amplitude and phase, and have identified the most useful physiological or hormonal markers.

The current challenge lies in developing simple markers for ambulatory use, which provide a reasonable estimate of circadian phase. Chronobiology requires long-term measurement over at least one 24-hour cycle. Polysomnography is the gold standard neurophysiological method for detection of sleep but is costly and requires subjects to attend a sleep laboratory and sophisticated technology for interpretative purposes. New microchip technologies permit non-invasive and continuous data collection over many days. Actigraphy monitors are data-loggers which record digitally integrated measures of gross motor activity. They record white light exposure, activity levels and are a

reliable indicator of activity status in healthy populations (94). Pollak et al (95) studied two commercial devices in adults and found that actigraphy correctly predicted sleep and wake at a rate of 86.6% compared with polysomnography (96), and demonstrated robust inter unit reliability and good validity (96).

1.1.3 Biological disruption

The importance of biological rhythms is clear as when they are disrupted, either by genetic or environmental insults, disorders of diverse physiological processes can occur. Biological rhythmic outputs can be either internally synchronised or desynchronised depending on the strength of a zeitgeber. Disruption to the biological clock is termed, 'internal desynchronisation'. This can occur in circadian timing, when there are phase differences resulting from period differences between two circadian output processes. For example, sleep and temperature can become non-entrained as a result of constant light exposure on a global and molecular level (see 1.6). Such internal synchronisation has been recognised as a risk factor for human disease (97).

1.1.3.1 Circadian typology

Chrono- from Greek khronos, time

Circadian rhythmicity differs amongst individuals and affects biological and psychological functioning (98). This variation is referred to as the chronotype. Some people are described as, 'early-morning larks', or, 'late-night owls', and these differences or changes in sleep-wake cycle behaviour are not always, although may be, a consequence of abnormal biological function (73).

Animal knock-out studies have provided a useful insight into clock gene function. Some information has been gained as to how clock gene mutations relate to abnormal rhythms of behaviour, activity and sleep. Much less is known about the effect of clock gene mutation in humans. Certain sleep disorders are associated with 'clock mutants' and a brief summary is given in appendix A.5.

1.2 The biological clock

1.3 History

1.3.1 The control of biological rhythms

The central control of biological rhythms lie in the suprachiasmatic nucleus (SCN), a small midline hypothalamic area, located just superiorly to the optic chiasm and divided in to two halves by the third ventricle. The SCN regulates rhythms in the periphery by the autonomic nervous system and the control of the pineal gland from where melatonin (see Melatonin 1.1.2.5.3) is produced.

The SCN was demonstrated to be a circadian modulator by animal studies in the 1970s when lesions in the SCN of rats permanently interfered with their previously entrained nocturnal drinking habits and activity rhythms(99). This in turn led to the understanding that damage to the SCN leads to the loss of biological rhythms, and the inability to respond to changes in the external environment.

1.3.2 The discovery of the biological clock

1.3.2.1 The central pacemaker

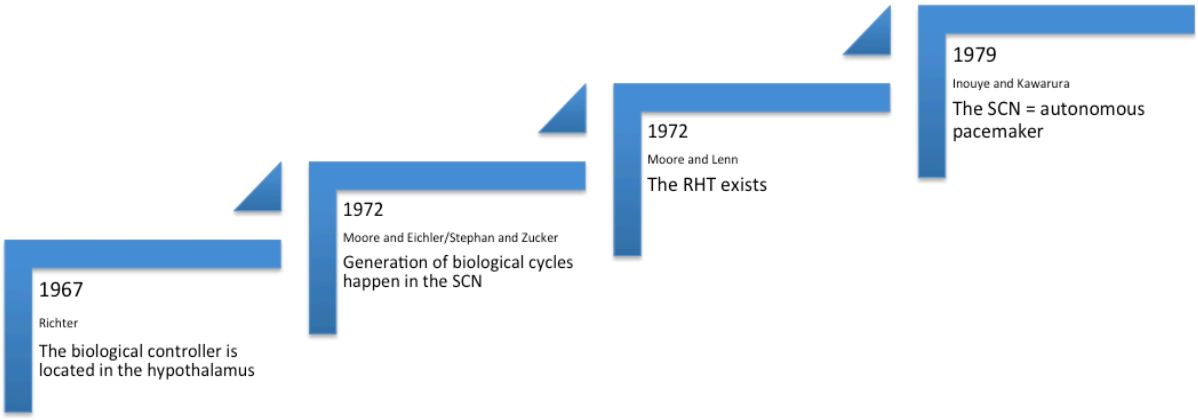
In 1967 studies which ablated a of part of the hypothalamus disrupted circadian rhythm in rats (100). This area was localised to the SCN in 1972 when it was shown that bilateral electrolytic lesions in the SCN permanently eliminated circadian rhythms in rats, so it was concluded that the generation and the entrainment of these was coordinated by neurons in the SCN region of the brain (101, 102). It was already established that environmental light played an important role in regulating circadian rhythm via hypothalamic-anterior pituitary output however, the mechanism behind this was not clear. Simultaneously to the discovery of the SCN as a central biological clock co-ordinator the retino hypothalamic tract (RHT) which is responsible for mediating light as a zeitgeber, was also first described (103). The RHT is a central retinal projection of cells and acts as a photic neural input pathway to the SCN. The intrinsically photosensitive retinal ganglion cells (ipRGC) comprising of this tract contain the photopigment melanopsin which feeds this environmental signal back to the SCN. The SCN then interprets information on environmental light to entrain the biological clock.

However, the SCN is a potent autonomous biological clock. This was demonstrated in experiments that created ‘islands’ of hypothalamic tissue containing the SCN, whilst

severing all afferent inputs from elsewhere in the brain. Circadian rhythmicity was lost at all brain locations recorded outside the island, but it persisted within the island that contained the SCN. The rhythmicity of the SCN is therefore not dependent on input fibres from outside brain areas (104) (see Figure 1-4).

Figure 1-4: Schematic representation of the discovery of the Suprachiasmatic Nucleus

References used to compile (100-104). SCN suprachiasmatic nucleus, RHT Retino Hypothalamic tract.



1.3.2.2 The discovery of the molecular clock

Whilst it was speculated that the SCN was responsible for biological rhythms, ongoing experimentation aimed to probe the mechanism. Biochemical oscillations were detected and measured outside the brain, but it took some time to prove that the biological rhythms were not simply reflections of the oscillations caused by the SCN.

Disrupting the periods of biological clocks was achieved with inhibitors of protein synthesis (105) and DNA-dependent RNA transcription (106). This constituted the early evidence of the biochemical nature of the pathway involved in a biological clock mechanism. In 1971 initial work done in *Drosophila melanogaster* demonstrated mutations involving a functional gene on the X chromosome, and this was the first evidence of genotype being implicated in SCN controlled circadian rhythms (107). Three mutations were studied that affected the period of both the pupal hatching and the activity rhythm of flies, suggesting a common control mechanism; the term, 'Clock mutant' was coined. Further molecular work with *Drosophila* identified a candidate gene responsible for this phenotype, period (*per*) (108), which also suggested that a circadian rhythm of gene transcription lay at the heart of the oscillator mechanism.

It was not until 1988 when accidentally, a single autosomal locus mutation, which dramatically shortened the period of the circadian locomotor rhythm of hamsters, was found in mammals (109). Almost a decade later the first mammalian clock gene, *clock*

*(*circadian locomotor out-put cycles kaput) was reported in a mouse model when the isolation of this mutation changed the period length and the persistence of rhythmicity (109). Since then, a series of core clock genes have been described which regulate their own expression through a transcription translation oscillating loop (TTO) (see Figure 1-5).

In 2001 the first human clock gene, '*per2*' was described, a human homolog of the period gene in Drosophila which mapped to the same locus (110). Affected individuals were found to have a missense mutation in *hper2* which interferes with casein kinase 1ε (CK1ε) function *in vitro* and manifests as a variant in human sleep behaviour (see 1.4.2).

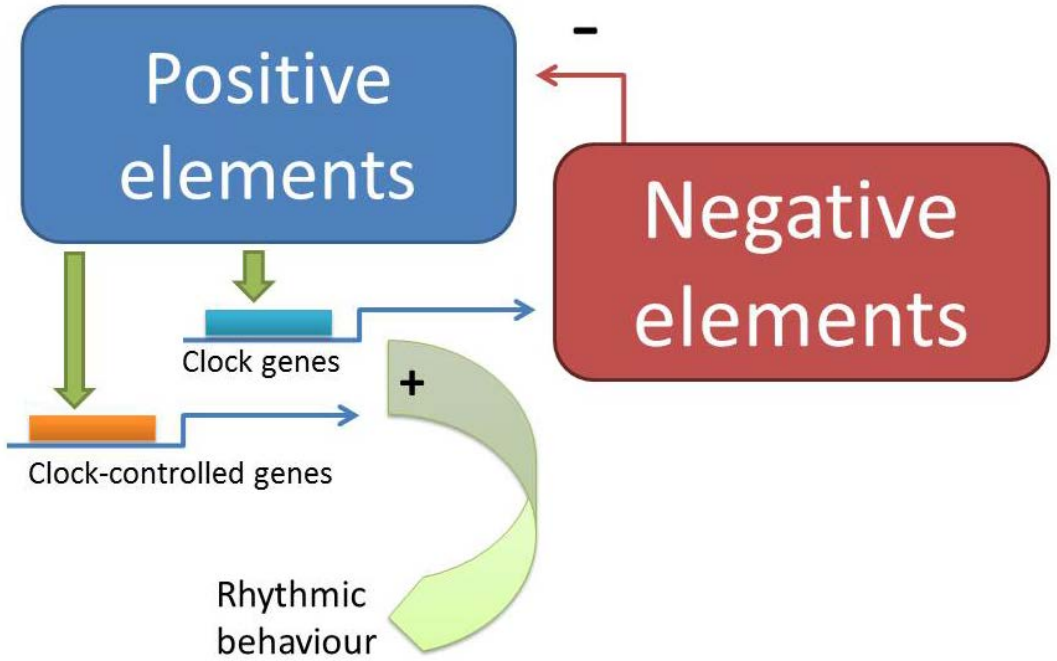
1.4 The molecular clock

1.4.1 The fundamental mammalian molecular clock

It is established that several genes, expressed in neurons of the suprachiasmatic nucleus (SCN), are activated or inhibited in a cyclical pattern and are at the core of the central mechanism for controlling genes that regulate biological rhythms. The actual mechanism by which these molecular oscillations work is via a negative feedback loop, consisting of interacting positive and negative transcription and translation feedback loops and post translational modifications (see Figure 1-5) (111).

Figure 1-5: Common Elements in the Design of Circadian Oscillatory Loops

A schematic representation of how the main components of the core molecular clock interact with the peripheral clock and effect downstream genes as well as system-wide effects. (+ represents a positive effect, - represents a negative effect). Adapted from: Dunlap JC. Molecular basis for circadian clocks. Cell. 1999;96(2):271 90. Epub 1999/02/13.



1.4.1.1 The action of the mammalian molecular cock

In mammals, the transcription factors CLOCK and BMAL1 (brain and muscle ARNT (arylhydrocarbon receptor nuclear translocator)-like protein 1)) form heterodimers which drive rhythmic gene transcription. Both proteins belong to the bHLH (basic helix-loop-helix)/PAS (PER (Period)/ARNT/SIM (single-minded)) family of transcription factors (112). The main target genes are the three *period (per1-3)* and two *cryptochrome (cry1-2)* genes, which are themselves, biological clock components. These genes also code for proteins that too form dimers, and repress CLOCK-BMAL1 transcription; thus completing the negative feedback loop. The core set of clock genes are described in the appendix. In short, in the nucleus CLOCK:BMAL1 proteins dimers bind to E-box elements at the promoter region of the genes *per1-3* and *cry1-2* which induces transcription of their proteins. This transcriptional activation results in the production of mRNA, which exits the nucleus through nuclear pores and is translated into protein by the ribosomes (these can be viewed as positive activators). PER protein is susceptible to degradation unless it forms a dimer.

PER/PER and PER/CRY dimers translocate back into the nucleus. These dimers interact with BMAL1/CLOCK to block activation; so as the levels of PER and CRY proteins rise the production of PER and CRY falls. A negative feedback loop is created as PER and CRY proteins (negative regulators) block transcription by their own genes. Over time, the PER/PER and PER/CRY dimers degrade and eventually too few dimers are available to block activation because no more proteins are being made. Inhibition is relieved, and transcriptional activation of BMAL1/CLOCK begins again.

Mammalian *ck*1ε/δ (*caesin kinase 1*) is a critical regulator of the molecular biological clock. This kinase enzyme is the mammalian equivalent of the *Drosophila* doubletime molecule (see supplementary data). *Ck*1ε/δ in the cytoplasm phosphorylates susceptible PER proteins making them less stable and resulting in their degradation. It also is involved in the translocation of dimers from the cytoplasm to the nucleus. In the nucleus, *ck*1ε/δ plays a role in the degradation of the inhibitory complex of PER and CRY dimers.

The CLOCK:BMAL1 dimers also control the transcription of a second feedback loop which acts in coordination with the BMAL1:CLOCK PER:CRY loop. This involves the orphan nuclear-receptor genes *rev-erb* (α and β) and *ror* (Retinoic acid-related Orphan, α and β) (113). REV-ERB and ROR proteins compete for Retinoic acid-related Orphan receptor Response Element (RORE) binding sites within the E-box promoter of *bmal1*. REV-ERB proteins inhibit, and ROR proteins initiate. The rev-erbs are necessary for normal circadian period regulation (113).

1.4.1.2 The peripheral clock

Individual SCN neurons are circadian clocks with their intrinsic oscillator consisting of a series of interlinked auto regulatory feedback loops (1.4.1). Molecular timekeeping in SCN neurons is synchronised and sustained by signals between neurones. However, a molecular clock mechanism comparable to that of the SCN neurons is present in most major organ systems (43).

As discussed in 1.3.2.1, damage to the SCN renders experimental animals arrhythmic. However, intracerebral grafts of perinatal SCN can restore behavioural circadian rhythms even if the SCN is destroyed (114) (115) and the SCN exhibits circadian rhythms both *in vivo* and in isolated culture as evidenced by their rhythms of electrical activity and gene expression (43).

Clock genes are expressed in many peripheral tissues in a circadian pattern, but they are not merely driven by the SCN has been demonstrated by cells that continue to express circadian rhythms of clock gene expression in long term culture (116, 117). These peripheral clocks are less 'resilient', than the SCN. With prolonged culture the rhythms weaken whereas SCN rhythms continue indefinitely. Individual cells continue to express circadian rhythms but cannot re-synchronise and so the cells drift out of phase from each other (118). Experimental models have used various chemical agents to 'reset' the clock to good effect. The clocks of peripheral cells use essentially the same molecular components as the SCN and show the same effects of circadian mutations (119) but the subtle differences are shown in Table 1-4.

Table 1-4: Differences between central and peripheral clocks in mammals

	SCN	Peripheral Clocks
Resilience (118)	Survive in prolonged culture indefinitely	Will maintain clocks but are less robust
Synchronisation (43)	Able to synchronise and sustain each other via interneuronal, circuit interactions	Cells unable to resynchronise and will drift out of phase unless reset in an artificial setting (e.g. cell culture)
Plasticity (120)	Possibly can an function without the *clock* gene (as other paralogs may compensate in mammals)	Cannot function without *clock, cry1* or *per1*

Thus the circadian clock can be divided into two major components: the SCN as the central clock, and the peripheral clocks. However, the circadian phenotype can be seen from individual neurons, to a tissue level and within whole organ systems.

1.4.2 The link between sleep and the circadian clock

Within neurons in the SCN the molecular changes regulating biological clocks is correlated with the normal sleep-wake cycle (121). Clock genes involved in circadian regulation also partake in sleep regulation (122). Behavioural rest poses less strain on metabolic demand and so circadian timing utilises periods of inactivity to link with availability of nutrients and enzymes. As a result the sleep mechanism is composed of multiple sleep regulatory substances that act over different time periods and comprise of a further complex series of feedback loops (123). This is demonstrated in Figure 1-6 in an ultra-simplified version. The system is complex, probably as a result of its long evolutionary development. The longer acting components, such as the transcription and translation loops of the Clock genes and proteins (left hand side of diagram) allow for 'sleep memory' by recalling previous sleep-wake events. The shorter acting components mediated by the systemic circulation such as prostaglandins, are known to collect during awake time and are more involved in direct sleep promoting activities (124). Because many physiological parameters will vary with sleep, certain criteria should be considered before a substance is deemed to be a sleep-regulatory (124). These are that the substance should;

1. Enhance sleep
2. If inhibited, reduce sleep
3. The level in the brain should vary with sleep propensity
4. It should act on sleep regulatory circuits
5. Be altered in pathological states which affect sleep.

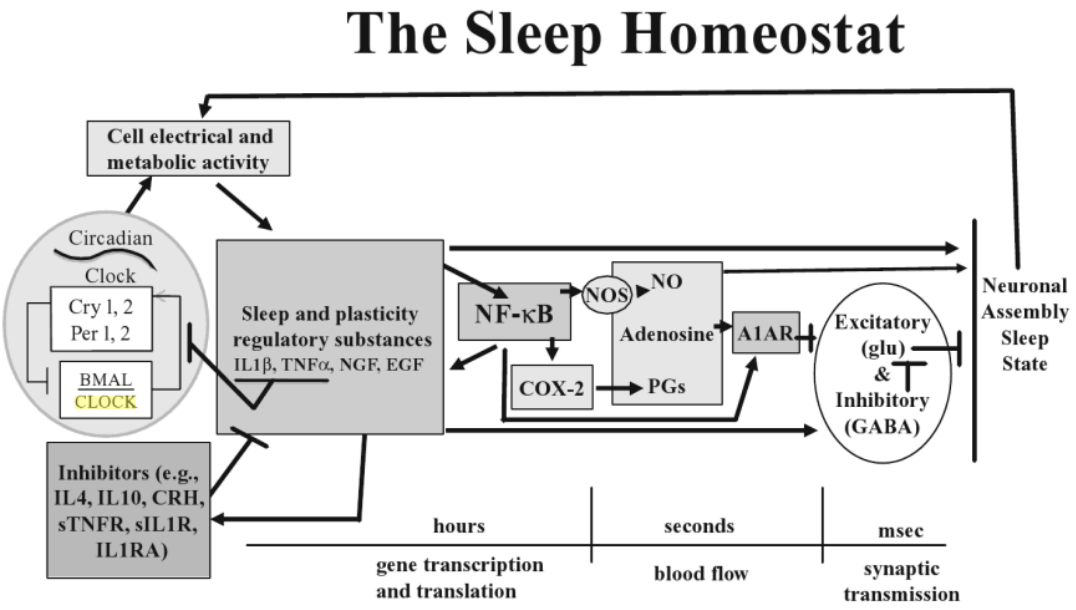
One such group of compounds which does this are cytokines. These are traditionally thought to belong to the immune system as immunomodulatory molecules, but they also have sleep-regulatory roles. Various hormones also affect their production and in this way they can mediate systemic effects (see section 1.1.2.5.3 Melatonin). For example TNF-α, IL1, growth hormone-releasing hormone (GHRH), vasoactive intestinal polypeptide (VIP) and prolactin (PRL), are involved in sleep regulation. These substances enhance sleep, inhibition of them inhibits sleep, and duration and timing of sleep affects their production (125).

The functions of many clock genes and proteins have been demonstrated in Drosophila, hamsters, and other rodents where the functions and structures of these nonhuman

genes are known. The human genome contains structurally similar genes that mimics these 'clocks'. One good example of this concept is the effect of the human period2 (per2) gene. In individuals with familial advanced sleep phase syndrome (FASPS) (a condition whereby individuals have a propensity to sleep and awaken much later than normal subjects such that they are sleep-deprived and find societal functioning difficult), they have a single base-pair mutation. This mutation alters the site in the hPER2 protein through which CK1ε acts (normally resulting in phosphorylation), which marks the protein as a target for degradation. Mutant individuals with prolonged hPER2 action (secondary to reduced degradation of hPER2 and subsequent accumulation in the cytoplasm) have increased levels of hPER2 protein entering the nucleus and restricting hPer2 gene transcription. This results in a shortened sleep cycle and is associated with pathological consequences.

Figure 1-6: The Sleep homeostat

The Sleep homeostat is composed of multiple sleep-regulatory substances that act over different time periods From: Krueger, JM, What exactly is it that sleeps? The evolution, regulation and organisation of an emergent network property in, McNamara P, Barton RA, Nunn CL. (Eds) Evolution of sleep : phylogenetic and functional perspectives. Cambridge: Cambridge University Press; 2010.



1.4.3 The evidence for a link with clock and reproduction

Circadian clock gene polymorphisms have been linked to sleep, mood and metabolic disorders. *Bmal1* and *npas2* (a member of the basic helix-loop-helix (bHLH)-PAS family of transcription factors, which may function as a part of a molecular clock operative in the mammalian forebrain) gene variants, have been associated with reproduction and with seasonal variation. BMAL1 deficiency has been linked to infertility in mice, and

polymorphism of *bmal1* and npas2 have been linked to pregnancy rates and miscarriages genes in humans (126).

At a tissue level, rat USC have undergone microanalysis and it has been shown that 11 implantation-related genes and 24 placenta formation-related genes display significant circadian alterations, suggesting that these genes are controlled by the circadian clock (127). Whilst the molecular machinery of a circadian clock in rat USC *in vitro* (after dexamethasone synchronisation) exists, it is not known if this is the case in humans and whether or not it alters with menstrual cycle phase.

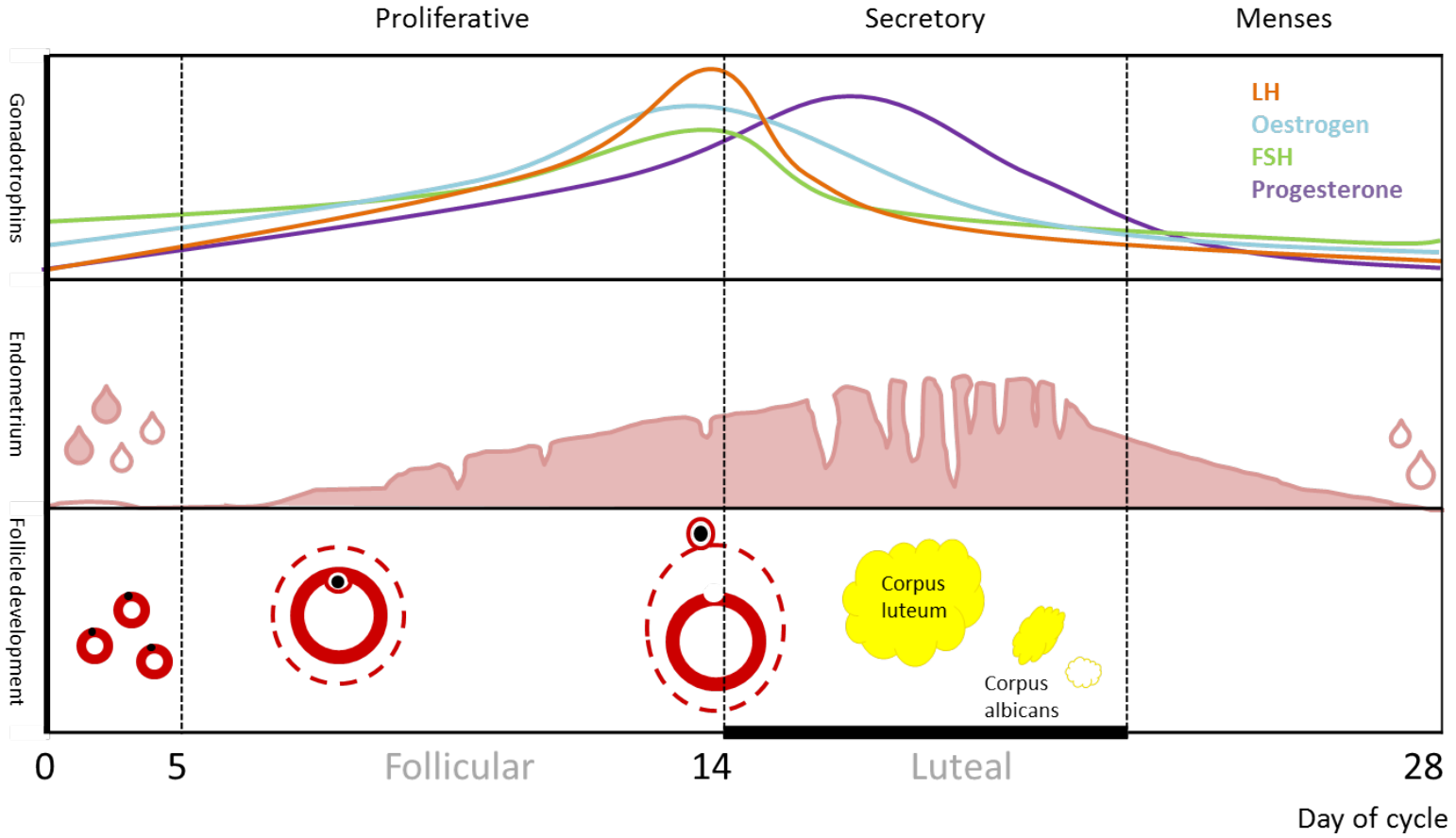
1.5 The biological rhythms of reproduction

1.5.1 Endocrine control

Circadian clocks are found at each level of the Hypothalamic-pituitary-gonadal axis (HPG) axis, and in particular the gonads. This suggests that successful fertility depends in part on synchronisation among these oscillators as otherwise they would be confined to one or a group of particular organs (related to ovulation or implantation in isolation) (see Figure 1-7 and Figure 1-9).

Figure 1-7: The human menstrual cycle

A graphical representation of the human menstrual cycle: The top line represents endocrine changes over the cycle, the middle line demonstrates relative endometrial thickness and the bottom line shows follicular changes. FSH, Follicle stimulating hormone; LH, Luteinising hormone. Time scales (days) is on the X-axis. Picture by LJS.



1.6 Clock genes in the reproductive tract

Many aspects of reproductive function are strongly circadian but the rhythmicity that happens within the reproductive organs themselves is less well understood. The majority of evidence from mammals suggests that female reproductive structures contain circadian clocks and Clock genes are expressed throughout the hypothalamic-pituitary-ovarian (HPO) axis.

Signals from the SCN synchronise the clocks that exist in most peripheral organs. It may be assumed that peripheral clocks contribute to the functions of the reproductive organs and, 'it is likely that such contributions will be highly organ-specific' (128).

1.6.1 The HPO axis

Hypothalamic oscillators affect the timing of reproductive biology. Pituitary gonadotrophins provide the biological timing control of ovulation (see Figure 1-8) (129) and LH secretion, itself driven by neuroendocrine releasing factors controlled by the circadian clock in the SCN. If there are disruptions to this central clock, such as an intrinsic disorder whereby women do not undergo an LH surge, for example in polycystic ovarian syndrome (PCOS), then oestrogen and progesterone do not undergo their normal biological rhythm, and so the endometrium is not synchronised. In other circumstances, such as is associated with the, 'luteal phase defect', the endometrium is retarded both histologically and functionally. Both these patient types will suffer poor reproductive outcomes; manifested by infertility, implantation failure and recurrent miscarriage. Endometrial dysfunction of this nature is also seen in Assisted Reproductive Technologies (ART), where patients undergo premature luteinisation, should they not receive timely progesterone supplementation. Here, the high levels of steroids have disrupted their intrinsic control and the ovarian-endometrial synchrony. In the early days of In Vitro Fertilisation (IVF) attempts at new technologies failed because this important regulator was overlooked. So, too much or too little hormone or regulatory stimulation, either from the pituitary or the ovary seems to dysregulate the synchrony of implantation.

The traditional model of the HPO axis relies on the timing of events being initiated by the pacemaker neurons in the SCN. Sellix et al (128) proposed an alternative scheme which emphasises the existence of circadian oscillators within each component of the axis. Autonomic circadian oscillators are found and produce effects in a variety of peripheral tissues, including in the heart, liver, kidney, lung, spleen, adrenal gland, skeletal muscles (130) (131), uterus (132) (133) and the ovaries . For the system to work, it involves synchronisation between the SCN pacemakers, GnRH neurons,

pituitary cells and ovarian cells (Figure 1-8). These documented clocks in the female reproductive system will be described in this chapter. It is likely that these peripheral clocks complete the commonly cited, 'feedback loop'. The question remaining is where exactly they exist, what and how they control in regards to reproductive functioning and how they network with the rest of the biological systems. Perhaps, most importantly of all is how scientists and clinicians may intervene to control these clocks to bring about molecular and physiological changes that can be clinically beneficial.

Figure 1-8 The Hypothalamic pituitary ovarian axis

Hypothalamic oscillators affect the timing of reproduction: GnRH, Gonadotrophin releasing hormone; FSH, Follicle stimulating hormone, LH, Luteinising hormone. Picture by LJS. Prior to ovulation, the increased oestrogen levels cause an LH surge which leads to rupture and oocyte release: GnRH acts on the anterior pituitary to release gonadotrophins, which in turn act upon the ovary to produce oestrogen (green arrows). Above a threshold value, this negatively feeds back (red arrows) to the pituitary and hypothalamus. Maturing follicles within the ovary also produce oestrogen which leads to a peak concentration. Above threshold levels, oestrogen has a positive feedback effect (green arrow) with a resultant LH surge which causes follicle rupture and ovulation

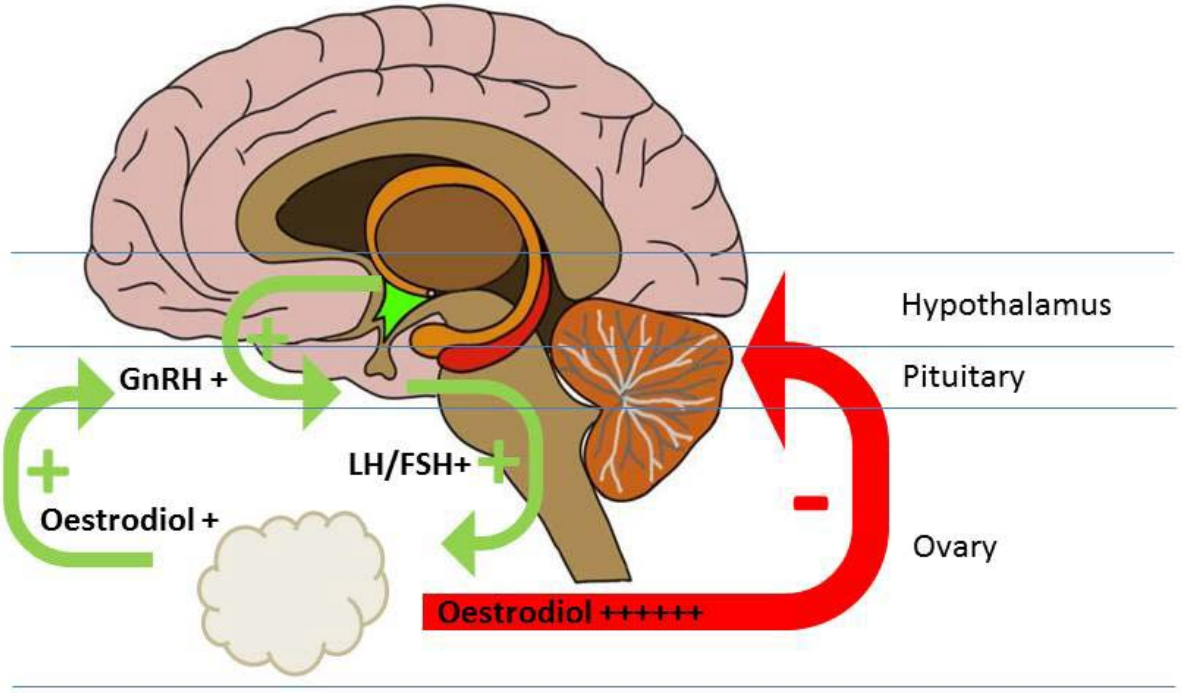
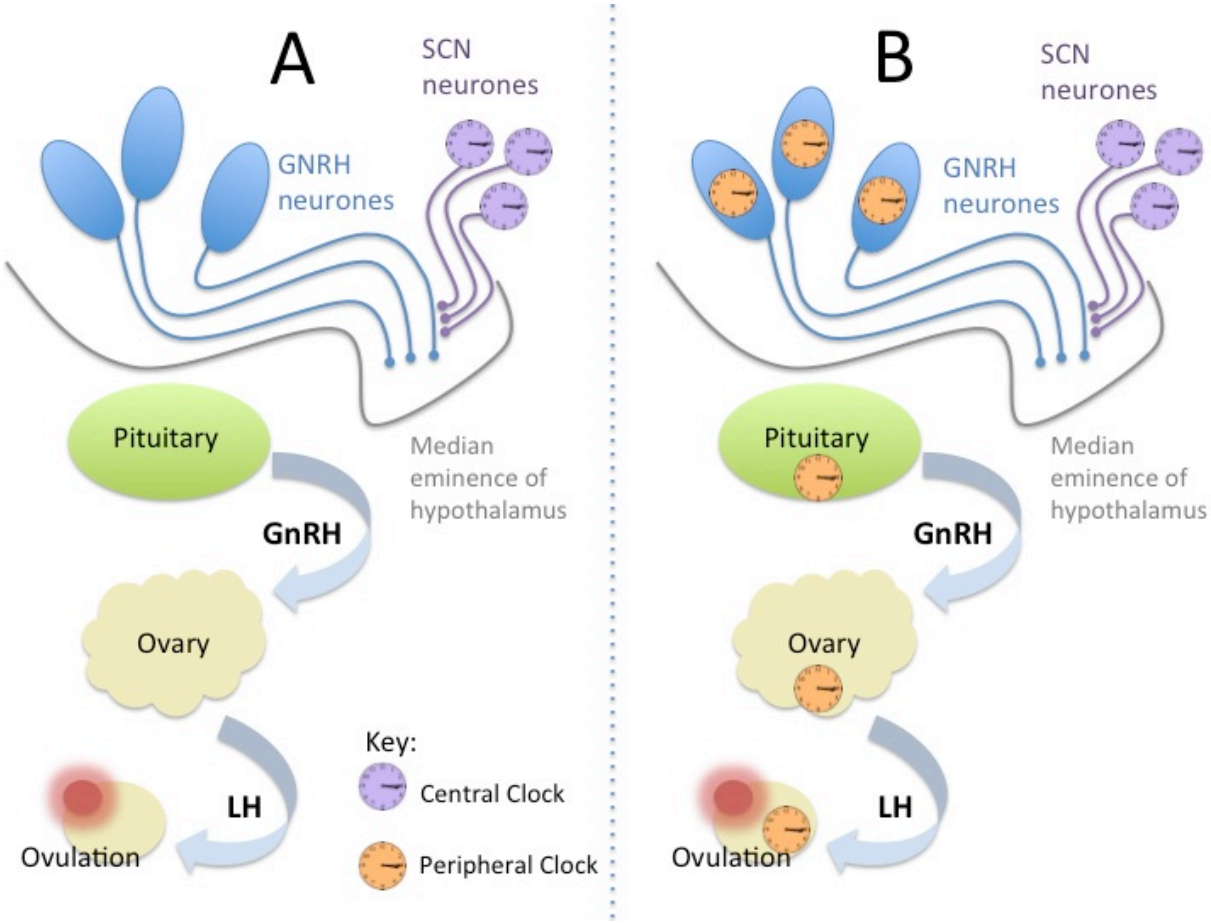


Figure 1-9: Revised version of the HPO axis

According to Sellix et al, artwork by LJS (128) ; Given it is now known peripheral oscillators exist (B), the classic HPO axis (A), is out of date. GnRH, gonadotrophin releasing hormone; LH, luteinising hormone; SCN suprachiasmatic nucleus. Artwork by LJS.



In vivo, the pre ovulatory LH surge initiates a 'cascade' of molecular events resulting in rupture of the follicular wall. This may be seen as an inflammatory reaction because it involves the secretion of enzymes such as cyclooxygenase-2 (COX-2), and prostaglandins (PG) (e.g. prostaglandin E2, and prostaglandin F2α) (134). Sellix and Mennaker 2010 (128) propose that the circadian clock in the ovary and the timing of ovulation could involve an increase in this prostanoid signalling. The activity of COX-2 is the rate-limiting step in PG synthesis. COX-2 transcription is regulated through the E-box DNA binding sequences located in the promoter region; the same E-box which is the target of the CLOCK:BMAL1 dimer) (135). E-box mediated transcription is also part of the activation of COX-2 that happens under the control of LH and FSH as its expression begins to increase in the peri ovulatory period. Therefore Sellix and Mennaker 2010 propose that CLOCK: BMAL1 heterodimers bind to and activate clock controlled transcription. Circadian rhythms of COX-2 mRNA expression may result in rhythmic accumulation of COX2 enzyme and accumulation of PGE2 and PGF2α and increase the amount of LH-inducible prostaglandins. So on the day of ovulation, the COX-2-induced production of PGs facilitates the action of LH surge and follicular rupture leading to ovulation.

In addition, COX-2 and other prostaglandin production may be facilitated by LIF (leukaemia inhibitory factor) which is thought to play a pivotal role in implantation as demonstrated in animal studies (136). It is likely that LIF also is an important regulator of implantation in humans, as a regulator of cell differentiation and cell survival. There is an increased production of LIF in the proliferative to secretory phase in women with RIF and subfertility and has been proposed as a treatment in women with implantation problems (137). ESC have been shown to be responsive to LIF and it is possible that LIF induces PG production through expression of COX-2 and prostaglandin receptors. If the molecular clock does not produce the expected rise in COX-2 and PG synthesis then even if causative, the production of LIF will not be as expected in LIF deficient implantation-failures.

In addition to the process of follicular rupture, recent evidence suggests circadian clock control of steroidogenesis, a facet of ovarian physiology critical for reproductive success. The timing of follicular rupture and steroidogenesis may both be regulated by the circadian clock in the ovary and act together to facilitate the timing of ovulation.

1.6.2 Clocks in the reproductive periphery

The reproductive clock could be seen as several levels - the most important one is the HPO axis, surrounded by the middle the ovary and the endometrium. The ovary and endometrium shall be discussed here.

1.6.2.1 In the ovary

Fahrenkrug et al 2006 (138) first demonstrated the presence of clock genes within mammalian ovaries and subsequent studies have demonstrated that these ovarian clocks may play a role in the timing of ovulation. A summary of the evidence behind a mammalian ovarian clock is shown in Table 1-5. This data has led to studies being designed to investigate whether the level of control of the timing of ovulation is entirely derived centrally from the HPO axis or whether more local regulation also occur

Table 1-5: Clocks in the mammalian ovary

Author	Model	Clock identified	Expression in ovary	Location	Absent expression/with the stimulation of gonadotrophins	Methodology (time points and duration of study)
Fahrenkrug 2006 (138)	Rat	*per 1, per 2*	Peak Per1 at onset of darkness, peak Per 2 midpoint of darkness; persists in constant darkness (regardless of phase of cycle)	Corpus luteum, granulosal, thecal and glandular tissue cells	No expression in the oocyte	RT-qPCR (seven time points over 22 hours(day 2-4 of cycle),10 time points over 20 hours (day 4 of cycle) and 9 time points in 20 hours(constant darkness) depending on stage and treatment of rats), in situ hybridisation and immunohistochemistry at each of the same time points except the latter group.
Karman 2006 (139)	Rat	*bmal1, clock, per 1, per 2, bmal1, cry 1*	Rhythmic expression of Per1 and Per 2 confirmed. Anti-phasic expression of BMAL1 (peak at ZT18). Prior to ovulation, BMAL1 levels are highest.	Corpus luteal, thecal, granulosa and stromal cells and antral follicle	Lack of rhythmicity within the corpus luteal or stromal cells	RT-qPCR and Western blot ((A) in adult animals every four hours over 24 hours, beginning at ZT 6 and ending at ZT 2 the following day, (B) in hypophysectomised animals five time points over 24 hours), imunohistochemistry (single time point) and in situ hybridisation (two time points) and Western blotting (seven time points over 24 hours).
He 2007 (140, 141)	Rat/ culture	*per 1*	Per 1 'constantly abundant'. Becoming cyclical with FSH/LH stimulation	Immature granulosa cells and stimulation in cultured immature and mature granulosa cells	Not able to demonstrate a rhythm in immature granulosa cells but able in luteal cells and mature granulosa cells	RT-qPCR (13 time points over 48 hours) and real-time monitoring of bioluminescence in culture (imaged every 8 minutes for 96 hours).

Author	Model	Clock identified	Expression in ovary	Location	Absent expression/with the stimulation of gonadotrophins	Methodology (time points and duration of study)
Yoshikawa 2009 (142)	Rat/ culture	*per 1*	Per1 rhythmic and remains so after ovarian nerve resection, heterotopic transplant and in culture.	Whole ovary and granulosal cell culture	Phase induced changes can be reset by gonadotrophins in granulosa cell cultures	Period1-luciferase (Per1-luc) transgenic rats, bioluminescence in culture (48 images every 24 h over 12 days).
Johnson 2002 (143)	Mouse	*per1-3, cry1-2, bmal1, clock, timeless*	Circadian genes are expressed in the early pregnant reproductive tract, oocyte and early embryo, but with different rhythms.	Oocytes, uterus, oviduct tissues and pre implantation embryos	N/A	RT-qPCR (single time point of mice on each of the first four days of pregnancy).
Gras 2012 (144)	Rat	*per1, bmal1*	Per1, Bmal1 rhythmic in secondary interstitial cells (and corpus luteum) (gonadotropin-independent)	Pre ovulatory granulosal, theca interna, secondary interstitial and corpus luteal cells	Low/arrhythmic during early gonadotropin-independent follicle development in granulosal, theca interna and secondary interstitial cells and in pre pubertal animals. Rhythmic with stimulation.	RT-qPCR (six time points in prepubertal animals, nine time points in hypophysectomised animals), in situ hybridisation (two and two time points) and immunohistochemistry (six and three time points).
Chu 2012 (145)	Mouse culture	*per 1, per2, bmal1, rev-erb* α	Per 1, Per2, Bmal1, Rev-erb α rhythmic in mature granulosal cells.	Immature and mature granulosa cells (culture)	Absent clock in granulosa cells at immature stage but gained a strong circadian rhythm with FSH. LH reset the clock of matured granulosa cells.	RT-qPCR (12 time points, ignoring the first 14 hours) and real-time monitoring of bioluminescence in culture (over four days).

The ovarian circadian rhythm has been interrogated by removing central control in *in vitro* experiments and by removing the LH surge (hypophysectomy) *in vivo* (140-142). The pre-ovulatory rise in *bmal11* does not occur without central control, but can be reinstated by LH replacement, with a resultant up regulation of *bmal1* and *per2* (141). It would therefore seem that LH is important circadian regulator of the clocks within the ovary. However, only post pubertal and mature granulosa clocks are inducible with gonadotrophins (141, 144, 145).

The roles of the two primary gonadotrophins in ovarian clocks appear to differ slightly. Stimulation with FSH causes initiation of clock rhythms, whereas LH resets the cellular

clock of matured granulosa cells (145). As a result, FSH seems to be to provide a cue for the development of the functional cellular clock in the immature granulosa cells (145) whereas LH would seem to play more of a regulatory role, possibly providing a functional link to androgen and progesterone production. In contrast, the corpus luteum, the main source of progesterone in the early luteal phase, has clocks which are gonadotrophin independent (144). The fact that different rhythmic expression of clock genes in specific ovarian compartments without central hormonal control suggests that environmental light/dark information is transmitted in a gonadotropin-independent way (144).

A circadian ovarian clock has clinically relevant implications; disruption of the clock in ovarian cells, or desynchrony between ovarian and circadian clocks elsewhere in the body may contribute to reproductive pathologies. Whilst there is no definitive evidence to demonstrate the presence of such control in humans, this is an area requiring further investigation. The significance of an internal modulator, dependent on hormonal control, would help explain the relationship between sleep and the secretion of ovarian hormones which has been described in observational studies (146).

1.6.2.2 In the uterus

Rhythmic clock gene expression in the uterus was described in 2002 (see Table 1-6). Subsequent investigations have determined that uterine cells have some element of self-regulation. That is they are not in necessarily in synchrony with other peripheral clocks but there is paucity of information as to the nature of this interaction. However, the process of implantation, embryo development and parturition appears to have some clock dependence (147-150).

Table 1-6: Clocks in the mammalian uterus

Author	Model	Core clock gene identified	Expression in uterus	Location within uterus	Significance and relevance to existing literature	Methodology (time points and duration of study)
Johnson 2002 (143)	Mouse	*per1-3, cry1-2, bmal1, clock,* *timeless*	Circadian genes are expressed in the early pregnant reproductive tract oocyte and early embryo, but with different rhythms	Whole uterus (pregnant)	Demonstration of uterine clock	RT-qPCR (single time point of mice on each of the first four days of pregnancy).
Horard 2004 (151)	Mouse	*bmal1, rev-erbα*	Cyclical rhythms in the uterus (comparable with those expressed in liver)	Whole uterus (non pregnant)	Uterine clock is rhythmic and autonomously functioning.	RT-qPCR (six time points over 24 hours).

Author	Model	Core clock gene identified	Expression in uterus	Location within uterus	Significance and relevance to existing literature	Methodology (time points and duration of study)
Nakamura 2005 (152)	Rats	*per1, per2*	Cyclical rhythms in the uterus; biphasic when with treated with oestrogen.	Whole uterus (non pregnant)	Oestrogen has different effects in uterus compared with other organs (liver/kidney)	Northern blots (6 time points over 20 hours).
Yeh 2005 (153)	Human endometrial cancer cells	*per 1*	Expression (single time point) of all clock genes studied	Sample of uterine specimen (cancerous and normal tissue)	DNA methylation of the *per1* likely to be involved in the early carcinogenesis process	Immunohistochemistry (single time point).
Shih 2005 (154)	Human endometrial cancer cells	*bmal1, clock, cry1, cry2, per1, per2, per3, tim*	Cyclicity all clock genes studied	Sample of uterine specimen (cancerous and normal tissue)	Different gene expression patterns within cancer cell population and possible multiple asynchronised circadian rhythms in the same cancer tissue	RT-qPCR (single time point) and Immunohistochemistry (single time point).
Dolotshad 2006 (132)	Mouse	*per2, cry1 and bmal1*	Cyclical rhythms (anti phase of *cry/per* with *bmal1*)	Whole uterus (pregnant)	Disruption to clock (clock mutants) affects oestrous cycle and increased rates foetal loss when housed in constant darkness	RT-qPCR (six time points over 24 hours)
He 2007 (140)	Rat cultured uterine stromal cells (USC)	*per 2*	Cyclical in USC	USC (pregnant)	Clock irregular in USCs undergoing decidualisation	RT-qPCR (13 time points over 48 hours) and real-time monitoring of bioluminescence in culture (imaged every 8 minutes for 96 hours).
He 2007 (133)	Rat and cultured USC	*per1*	Cyclical in endometrium, myometrium, luminal and glandular epithelium	Endometrium, myometrium, luminal and glandular epithelium (pregnant)	Clock up regulated by oestrogen and progesterone.	RT-qPCR (6 time points over 20 hours) and In situ hybridisation (single time point).
Hirata 2008 (155)	Rat uterine stromal cells USC	*per 2*	Cyclical in immature and mature animals, and in pregnancy. Amplitude strengthened with decidualisation and by the addition of	USC (immature, mature and pregnant)	Uterine *per2* oscillation is under the regulation of ovarian steroids (oestrogen and progesterone) in mature endometrium	Real-time monitoring of bioluminescence in culture (imaged every 8 minutes for 96 hours).

Author	Model	Core clock gene identified	Expression in uterus	Location within uterus	Significance and relevance to existing literature	Methodology (time points and duration of study)
			oestrogen.			
Ratajczak 2010 (148)	Mouse and mouse explants	*bmal1*, *clock*, *cry1*, *cry2*, *per1*, *per2*	*Cry1*cyclical in mouse model, *per2* in uterine explant	Whole uterus (pregnant; during late gestation)	Clock expressed throughout the final third of gestation, is rhythmic and regulated with respect to progression through gestation (peripheral molecular clocks exist in tissues relevant to parturition)	RT-qPCR (nine time points over 20 hours in non-gravid and six time points over 20 hours in gravid uteri) and real-time monitoring of bioluminescence in culture (imaged every 8 minutes for 96 hours).
Akiyama 2010 (156)	Mouse explants	*per1*	*per1* peaks at dusk, maintained in darkness	Whole uterus (non-pregnant, early and late pregnancy)	Absent in foetal derived placenta (so clock signals to foetus must come from mother)	Real-time monitoring of bioluminescence in culture (imaged from 12 to 84 hours).
Uchikaw 2011 (157)	Rat and cultured ESC	*per2*	In contrast to the implantation stage, no significant rhythm of *per2* protein was found during decidualisation.	Whole uterus and ESC (pregnant)	Clocks are down-regulated during decidualisation suggesting that cellular differentiation in USCs interferes with clock	RT-qPCR (six time points over 20 hours), fluorescent immunohistochemistry (two time points over 12 hours), Western blotting (six time points over 20 hours) and real-time monitoring of bioluminescence in culture (imaged for 96 hours).
Isayama 2014 (158)	Bovine USC and uterine epithelial cells	*per1*, *bmal1*	Weak oscillation compared with rodents in ESC and UEC	USC and UES (luteal phase)	*bmal1* and *per1*expression altered by ovarian steroids. Significant expression of clock genes in ESC exposed to progesterone. Clock genes showed no expression without progesterone.	RT-qPCR (nine time points over 24 hours) and real-time monitoring of bioluminescence in culture (imaged for 120 hours).
Muter 2015 (159)	Human endometrial stromal cells (HESC)	*bmal1*, *clock*, *cry1*, *cry2*, *per1*, *per2*	Cyclical expression of all genes investigated. Dampened oscillations on decidualisation.	USC	Loss of *per2* expression silences circadian oscillations in decidualised cells. Inverse correlation between *per2* levels and the number of miscarriages	RT-qPCR (eight time points over 26 hours), Western blot (4 time points over eight days).

Author	Model	Core clock gene identified	Expression in uterus	Location within uterus	Significance and relevance to existing literature	Methodology (time points and duration of study)
Tasakil 2015 (160)	Rat USC	*bmal1 ,per 2, rev-erbα*	*bmal1 ,per2, rev-erbα displayed in cyclical manner*	USC	*bmal1* and *rev-erbα* have decreased expression with decidualisation but circadian rhythm persists. Decreased amplitude of *per2* in the first period decidualisation but not subsequently.	DNA microarray analysis (4 time points over 48 hours), RT-qPCR (four time points over 24 hours), and real-time monitoring of bioluminescence in culture (imaged for 72 hours).
Zhao 2016 (161)	Rat USC	*bmal1, per2*	Cyclical expression of *bmal1* and *per2*	USC from pregnant rats	*bmal1, per2* not detected in decidualised cells	RT-qPCR (4 time points over 48 hours), and real-time monitoring of bioluminescence in culture (imaged for 120 hours).

Clock gene expression in the uterus appears to be affected by the reproductive cycle and stimulation with ovarian steroids (141, 155). This may be unsurprising given the plasticity of the cells within the endometrium and the recognised diurnal variation in gonadotrophins. However, circadian clock gene expression in endometrial stromal cells is impeded during decidualisation in rodents. This is not what is seen in ovarian and testicular tissue, where molecular clock function is linked to differentiation and maturation of cells (157). It is plausible therefore, that the 'silencing' of the clock in uterine stromal cells may be necessary for cellular differentiation, maturation and/or implantation. This 'silencing effect' has been described in an animal study, where circadian clocks are enhanced during implantation and impeded during decidualisation (157).

However, in terms of reproductive potential, destruction of the core clock may disrupt implantation, alters the level of steroid hormone synthesis, compromises fertility and prevents normal implantation in animal models (149, 150). It follows that a function core clock network plays an important regulatory role in gating the implantation process. The endometrium as the site of embryo interaction acts at the maternal-embryo interface and can be seen as the, 'gateway' for implantation (see 1.7.2 The role of the endometrium). There is an obvious dialogue that occurs between the embryo and the endometrium and the timing of their intricate interaction is as yet, not understood. The underlying mechanism could well be due to the molecular clock of the endometrium, the embryo or both.

1.6.3 The malfunctioning reproductive clock hypothesis

The reproductive rhythm is a highly orchestrated process whereby the endometrium develops from a non-receptive to a receptive state. During the follicular phase the thin lining of epithelial cells, a hostile environment for the embryo, is made favourable for implantation by the action of the steroid hormones, oestrogen and progesterone. This is termed decidualisation. The endometrium is described as altering from a proliferative phenotype to a secretory one.

Reproductive disorders may be the result of the lack of synchrony between the ovary and the endometrium. Mismatches in the timing of events within the endometrium and the ovary, could result from an incorrectly timed HPO axis (i.e. hormones and endocrine signals being incongruous). That is, if the timing of the release of steroid hormones is out of sync with the timing of ovulation, implantation may not occur. These processes are also timed by the clock centrally, and potentially peripherally.

During implantation the embryo-endometrial interface must demonstrate synchronicity. It is ultimately important that a good quality embryo is exposed to the well-equipped endometrium, i.e. at the correct time in the menstrual cycle. Such synchrony has been shown to be disrupted in those with RM and RIF (162). Whether this disruption is reversible, i.e. the lack of synchronous timing between an optimally prepared endometrium and the timing of implantation is still being evaluated. Even so, whether this 'resetting' of timings is clinically beneficial is also unknown.

The endometrium is highly responsive to external stimuli, such as endocrine influences. It may also be manipulated to provoke an inflammatory response (163). Both the endocrine and inflammatory environment of the uterus may change on a daily basis. It is plausible that the altered timings of these may have an effect on the success of implantation. Whether the changes occurring monthly within the endometrial menstrual cycle are linked at a molecular clock level is not known. Nor is the phenotype of these women's chronobiology (164, 165).

This thesis therefore aims to:

1) Explore the association between disrupted circadian biology and reproductive outcomes by systematically reviewing the literature assessing the reproductive impact of shift work on early reproductive outcomes.
2) Establish if there is an association between reproductive pathology, namely implantation failure and recurrent miscarriage, and the peripheral clocks within the endometrium through exploring the circadian expression of clock genes,

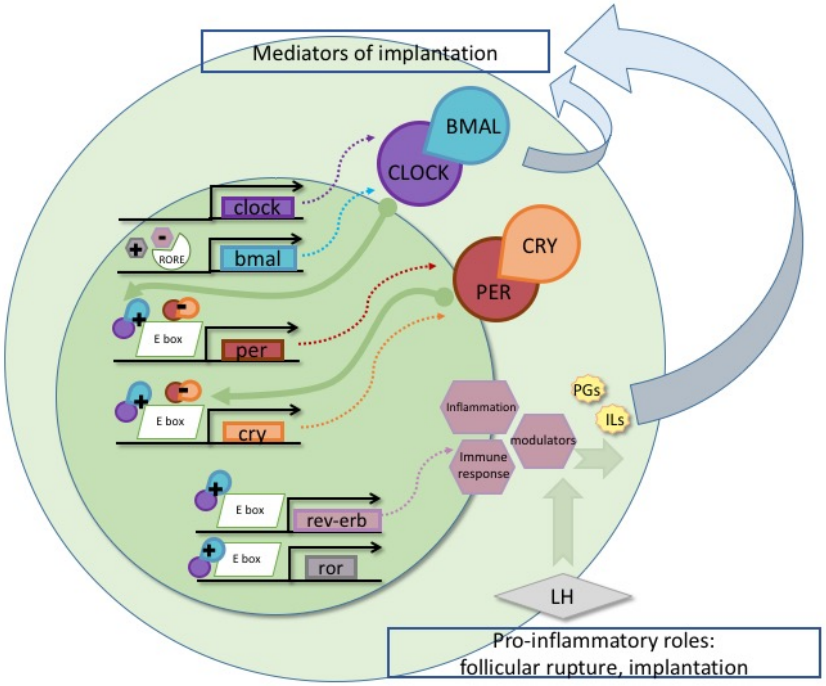
clock controlled mediators in endometrial secretions and the establishment of endometrial stromal cell cultures.

3) Assess whether certain circadian biological characteristics assessed by activity, sleep patterns, demographics and questionnaires are associated with reproductive pathological phenotypes.

Here, a model is proposed whereby malfunction clocks; whether as causative factors or as a downstream product of altered physiology, could be liable for the poor reproductive outcomes seen in clinical practice. This is shown in the diagrams that follow:

Figure 1-10: The peripheral clock: a proposed mechanism

The proposed methodology whereby the peripheral clock regulates peri conceptual and later pregnancy and reproductive outcomes, artwork by LJS. Dotted lines represent movement of mRNA for translation into protein in the cytoplasm. The green arrows represent the trans-nuclear movement of proteins and binding to E-box promoters or Retinoic acid-related Orphan receptor Response Element binding sites (RORE) and having either a positive (+), negative (-) effect on transcription. The large blue arrows represent hypothesized down-stream effects. See 'Abbreviations' section).



In Figure 1-10 some core clock components are drawn with proposed relationships to mediators of reproductive functioning. *Bmal1* and *clock* genes are shown being activated. Following translation, the trans nuclear transfer of protein from the cytoplasm has a positive effect on the E-Box premotor of *per* and *cry*, resulting in the rhythmic expression of *per* and *cry* genes, which then act as repressors, having been phosphorylated by casein kinases (CKε/δ). A new element has been added here, the downstream response of rev-erbs and other core clock components (e.g. NF-κB, COX-2) which may be critical components of the molecular clock. *Rev-erbα* is known to have an important regulatory role in inflammatory responses and may be a key mediator between clock and inflammation. Pro-inflammatory disease processes such as inflammatory lung disease (166), rheumatoid disorders (167), dermatological complaints (168) as well as macrophage response (169) and general clinical conditions associated with chronic and systemic inflammation, such as aging or obesity, show dampened *rev-erbα* gene expression.

The inadequate secretory transformation of the endometrium, resulting from deficient oestrogen and progesterone production, has been implicated in both infertility and recurrent pregnancy loss (170, 171). Figure 1-11aims to show how the endocrine control of the clock circuitry could also contribute to these reproductive pathologies. The role of steroid hormones and the inflammatory response related to implantation can be compared to endometriosis, a chronic pelvic inflammatory condition characterised by increased numbers of activated macrophages (172). Chemokines, whilst involved in many physiological and pathological processes, are thought to be implicated the ectopic ESC invasion seen in endometriosis and the production increased oestrogen is thought to be contributory to this. (173). Oestrogen also supports motility in endometriotic ESCs, promotes extracellular matrix (ECM) degradation and supports invasion. This process is thought to be similar to the events at the embryo-endometrial interface during the process of implantation (174).

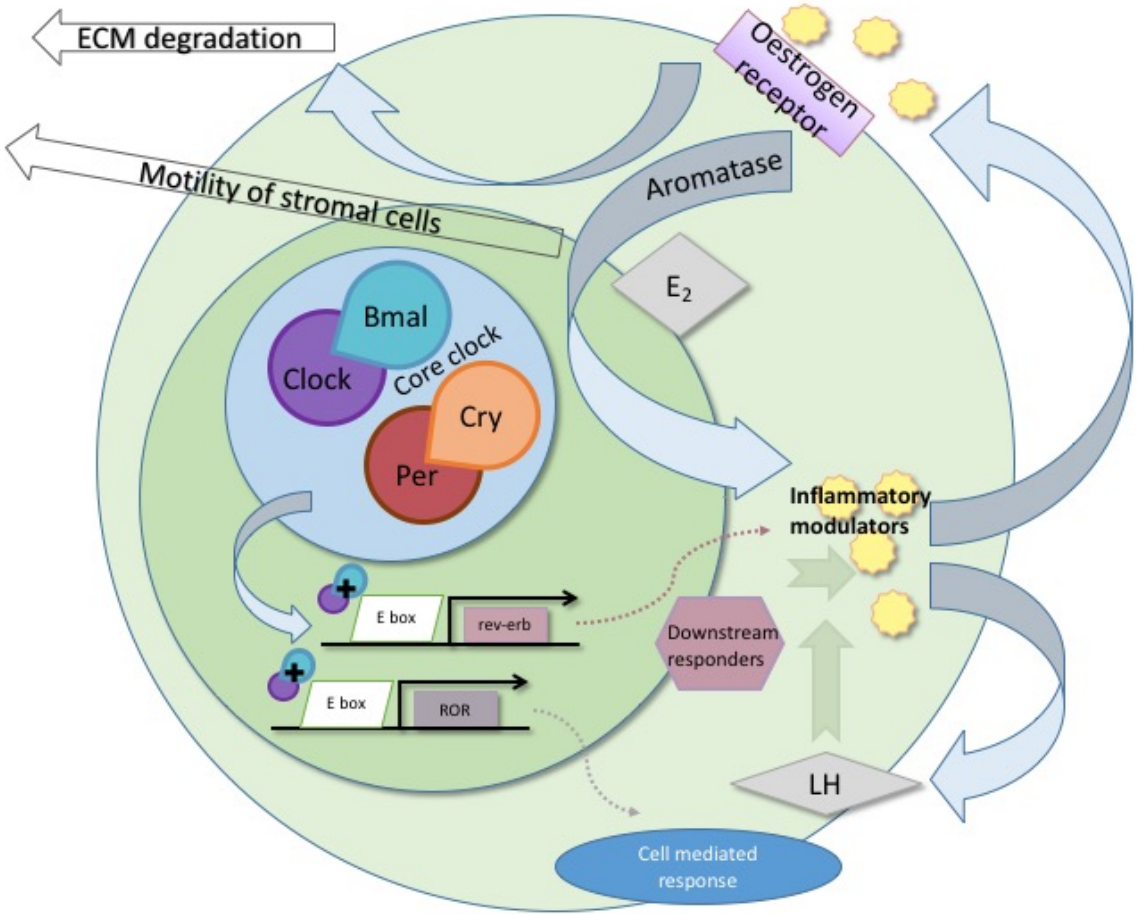
The same basic clock mechanism is shown in Figure 1-11 as in diagram Figure 1-10 but the difference is that the influence of oestrogen can be seen within the context of its possible action upon the up-regulation of pro-inflammatory mediators such as prostaglandins, interleukins, cytokines and other immune-modulators, as well as a proposed role within the clock mechanism. Conversely, clock-mediated effects upon immune modulatory production could mediate effects via steroid membrane receptors. In both the ovary and uterus, hormonal exposure induces expression of various oestrogen receptor subtypes in specific cells and shows a cyclical pattern of expression. Inflammatory mediator expression changes have been demonstrated to occur in a similar pattern to oestrogen receptor up regulation upon gonadotropin stimulation

(175). What this demonstrates is that the link between clock and endometrial function may be inexorably linked, and it would reasonable to assume that reproductive hormones may be upstream and/or downstream of these effects. Although it is important to remember that whilst the central clock can exert effects via down-stream products such as hormones and immune related activities, the peripheral clock may interact with the central clock. The peripheral clock may also exert effects independently (see Clocks in the reproductive periphery). Therefore, should manipulation of the endometrium be undertaken, for example with exogenous hormones it would appear that the central clock may be over-ridden, see Figure 1-11.

.

Figure 1-11: The proposed interactions of the molecular clock

This diagram shows the interactions between systems-wide endocrine control, cytokines and membrane receptors in the cellular clock and how this may be involved in implantation. Artwork by LJS.



1.7 The burden of reproductive disease

1.7.1 Overview

Pregnancy is a unique phenomenon and represents the time from implantation of the embryo until birth. The embryo is formed when the fusion of an oocyte by a spermatozoon which occurs in the fallopian tube at 24-48 hours after ovulation. The onward division of cells form the blastocyst, which enters the uterine cavity on day 4 of development. The outer cells of the blastocyst will form the trophoblast (and ultimately placenta), and the inner cells the embryo. The implantation phase then occurs and can be subdivided into three stages of apposition, adhesion and invasion.

1.7.2 The role of the endometrium

The uterus, a highly dynamic tissue, undergoes profound remodelling and cyclic proliferation and differentiation. During this time, differential gene expression of the endometrial cells relates to the levels of circulating sex steroid hormones (depending on the phase of the ovarian cycle) as well as other external stimuli. An endometrium which permits invasion is vital to the implantation process. This is a complex biochemical process because, in effect the endometrium must facilitate the implantation of a, 'foreign body' in to the underlying maternal tissue.

The reason the human endometrium undergoes hormonal-mediated cyclic changes is in order to support potential pregnancy. In the pre ovulatory phase, oestradiol primes the uterine epithelial cells which in turn up-regulates the expression of oestrogen and progesterone receptors. Following ovulation, the corpus luteum provides progestogenic support and 'decidualises' the endometrium. Decidualisation, that is the exposure of the endometrium to progesterone, involve transformation from a proliferative to a secretory phenotype. The secretory endometrium mediates the interaction of immune cells and vascular remodelling. This results in a receptive state and is termed the, 'window of receptivity', whereby implantation of a potential embryo is facilitated. This is different from rodent models, whereby these changes are only brought about by the presence of a pregnancy.

In humans, little is known about the role of the clock in the endometrium. Core clock genes were first described in human uterine cells in 2005 when a cyclical rhythm of core clock genes (*bmal1, clock, cry1, cry2, per1, per2*) was noted in women with endometrial cancer (153). The demonstration of different gene expression patterns within cancer cell population compared with normal cell populations, and possible multiple asynchronised circadian rhythms in the aberrant cancer tissue is an indication

that disrupted cellular clock could play a role in the clinical behaviours of the endometrium. Whilst rodent studies first examined the role of decidualisation in endometrial clock genes, *bmal1, clock, cry1, cry2, per1, per2* were shown to have dampened oscillations upon decidualisation in the first in vitro study of the progesterone effect on human endometrial stromal cells. This study also found that loss of per2 expression silences circadian oscillations in decidualised cells. Subsequent studies have disagreed on the effect of decidualisation of the stromal cells with some studies claiming dampening or loss of circadian rhythms after the addition of progesterone, whereas some claim up-regulation of certain genes (but these have all been in animal models).

1.7.3 Adverse reproductive outcomes

Whilst the basics of implantation are fairly well understood, many factors influencing implantation and the continuation of pregnancy still remain elusive. For example, the viability of an embryo is determined only at the advent of pregnancy despite rigorous selection criteria during Assisted Reproductive Technology (ART). What happens during the remodelling of the endometrium in the peri-implantation phase remains poorly understood. It is not clear which maternal or embryonic factors regulate adaptation of the maternal immune system to be able to allow pregnancy to occur. The focus of this study will focus on this peri-implantation period and the two key pathologies of interest are briefly described below, but are also described in further detail during relevant parts of the text.

1.7.4 Implantation failure

When no pregnancy is reached after the transfer of a good quality embryo during ART, implantation failure has occurred. This also happens in natural cycles but is difficult to quantify. The generally accepted rate of implantation during ART is between 30-50%. For all of these patients wishing to achieve pregnancy, this is extremely frustrating. Discriminating between those with reproductive pathology from those without, has proved challenging to the practising ART doctor. Multiple aetiologies for implantation failure have been proposed but until recently, the primary focus has been on the embryo (176). More recently the endometrium has become the focus and is thought to have a key role in implantation, with even a possible role in differentiating between good and poor quality embryos and responding appropriately (177).

Foetal clocks are known to develop early in the early embryo, and are probably expressed in the oocyte. These persist until late development in embryonic and foetal organs. Embryonic peripheral circadian clocks develop much earlier than the SCN.

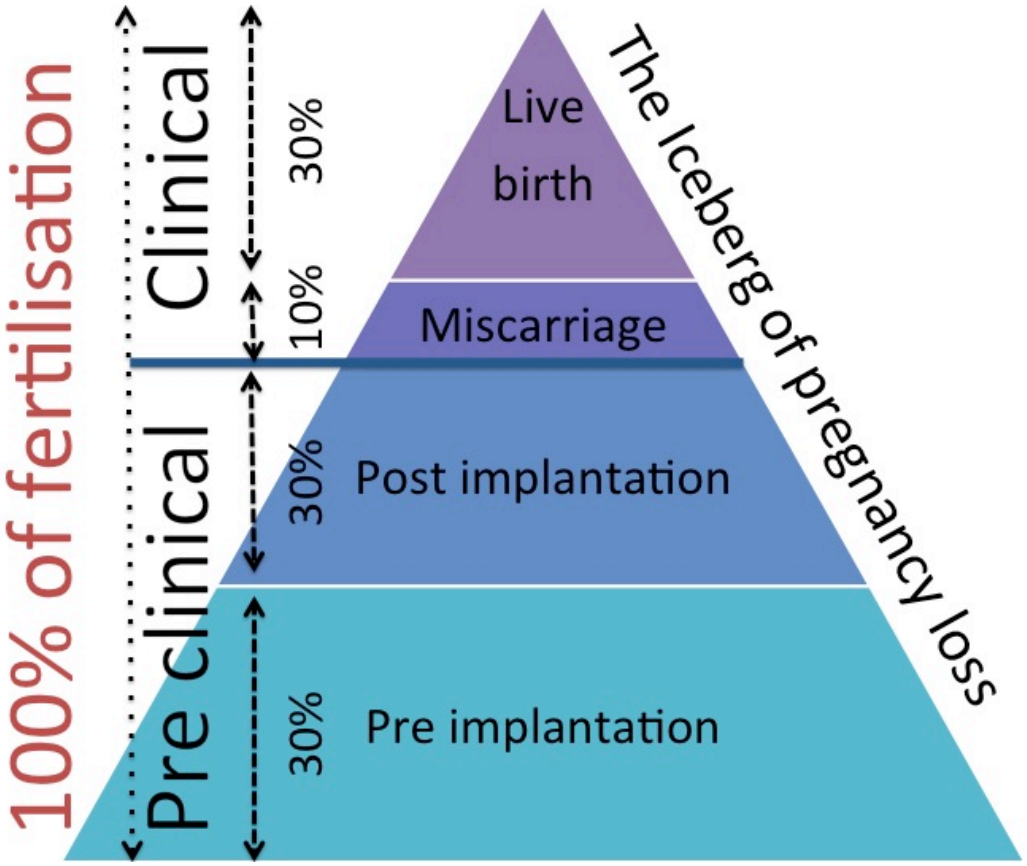
Experiments in rodents have demonstrated that within a litter, synchrony is lost between the pups after maternal SCN ablation (178). Rhythms also persist, but are not synchronised, in heterozygote foetuses of arrhythmic mothers (179). It therefore follows that an independent pathway allows the development of circadian rhythmicity in the foetus, but there is central control by the maternal clocks. Whether this is from self-sustaining clocks in the uterus and endometrium, or come directly from the SCN is unclear. The ability of the maternal interface to select a 'good' or 'bad' embryo may be in part related to the synchrony of clocks between mother and foetus. Where the clock in the uterus is out of sync, either with maternal or embryonic central clock signals, it may render the endometrium impenetrable to implantation by an otherwise good quality embryo.

1.7.5 Pregnancy loss

The relatively high rate of pregnancy loss in humans is thought to be because humans have developed the capacity to become expert in discriminating between good and poor quality embryos. However, if the maternal selection process is too rigorous this can lead to pathology and either the repeated loss of clinical pregnancy (recurrent miscarriage) or the failure to conceive due to pre-clinical pregnancy loss. As described above, this may be because the endometrium is too selective. However, in recurrent miscarriage it may be because the endometrium is not selective enough. This is the so-called iceberg of pregnancy loss, see Figure 1-12. It is estimated that 70% of conceptions are lost prior to live birth (180). The majority of these losses occur prior to implantation or before the missed menstrual period, and since they are not revealed to the woman they are termed preclinical. In the pregnancy loss 'iceberg', they are therefore below the 'waterline'.

Figure 1-12: The iceberg of pregnancy loss

Only 40% of clinical pregnancies are witnessed by the patient. The 'post implantation' losses are demonstrated in, 'biochemical pregnancies', whereby the βHCG level rises, but the patient never has clinical pregnancy demonstrated by imaging. Adapted from Larsen EC, Christiansen OB, Kolte AM, Macklon N. New insights into mechanisms behind miscarriage. BMC medicine 2013;11:154 (181)



It has been hypothesised that because more metabolically active embryos are seen as, 'noisy' to the endometrium, it is this, as much as their quality that can alert the endometrium to resist implantation (162). If recurrent miscarriage (RM), is not driven by the underlying quality of the embryo, the endometrial cells may instead be pathologically 'attracted' to embryos that will result in a poor pregnancy outcome. These pregnancies then miscarry as the pregnancy progresses. If the clock of the embryo is out-of-sync with the clock of the endometrium, for example because of a disease state within the developing foetus, the embryo may 'appear' falsely attractive for implantation.

Alternatively, a disrupted clock in the endometrium may be inappropriately able to allow implantation (of either good or poor quality embryos) but not facilitate successful placentation. It has been shown that there is an Inverse correlation between PER2 transcript levels in the endometrium and the number of miscarriages in decidualised cells, i.e. those receptive to implantation (159). This suggests perhaps that the endometrium having a faulty clock is in part responsible for successful embryo selection.

1.7.6 Uterine and embryonic synchrony

The data available raise the possibility that the embryonic SCN and foetal tissues may be peripheral clocks commanded by separate maternal signals. If the maternally signalling is incorrect, either from the central or peripheral clock, then reproductive functioning may not occur. The methodological and conceptual purpose of this thesis is to further explore how the reproductive clocks in human female reproduction may have a role in successful early reproductive outcomes.

Chapter 2 Shift Workers and early reproductive outcomes

2.1 Shift working

2.1.1 The biological impact of sleep deprivation

Despite a likely positive reporting bias, sleep deprivation has been associated with numerous adverse manifestations and disease processes, in the short and long term (182). However the frequent claims as to the negative implications of sleep deprivation are also driven by social and economic pressures.

Short-term lack of sleep is demonstrated by limited periods of insomnia or deliberate sleep deprivation for periods of more than 24 hours. It decreases cognitive ability short term memory and emotional lability (183, 184). Some of the most commonly reported effects are on altered metabolism, diabetes, obesity and metabolic syndrome, cardiovascular disease and cancer (185-188). These are high impact diseases leading causes of mortality in the developed world. In addition, there are also associations made with oxidative stress, psychological dysfunction, neurodegenerative disease, as well as negative reproductive outcomes (189-191).

2.1.2 Shift workers

In humans the long-term effect of altering sleeping patterns is inherently difficult to study. However as a proxy measure, the chrono disruption demonstrated by shift workers creates short and long term physiological disturbances. Altered psychological, social and biological functioning results, posing risk factors for disease including cardiovascular pathology (192), metabolic disturbances and cancer (193).

The underlying pathophysiology of such conditions is still not clearly understood. Much work in this area has included healthcare, factory and airline staff as people who exemplify a range of poor sleep hygiene over prolonged periods of time. Fortunately these studies include a relatively heterogeneous group of people, thus including various ethnic groups, education levels and ages and as a result limiting confounding variables from a potentially narrow range subjects.

Shift workers have activity levels that are de-synchronised from their circadian rhythm. In addition, because individual biological rhythms will restore themselves at different rates, shift workers who work altering shifts or who are frequently having to re-adjust

their clock, will have a lag time whereby the biological rhythms may become further disrupted. For example, the sleep-wake cycle is more readily restored than the body temperature circadian rhythm (194). All circadian rhythms will be more easily restored after a time shift, if all the important zeitgebers are synchronously shifted such as occurs with trans-meridian flights. Therefore airline industry workers, who are included in these studies, may have fewer disruptive physiological manifestations than ordinary night-workers. However, overall shift workers never properly adapt their biological rhythms to night work (195).

Whilst the mechanisms behind this are largely unknown the greatest physiological problem is sleep alteration. Non-circadian activity encourages poor sleep hygiene and sleep deprivation. Internal timekeeping is maintained in 24-hour oscillations despite out-of-synchronicity with the external environment (102) unless long term circadian disruption persists (196). Exogenous alterations to these tightly entrained processes are produced by altered light and working schedule (197, 198).

2.1.2.1 Female shift work and pathology

Women account for almost half the working population and the majority are of reproductive age (199, 200). Shift work occurs across occupations and social classes (199) with almost 20% of women performing duties outside standard hours (199).

The hypothalamic-pituitary axis is under circadian control and affects timing of ovulation and hormone secretion (147). Deregulating circadian rhythms by inappropriate light exposure or manipulating the body clock at a molecular level, negatively affects implantation and pregnancy success in animals (201, 202). If reliant on circadian timings, early reproductive outcomes may be disrupted by shift work.

Reviews have associated shift work with increased risks of preterm birth, low birth weight and foetal loss (203, 204) but less work has examined evidence surrounding early reproductive outcomes and the impact is still largely unknown.

2.2 Methods

2.2.1 Objective

The objective was to determine whether there is an association between shift work (any work outside 08:00–18:00) and the early reproductive outcomes of menstrual cycle disruption (<25 days or >31 days), infertility (a time-to-pregnancy interval exceeding 12 months) and spontaneous loss of pregnancy before 25 weeks.

2.2.2 Sources

A systematic review was performed (October 2012-July 2013) according to a predetermined protocol and reported in accordance with Meta-analysis Of Observational Studies In Epidemiology (205) (see supplementary data). Data generation was consistently performed independently by two assessors (LJS, YCC). This included searching, data selection, extraction and grading), with discrepancies resolved by arbitration.

All human studies that may have described shift work and menstrual disorders, infertility and pregnancy loss until July 2013 were eligible with no restriction on publication date, study design status or language (initially search was limited to English language, but a subsequent unrestricted search was performed). Authors of published studies were contacted for clarification of specifics where necessary. No formal attempt was made to retrieve unpublished data.

Electronic database searches were performed and all titles/abstracts of returned articles were reviewed. References were hand searched and experts were contacted to obtain additional data where required. Search terms were combined with Boolean logic and special features were used to identify synonyms and broaden the search. Keywords included 'work schedule tolerance', 'shift work', 'fertility', 'reproduction', 'menstruation', 'spontaneous abortion', 'pregnancy', 'pregnancy loss', 'infertility' and 'fecundity' (see supplementary data for an example search string).

2.2.3 Study selection

Studies reporting on women from any occupation were included. Shift work was defined as any work executed outside normal working hours (08:00-18:00). This included rotating, mixed or fixed shift patterns and not women who only worked occasionally outside working hours. A description of shift terms was created (206) and authors' definition of shift was recoded. The comparison group were women with normal working hours or who did not work. The number of subjects in each group had to be known.

Outcomes had to occur at the same time as shiftwork was undertaken and were defined as

1. Menstrual disruption: A short (< 25 days) or long cycle (>31 days) (207), excluding studies describing 'irregularity' or changes in cycle length. Metrorrhagia was excluded as it is often associated with an underlying structural pathology, while variation in cycle length is more commonly associated with infertility.

2. Infertility: Time-to-pregnancy interval exceeding 12 months (208) (studies reporting number of menstrual cycles had to be quantified in months).

3: Early spontaneous pregnancy loss: Spontaneous loss of pregnancy before 24 completed weeks (209). Data was included if the definition of early spontaneous pregnancy loss was fewer, but not greater than 24 completed weeks of pregnancy. Terminations of pregnancy were excluded.

All studies were screened, reviewing full papers where required and disregarding those ineligible. Authors were contacted if it was impossible to extract or calculate numbers from the data and where fruitless, these were excluded the study. Compliant subsets of data were included from larger series.

Data was extracted with a template adapted from the Cochrane Collaboration (see supplementary data) (210). Authorships and data sources were scrutinised to prevent data duplication. Original numbers were extracted for consistency of data handling. Where available, adjusted effect measures (odds ratios, relative risks and risk ratios with 95%CI or p values) were recorded.

Studies were rated for quality using the Newcastle-Ottawa scale (211), as recommended by the Cochrane Collaboration (210) (see supplementary data). Points were awarded in three domains: study group selection, comparability of the groups and ascertainment of outcome (maximum scores of four, two and three respectively). Using this scoring system, studies were graded low, intermediate or high quality by the percentage of possible points scored (1-33%, 34-66%, 67-100% respectively). Low-quality studies and those not achieving at least one point in each domain were excluded.

Review Manager (an Information Management System) Version 5.2. (RevMan computer program, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012) was used to combine studies, calculating odds ratios (OR) and 95%CIs for individual study results. Meta analyses were performed using fixed- (Mantel-Haenszel) (212) and random-effects methods (DerSimonian and Laird) (213) reporting both when conclusions differed without substantial heterogeneity. In the adjusted analyses, pooled multivariable adjusted risk estimates were preferentially used. Units of analysis were number of women (menstrual disruption and infertility) or pregnancies (miscarriage).

Heterogeneity was measured with the I^2 test, a validated test measuring consistency, was used to identify inter-study variation with results denoting low (<25%), medium

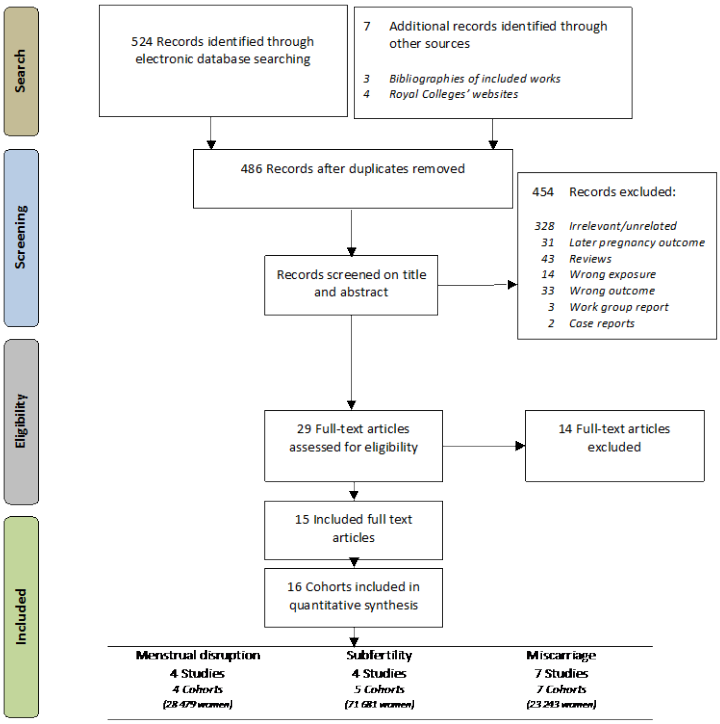
(25-50%), high (50%-74%), or considerable (>75%) heterogeneity (210, 214). In cases of high/considerable heterogeneity, random-effects analyses were reported.

As night shifts have been hypothesised to produce poorer outcomes (206) Predetermined subgroup analyses on women only working nights was performed . Night shifts were any shift stated by the author as such (see supplementary data). Pooled estimates were subjected to influence-analyses of study design, size and confounders to confirm robustness and identify sources of heterogeneity/bias. If adjusted data were available the natural logarithms of the effect measure was calculated with corresponding standard errors; otherwise the unadjusted estimate was included. Pooled adjusted and unadjusted data were weighted for the inverse of the variance.

2.3 Results

Of 524 screened studies (517 journal articles, 7 reports), 454 were disregarded on the information or data provided in the title and abstract (see Figure 2-1). 29 articles were considered for analysis, of which 13 met the eligibility criteria (215-227). One further study was able to be included following communication with the authors (228). The 15 included and excluded studies can be seen in supplementary data. All data were dichotomous.

Figure 2-1: A flow diagram to show selection process



All 15 included cohorts had a parallel design: 8 cross sectional (215, 217-220, 223, 228, 229), 2 case control (226, 227) and 5 cohort studies (three prospective (216, 225, 230) two retrospective (221, 224)). 12 countries were represented (largely USA and Europe) although two were from Taiwan and one Thailand (see supplementary data) shows the characteristics of the 123,403 included women; 71,681 from studies reporting on menstrual disturbances, 28,479 from studies on infertility and 23,243 women on early spontaneous pregnancy loss (from 23,604 pregnancies).

One infertility study used two populations of women; a 'pregnancy' (a group of women who had achieved pregnancy and were retrospectively asked about time to fertility) and a 'population' group (a group of shift workers who may or may not have achieved pregnancy) (218). There were significant differences in each arm of the study design so the data was treated as two entities (Bisanti 1996 (218)(1) 'population group' and

Bisanti 1996 (2) 'pregnancy group'), hereafter referred to separately and bringing the number of included cohorts to 16.

Nine of the 15 included studies had a primary objective of assessing the effect of shift work on early reproductive outcomes (217, 218, 220-223, 225, 228) ((218)1&2). Five examined shifts as one of various work-related risks (215, 216, 224, 226, 227, 230) and one as subgroup analyses (219) (supplementary data). Employed women included nurses, midwives, factory and office workers and were the comparison group in 11 cohorts (supplementary data). Eight studies matched for occupation using women who worked exclusively during the normal working day as the comparison group (215, 216, 222-225, 227, 228). Of the three unmatched cohorts, one compared office workers working only during normal working hours and factory employees working shifts (217) and two used mixed occupations (221, 226).

Four studies (218-220, 230) ((218) 1&2) combined working and non-working women as comparison groups. No studies were rejected due to poor quality. 14 out of 15 (215-222, 224-228)(1) were of high and two of medium quality (218, 223)(2) (for scoring see supplementary data, and see 2.2.3 for definition of quality). All studies scored 50% or above for 'selection'. Blinding was not possible for the case-control studies. Performance bias was most likely in studies including non-working women in comparison groups (218-220, 230) (27 1&2), but was otherwise low.

Despite efforts to contact authors who may have had includable data, not all were contactable so data was excluded (231, 232) (a potential n=579, 2% of the total women in the infertility group, and n=698, 4% in the early spontaneous pregnancy loss group). The response rate and participation rate of women in the included studies was high. Most studies had response rates of 75-84% (n=9). Three studies had rates of $\geq$85% and two studies of 50%-74%. The multi-country cohorts reported ranges of 54-88% (218)(1) and 70-98% (218)(2). Three early spontaneous pregnancy loss studies ascertained differences between the outcomes of women who responded (222, 226, 227) with higher rates (88.4% vs 81.4%) (226) from women not miscarrying, although similar complete data (87.8% vs 87.1%) (227). There were higher early spontaneous pregnancy loss rates in the non-responders (222) (11.3% vs 9.9%) suggesting that women suffering poor outcome were less likely to engage in the studies. Studies involving menstrual disruption and subfertility did not divide the results of response rate between affected and unaffected women so this comparison is not possible.

Outcome was determined by telephone (221), face-to-face interviews (218-220)(1), diary keeping (215, 216), data directly from medical records/discharge registers (226, 227, 230) or written questionnaire (218, 223-225, 228)(2). One study used monthly

instalments (217) and one checked medical records (222). The minority relied upon written recall alone, minimising detection bias.

Exposure to shift work was assessed by questionnaire in 14 studies. One asked employers to complete this information (227), and two confirmed the participant-provided information by referencing employment records (222), and asking women to keep a contemporaneous diary (215), thus minimising reporting bias. Those studies assessing working pattern by recall alone used written questionnaires (216-218, 223-225, 228)(2), face-to-face interviews (218-220)(1), telephone interview (221, 226, 230) or a combination of diaries and written questionnaires (215) (see supplementary data).

Publication bias (the tendency towards publication of a positive association) was not formally measured nor meta-regression performed because the number of studies (fewer than 10) for comparison within each outcome was small (210).

Three out of four menstrual disruption studies excluded women using hormonal contraception or endocrine disturbances (216, 217, 228). One study made no mention of this in their methods, potentially including women with endocrine manipulation or pathology (215). Four out of five fertility studies (218-221) (2) included only women who had conceived a pregnancy, not accounting for women who were infertile. Three out of six early spontaneous pregnancy loss studies excluded terminations, ectopic and multiple pregnancies (224, 225, 230). One study excluded women with recurrent early spontaneous pregnancy loss (223) and one with pregnancies affected by congenital malformations (227), slightly altering total pregnancy rates.

Four pooled studies (215, 216, 221, 224) showed an un weighted rate of menstrual disruption of between 13.4% (955/71,077)(228) and 40.0% (130/329)(217). Raw data showing an increased rate of menstrual disruption in shift workers (2270/13749 shift-working women, 16.05%) compared with women not working shifts (7561/57932 non shift-working women, 13.05%) supported the unadjusted analysis (OR 1.22 95%CI 1.15-1.29, I^2 0%) (Figure 2-2).

Regarding menstrual disruption the effect of night shift work was non-significant in the unadjusted subgroup of women who worked nights (10 of 28 night working women, 35.71%) compared with women who did not work shifts (39 of 155 non shift working women, 25.16%). However, heterogeneity (the percentage of variance in a meta-analysis that is attributable to study heterogeneity) was substantial and the analysis included only small numbers (n=183, OR 1.72, 95%CI 0.33-8.95, I^2 69%) (Figure 2-3).

Five cohorts showed an un weighted infertility rate of between 10.5% (295 of 3,092)(218)(1) to 16.3% (135 of 907)(220). Women working shifts had higher rates of

infertility (529 of 4,668, 11.3%) than women who did not (2,354 of 23,811, 9.9%) (OR 1.80 95%CI 1.01-3.20, I^2 94%) with considerable heterogeneity (Figure 2-4). Only one cohort (218) (1) included women who had not managed to conceive a pregnancy (n = 3,092 OR 2.54 95%CI 1.73-3.74).

Only one infertility study provided data for a mutually exclusive group of night shift workers so subgroup analysis assessing the effect of night shifts on infertility was not possible. There were higher rates of infertility in night shift workers compared with women not working shifts (29 of 177, 16.6% vs 1,797 of 17,531, 10.3% crude OR 1.72, 95%CI 1.15-2.56).

The combined data from seven studies showed an un weighted rate of early spontaneous pregnancy loss of between 10.1% (410 of 4051)(230) and 35.4% (432 of 1,341)(226) with no difference in early spontaneous pregnancy loss rates in shift workers (939 of 7,931, 11.84%) vs nonshift workers (1,898 of 15,673, 12.11%) (OR 0.96, 95%CI 0.88-1.05, I^2 0%) (Figure 2-5).

Figure 2-2: Comparison of shift work with non-shift work: outcome menstrual cycle disruption (unadjusted)

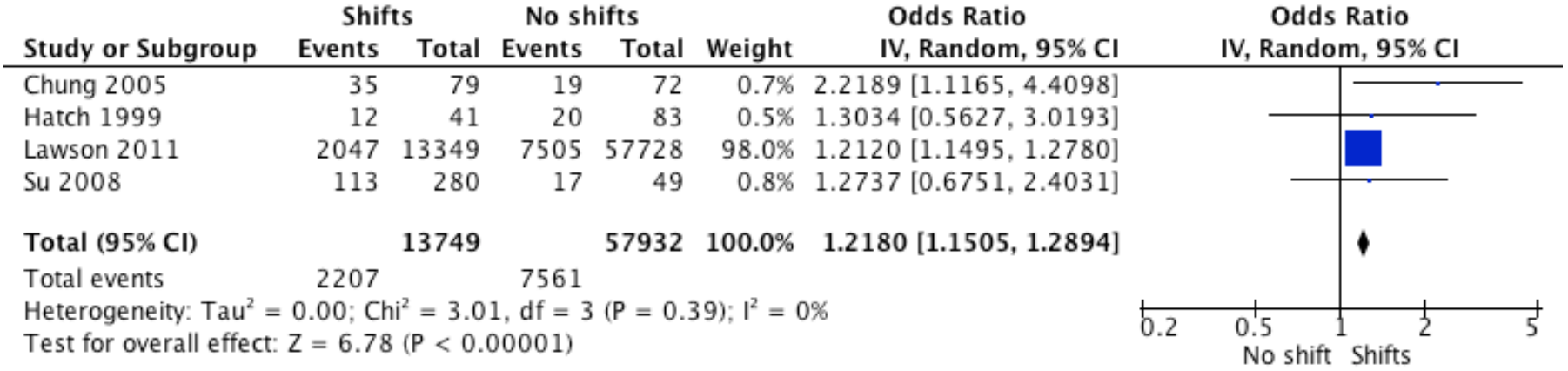


Figure 2-3: Comparison of night work with non-shift work: outcome menstrual cycle disruption)

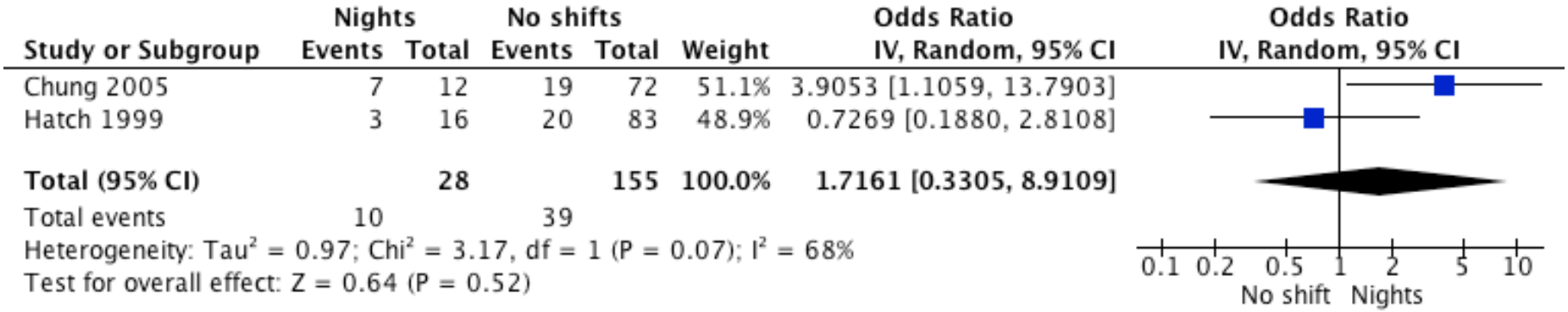


Figure 2-4: Comparison of shift work with non-shift work: outcome infertility (unadjusted)

Study or Subgroup	Shifts Events	Shifts Total	No shifts Events	No shifts Total	Weight	Odds Ratio IV, Random, 95% CI
Bisanti 1996 (1)	36	181	259	2911	20.2%	2.5422 [1.7270, 3.7421]
Bisanti 1996 (2)	44	211	156	2209	20.4%	3.4674 [2.3953, 5.0192]
Spinelli 1997	12	67	58	555	17.0%	1.8696 [0.9461, 3.6945]
Tuntiseranee 1998	51	302	84	605	20.3%	1.2602 [0.8626, 1.8412]
Zhu 2003	386	3907	1797	17531	22.1%	0.9599 [0.8549, 1.0778]
Total (95% CI)		**4668**		**23811**	**100.0%**	**1.7977 [1.0133, 3.1894]**
Total events	529		2354			

Heterogeneity: $Tau^2 = 0.38$; $Chi^2 = 61.80$, df = 4 ($P < 0.00001$); $I^2 = 94\%$

Test for overall effect: $Z = 2.01$ ($P = 0.04$)

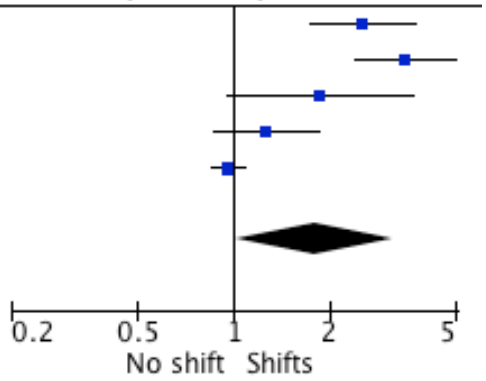
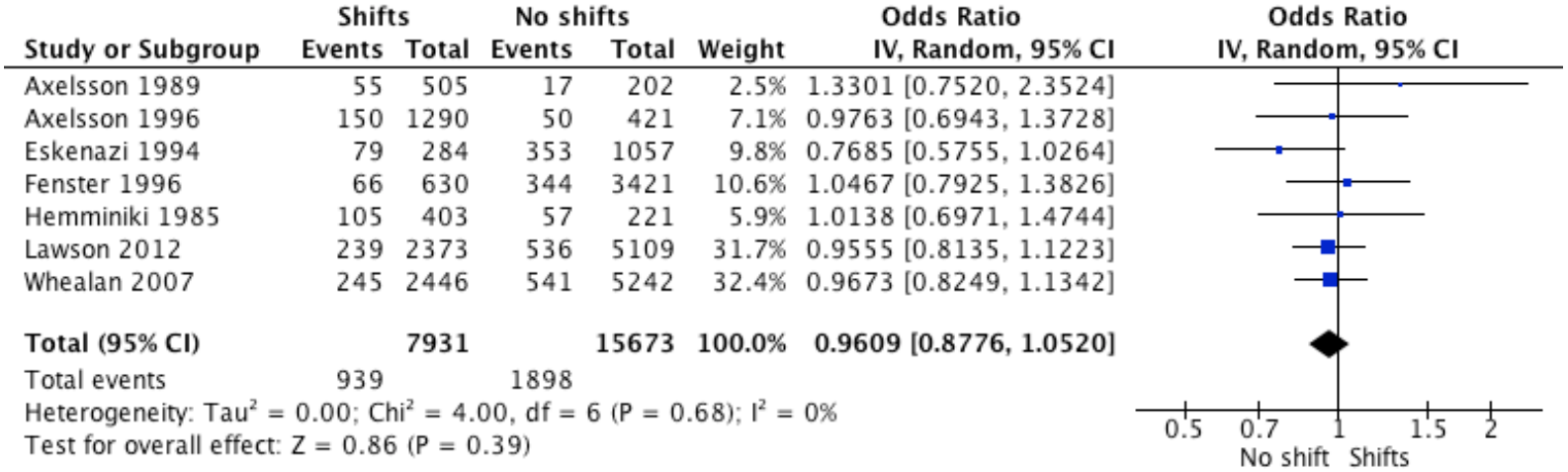


Figure 2-5: Comparison of shift work with non-shift work: outcome ESPL (unadjusted)

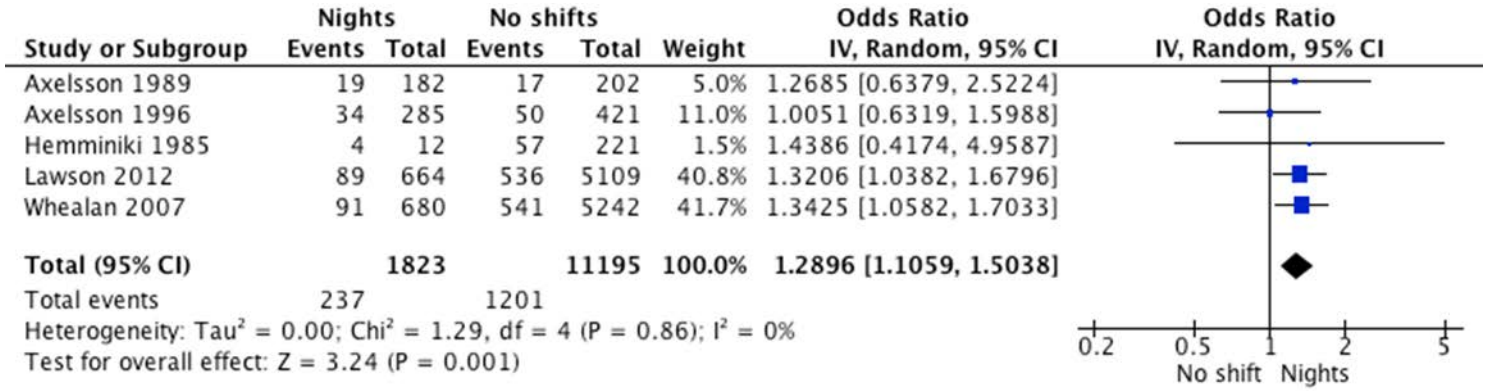


Study or Subgroup	Shifts Events	Shifts Total	No shifts Events	No shifts Total	Weight	Odds Ratio IV, Random, 95% CI
Axelsson 1989	55	505	17	202	2.5%	1.3301 [0.7520, 2.3524]
Axelsson 1996	150	1290	50	421	7.1%	0.9763 [0.6943, 1.3728]
Eskenazi 1994	79	284	353	1057	9.8%	0.7685 [0.5755, 1.0264]
Fenster 1996	66	630	344	3421	10.6%	1.0467 [0.7925, 1.3826]
Hemminiki 1985	105	403	57	221	5.9%	1.0138 [0.6971, 1.4744]
Lawson 2012	239	2373	536	5109	31.7%	0.9555 [0.8135, 1.1223]
Whealan 2007	245	2446	541	5242	32.4%	0.9673 [0.8249, 1.1342]
Total (95% CI)		**7931**		**15673**	**100.0%**	**0.9609 [0.8776, 1.0520]**
Total events	939		1898			

Heterogeneity: $Tau^2 = 0.00$; $Chi^2 = 4.00$, df = 6 (P = 0.68); $I^2 = 0\%$

Test for overall effect: Z = 0.86 (P = 0.39)

Figure 2-6 Comparison of night work with non-shift work: outcome ESPL (unadjusted)



Study or Subgroup	Nights Events	Nights Total	No shifts Events	No shifts Total	Weight	Odds Ratio IV, Random, 95% CI
Axelsson 1989	19	182	17	202	5.0%	1.2685 [0.6379, 2.5224]
Axelsson 1996	34	285	50	421	11.0%	1.0051 [0.6319, 1.5988]
Hemminiki 1985	4	12	57	221	1.5%	1.4386 [0.4174, 4.9587]
Lawson 2012	89	664	536	5109	40.8%	1.3206 [1.0382, 1.6796]
Whealan 2007	91	680	541	5242	41.7%	1.3425 [1.0582, 1.7033]
Total (95% CI)		**1823**		**11195**	**100.0%**	**1.2896 [1.1059, 1.5038]**
Total events	237		1201			

Heterogeneity: $Tau^2 = 0.00$; $Chi^2 = 1.29$, df = 4 (P = 0.86); $I^2 = 0\%$

Test for overall effect: Z = 3.24 (P = 0.001)

The early spontaneous pregnancy loss subgroup analysis (night work vs non-shift work) of 13,018 women from five studies showed a significantly increased rate of early spontaneous pregnancy loss in night shift workers compared with non-shift workers (237 of 1,823, 13.0% vs 1,201 of 11,195, 10.7% OR 1.29 95%CI 1.11-1.50, I^2 0%) (see Figure 2-6)

Heterogeneity was generally low excepting for the infertility group (I^2 94%). Cohorts were removed sequentially to investigate individual study effects. Two cohorts (220, 221) not specifying the period of exposure dramatically affected the I^2 value for the shift work comparison (OR 2.75 95%CI 2.02-3.75, I^2 30%). Data interrogation of the menstrual disorders was not possible in this manner (only two cohorts).

A large study was omitted (n= 71,077) (228) in the shift work and menstrual disruption analysis because of the potential bias posed by such large numbers. This large study also had the lowest rate of menstrual disruption (13.4% compared with 25.8%(216), 36.0%(215) and 40.0%(217)). The relationship remained stable (n=604 OR 1.56 95%CI 1.04-2.34, 1^2 0%). Similarly, when removing a particularly large cohort (n=21,438) (221) from the infertility analysis, the association with shift work persisted (OR 2.16 95%CI 1.33-3.50, I^2 80%).

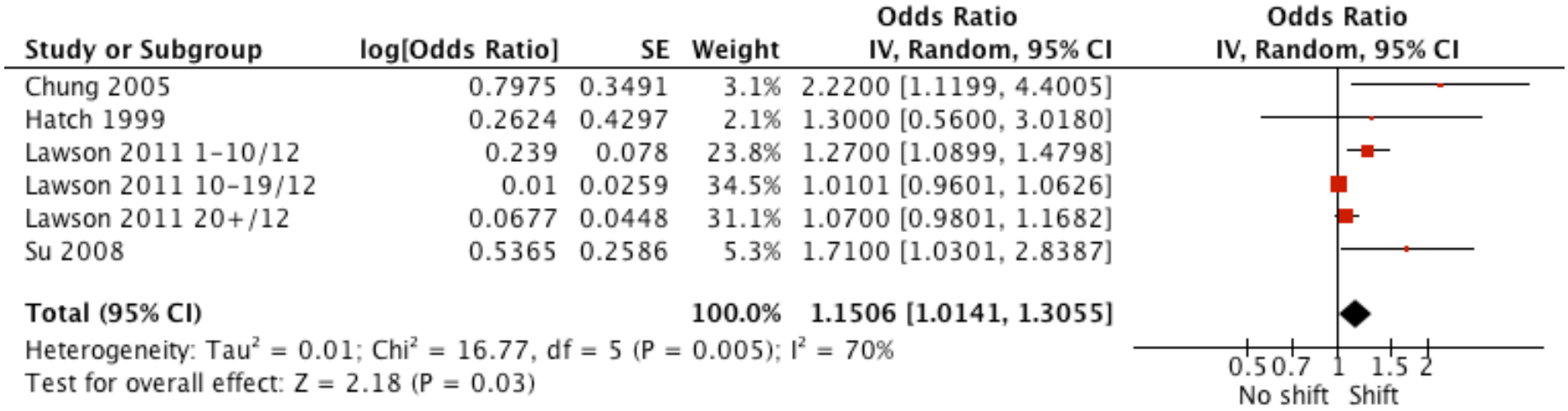
Restriction to cross sectional studies (215, 217, 228) suggested no significant menstrual disturbance in shift workers (n= 71,557 OR 1.33, 95%CI 1.00-1.75, I^2 33%). Infertility rates remained increased, but this comparison is equivalent to removing the large aforementioned study (n=7,780 OR 2.16 95%CI 1.33-3.50, I^2 80%). No other study design analyses were possible.

Restriction to cross sectional studies (222, 223) showed no effect of shift (n=2,418 OR 1.06 95%CI 0.79-1.42, I^2 0%) nor night work (n=1,090, OR 1.08 0.74-1.59 I^2 0%) on early spontaneous pregnancy loss rates. Similarly, neither case-control (226, 227) nor cohort studies (224, 225, 230) of shift work had an impact on early spontaneous pregnancy loss (n=1,965, OR 0.86 95%CI 0.66 -1.12 I^2 24%; and n=21,639, OR 0.98 95%CI 0.89 -1.08 I^2 0% respectively). However, cohort designs (224, 225) showed an augmented effect of night work on early spontaneous pregnancy loss rate (n=11,695 OR 1.33 95%CI 1.12 -1.58 I^2 0%).

One study defined early spontaneous pregnancy loss as pregnancies ending before 28 weeks gestation (222), stating that 99% of these occurred at fewer than 16 weeks but the authors were unable to provide the number of pregnancy losses between 25-28 weeks. This would have been a maximum of seven women and calculation demonstrated no impact on overall effect. Thus, this initially excluded study was retained. Excluding this study did not alter results for the shift workers' comparison

(n= 18,846 OR 0.94 95%CI 0.85-1.04, I^2 0%) nor the subgroup (night work vs non-shift work) of night workers (n=12,634 OR 1.29 95%CI 1.10 -1.51 I^2 0%).

Figure 2-7: Comparison of shift work with non-shift work: outcome menstrual cycle disruption (adjusted)



The menstrual disturbances study not specifying hormonal manipulation or disturbance (215) was removed in a sensitivity analysis, and the result was stable (n=71,681 OR 1.22 95%CI 1.15-1.29 I^2 0%).

Included studies adjusted for various occupational factors (work related factors, working hours, employment duration, shift work history), demographics (age, marital status, education, menarche, race, parity, pre-employment menstrual irregularity, previous miscarriage, medical insurance status, infection, nausea, gestation at interview, pregnancy history) and modifiable risk factors (smoking, alcohol, caffeine, physical activity, tap water consumption, BMI, medication, hormonal use in past) as detailed in supplementary data. The fertility studies included paternal data for occupation, work related factors and smoking.

Two studies examining menstrual disturbance provided adjusted effect measures (217, 228) (Table 1). Pooled multivariable analysis using adjusted risk estimates when available showed the effect of shift work remained significant (AOR 1.15 95%CI 1.01-1.31, I^2 70%) but not when restricting to adjusted data (AOR 1.12 95% CI 0.99-1.25 I^2 75%) (Figure 2-7). This comparison was not possible for the subgroup of night workers.

All infertility cohorts provided adjusted effect measures (see supplementary data). Pooled analysis was non-significant (AOR 1.11 95%CI 0.86-1.44, I^2 61%) (Figure 2-8), remaining similar with exclusion of the study reporting individual effect measures depending on type of shift worked (AOR 1.25 95%CI 0.82-1.91)(221) and in the one cohort (218)(1) providing data for women not managing to conceive (n= 3,092 AOR 1.2 95%CI 0.2-8.3). This comparison was not possible for the cohorts of night workers (n = 2 cohorts).

Six early spontaneous pregnancy loss studies provided effect measures for confounders (222, 223, 225-227, 230) (see supplementary data). When pooling these, the effect of shift work remained non-significant (AOR 1.04 95%CI 0.89-1.22, I^2 43%) (Figure 2-9). In a pooled multivariable analysis using adjusted risk estimates where available, the effect of night work was still significant (OR 1.41 95%CI 1.22-1.63, I^2 0%) with similar results if only adjusted data was used (n= 3 cohorts, AOR 1.40 95%CI 1.07-1.82 I^2 29%) (Figure 2-10).

Figure 2-8: Comparison of shift work with non-shift work: outcome infertility (adjusted)

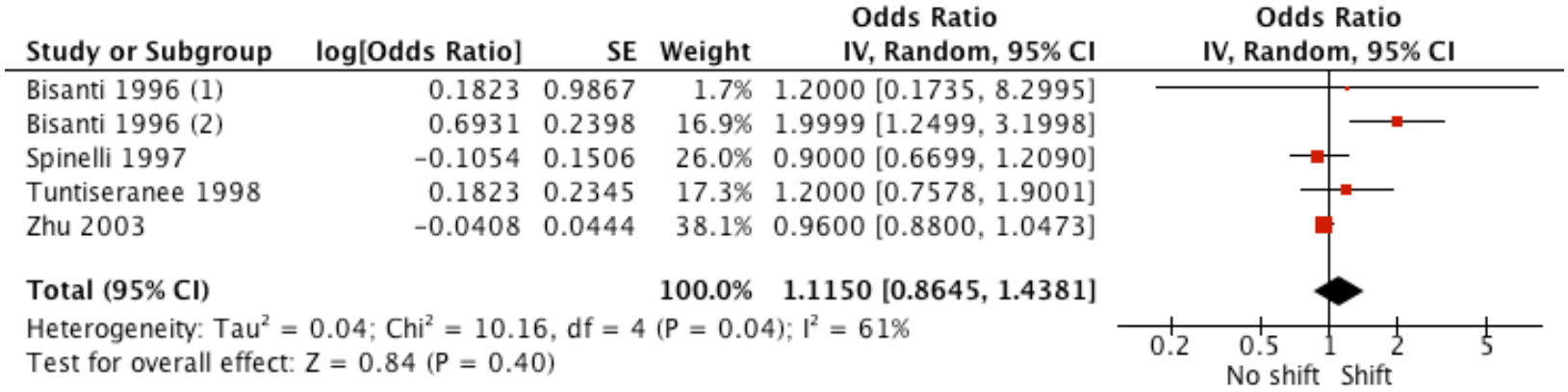


Figure 2-9: Comparison of shift work with non-shift work: outcome ESPL (adjusted)

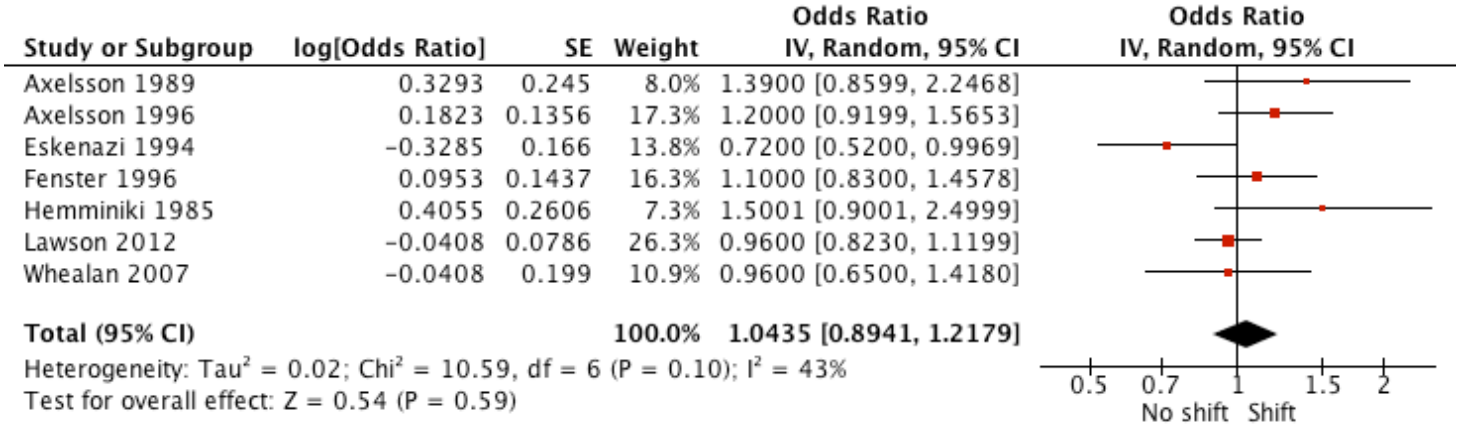
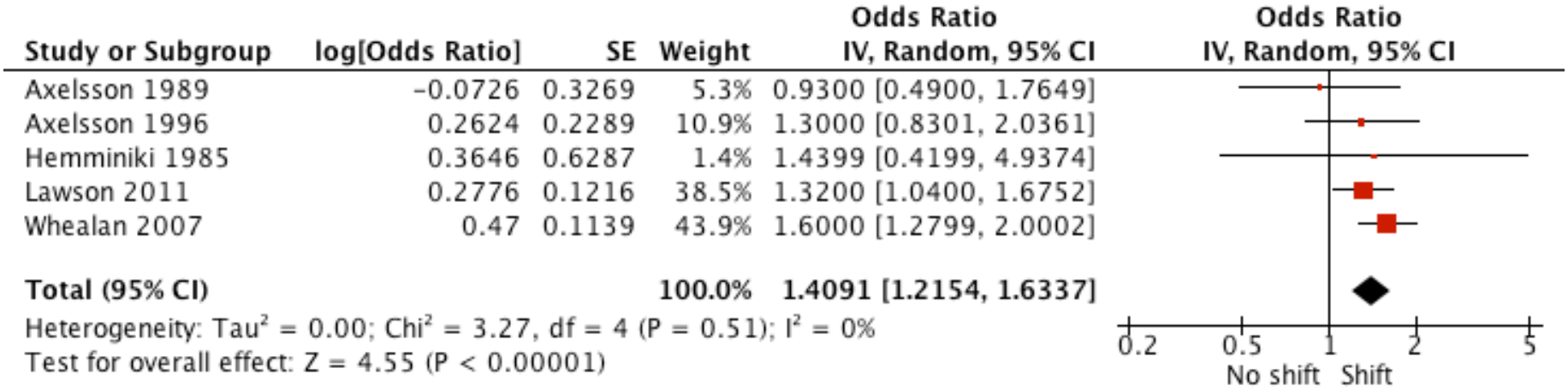


Figure 2-10: Comparison of night work with non-shift work: outcome ESPL (adjusted)



2.4 Discussion

This review provides evidence for an association between shift work and early reproductive outcomes, consistent with findings in later pregnancy (203, 204). Clinicians may advise that there is currently insufficient evidence to restrict shift work in women of reproductive age.

Strengths of the methodology included comprehensive searching, robust data-synthesis and validated quality assessments. Unadjusted data was used from methodologically strong studies, as demonstrated by the The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses. This ensured adherence to current and standardised meta-analytic practice. Adjustments for work-related parameters, demographic differences and risk factors broadened applicability of evidence. Analysis included large numbers, spanning countries and occupations thereby increasing generalisability.

Differences between studies persist despite strict inclusion criteria. The exposure to shift work (type, length and duration) varied between studies due to national and cultural variation and the literature fails to consistently address the distinction between constant/fluctuating shifts. Narrowing inclusion criteria compromises breadth and application of results without necessarily comparing equivalent shifts, as demonstrated by a recent study (233). 'Normal hours' (08 00-18 00) creates arbitrary cut-offs, an inevitable compromise of categorical comparisons. Even small differences in awakening time impacts on daylight exposure with plausible biological consequences. 'Out-of-hours' work may differ in type/intensity but including broad variation in occupations ensured coverage.

Outcomes varied between studies, possibly contributing to heterogeneity. The small number of studies available may explain inability to ascertain major sources for the heterogeneity across infertility studies and may limit reporting reliability with night shifts and menstrual dysfunction. Only one infertility cohort included women not conceiving, rendering analyses susceptible to underestimation (by excluding sterile women). Difficulty in recall may contribute bias and explain lack of effect when restricting to cross-sectional studies.

Observational studies cannot demonstrate causation. Adjusting for confounders attenuated but adverse outcomes persisted in studies adjusting for unhealthy behaviour (217, 218, 220, 222, 223, 225, 228)(27 1&2) and demographic differences, possibly because shift work selects for poor lifestyle-habits. Certain confounders will be more relevant to particular pathology, for example age and early spontaneous

pregnancy loss, smoking with fertility. Individual participant data was not available (IPD) to compare inherent discrepancies so certain comparison assumptions were made, such as baseline rates of pathology and year of data collection. Given these caveats, the ability to demonstrate that shift work confers an effect without residual confounders, remains elusive.

Working shifts for ≥30 years increases rates of breast cancer, (OR 2.21, 95% CI 1.14 to 4.31) (234)), thus prolonged exposure might amplify effects thus duration and work parameters may be important. Conversely, the 'Healthy Worker Effect' ("consistent tendency of the actively employed to have a more favourable mortality experience"), (235) might impact upon findings. Working populations exclude the severely ill, while employment affords a better life-quality and women unable to tolerate shift work may select jobs with routine hours (206). These would attenuate these results.

Menstrual disruption rates were widely distributed (13.4%(228) - 40.0%(217)) with the largest study possibly having lowest prevalence (236). This will underestimate any shift work effects and is an important consideration given that menstrual cycle function may affect fertility, early spontaneous pregnancy loss and chronic disease risk (237).

It is likely observed effects are multifactorial, mediated by medical, social and psychological factors. Shift workers are exposed to risks for adverse reproductive functioning (192, 206) such as obesity (238), caffeine (239) nicotine/drug use (240, 241)), sedentary-lifestyles (242), and poor diet (243). In addition, the size of the odds ratios (OR 1.22-1.80 and AOR 1.15 -1.41) were modest compared with some of these other recognised risks for early reproductive outcomes. Environmental factors may also modify individual differences in circadian 'typology' and influence diurnal 'preference' (98). However, most cohorts provided AOR, suggesting that confounders are not solely accountable.

For these reasons it cannot be concluded that avoiding shift work would lessen adverse outcomes without interventional trials. Nevertheless, if replicated these findings have implications for those attempting pregnancy, working women and employers. Disruptive shift work effects cannot be negated but coping strategies may be utilised, as facilitated by Working Time Directives and an awareness of womens' rights. However shift work has financial and social benefits for many women.

The magnitude of these results should not be overstated. Any increases in adverse risk will be small relative to background: 44% of women experience menstrual disruption (236), 14% of couples infertility (208), and 20% of pregnancies early spontaneous

pregnancy loss (244). Thus if a woman experiences these outcomes, it is unlikely to be shift work related.

Research should be performed to validate these findings. Prospective long-term cohorts or cross sectional studies of IPD would provide useful information. Ways to modulate circadian disruption should also be investigated.

Chapter 3 Biological rhythms in clinical practice

3.1 Introduction

The relationship between diurnal rhythms and fertility is largely unknown. This paucity of research is surprising given that many of these circadian rhythms, for example sleep and nutrient intake, are critical components to in human wellbeing. Sleep disorders contribute to the malfunction of reproductive biological rhythms, including premenstrual dysphoria syndrome, postpartum depression and the menopause (245). While there appears to be a relationship between sleep disturbance and reproductive health (246), little is known about which form of sleep disturbance are related to reproductive capacity and which specific aspects of fertility are particularly affected.

Chapter two has shown that shift work increases the risk of adverse early reproductive outcomes. Shift workers adopt patterns of activity outside their normal circadian rhythm, which encourage poor sleep hygiene and sleep deprivation. These are risk factors for disease, cause alterations to psychological and social functioning as well as short and long-term biological changes (182). For example, shift workers experience higher rates of cardiovascular disease, (192, 247, 248) metabolic disturbances (249, 250), cancer (193) and disrupted circadian rhythms are commonly found to be associated with clinical disorders such as diabetes, mental health disorders (192, 193) and associated metabolic derangements such as insulin resistance, and inflammation.

In addition, people with cardiovascular disease and with metabolic disorders are more likely to have disrupted clock genotypes (251). It follows that other pathologies including those associated with reproduction, for example RIF and RM may also have altered molecular clock functioning. The new paradigm may be that, rather than shift work affecting biological rhythms and causing disease, that the women suffering poor reproductive outcomes in chapter two, also have malfunctioning clocks. This could be a cause (their clocks are 'out-of-sync and so they prefer to work shifts) or affect (shift work has caused then to have disrupted clocks) of working shifts. Ultimately, whether a disrupted sleep wake cycle is associated with reproductive disorders is a hypothesis yet to be tested. To this end, this chapter will present data from the Actiwatch, actigraphy-based data loggers that record sleep and activity habits, as well as self-reported measures of circadian disruption in the form of sleep questionnaires.

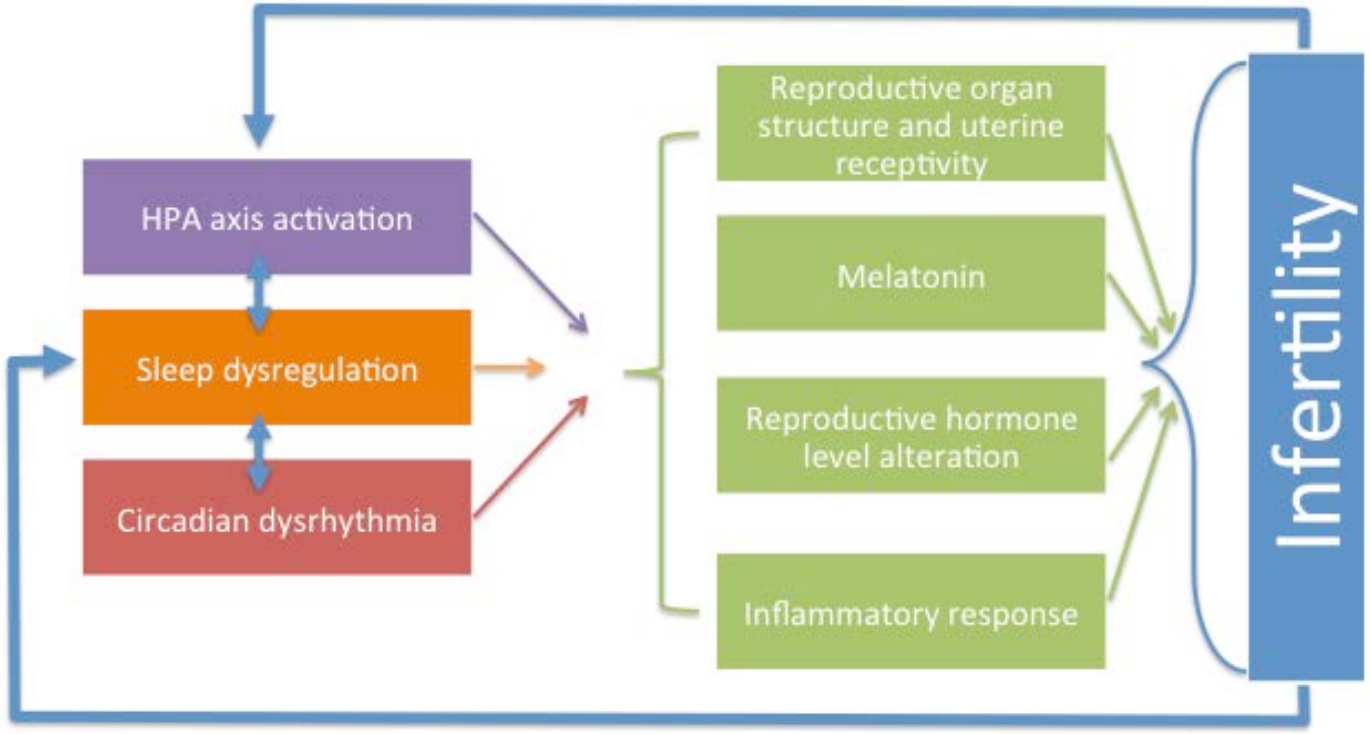
Figure 3-1 is an illustration of the hypothesis that circadian rhythm, and specifically the sleep/wake and feeding profile is crucially linked to reproductive function. Of these,

many factors have been implicated to be contributory factors, such as poor dietary habits, increased levels of obesity, higher intake of drugs and alcohol, unhealthy social and psychosocial functioning and circadian disturbance. Sleep disturbance (for example disrupted sleep continuity, short or long sleep duration, circadian dysrhythmia, and even hypoxia) itself has not been investigated. Problems with fertility, conception, implantation, gestation and parturition could also be reciprocal, such that sleep disturbances and their associated sequelae may not only be caused by, but also interfere with, reproductive processes. To date, the majority of evidence for the association between biological disruption and diminished reproductive capacity has been within the area of shift work. In general, adverse reproductive health outcomes have been observed such as menstrual irregularities, menstrual disruption, increased time to, and reduced rates of conception, increased miscarriage risk and lower birth weights.

Various pathways may link sleep and fertility, either involving or independently of the HPO axis as described in 1.6.1, or the Hypothalamic Pituitary Adrenal axis (HPA) (252). The HPA axis is a complex set of direct influences and feedback interactions (for the basic structure see Figure 3-2). Altered sleep may cause HPA activation and thereby interfere with reproduction directly by exerting its effect on reproductive hormones (245). For example, prolactin production is altered by Rapid Eye Movement Sleep (253) and hyperprolactinaemia is an established cause of infertility. However, these effects may be far reaching as sleep alterations have been found to modulate thyroid stimulating hormone (TSH), LH, prolactin, testosterone, oestradiol, anti-Mullerian hormone (AMH), and progesterone. Indirect HPA effects could also play a role, for example mediated by heightened levels of melatonin seen in poor sleep (254) and those with amenorrhea, GnRH suppression and altered ovulation (255, 256).

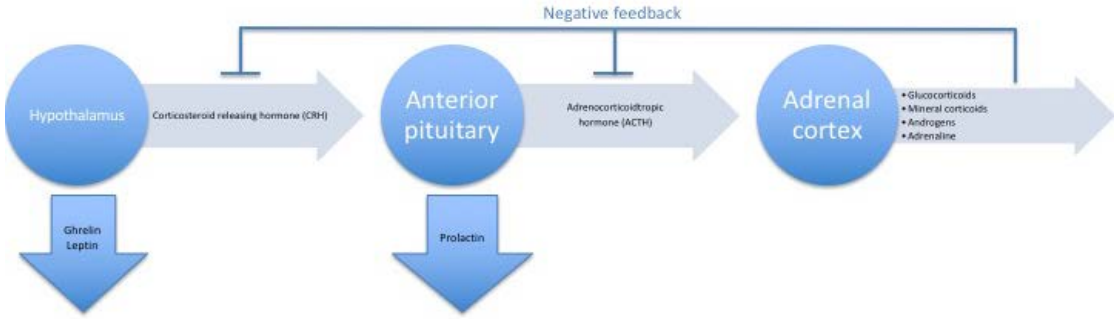
Figure 3-1: Sleep and possible causal links for subfertility

A schematic to show how sleep may result in infertility. 1) The HPA activation that precipitates sleep disturbance may also interfere with reproduction. 2) Altered sleep may interfere with reproduction or result in further increased HPA activation. 3) Circadian dysrhythmia, independent of (or in interaction with) HPA axis activation, sleep duration and/or sleep continuity disturbance, may result in infertility.



Polycystic ovary syndrome (PCOS), as one of the most common causes of female infertility, is associated with poor sleep and obstructive sleep apnoea (OSA) (257). Sleep dysregulation has been associated with multiple metabolic changes, including increased levels of adrenal cortisol and hypothalamic ghrelin and decreased levels of hypothalamic leptin, resulting in impaired glucose metabolism (258) and non sex-steroidal hormonal upregulation (259). OSA is also believed to contribute to the metabolic abnormalities seen in women with PCOS (260). The altered sleep seen in PCOS may be multifactorial in its effects on the underlying pathophysiology of the disease as well as fertility outcomes. This suggests that the underlying cause that precipitates sleep disturbance may also interfere with reproduction in other reproductive disease states.

Figure 3-2: The Hypothalamic Pituitary Adrenal axis



On a simplistic level, a cause-and-effect model is biologically plausible, such that sleep dysrhythmia, independent of HPA axis activation, may result in subfertility. Sleep physiology during the 'fertile window' is altered and a disruption to this could have negative effects (261). As implantation and embryo development is known to be orchestrated by a highly complex network of inflammatory mediators and immune cells (262), the disruption of these systems secondary to sleep deprivation (263) can have up and downstream effects.

However, sleep disturbance may also be a consequence of the psychological impact of subfertility, and a number of neurobiological pathways are postulated as modifiers in the risk of subfertility (255, 256, 264, 265). Interventions directed to reduce the effects of anxiety, have been suggested to improve fertility treatment outcomes (266, 267) but the evidence for this providing clinical benefit is weak.

Sleep has the potential to affect the reproductive capacity of women through several theoretical mechanisms but the data to date do not consistently substantiate any of these pathways. Whether a disrupted sleep-wake cycle is directly associated with reproductive disorders is a hypothesis yet to be tested.

3.2 Objective

To investigate whether there is difference between the sleep and wake activity of women suffering with poor reproductive outcome compared with controls.

3.3 Hypothesis

Disrupted sleep and wake phenotypes are associated with women with the recurrent implantation failure and recurrent pregnancy loss, compared with more normal sleep-wake functioning of fertile controls.

3.4 Method

3.4.1 Patient selection

Women attending gynaecology outpatient clinics at a tertiary university hospital were invited to participate in this cohort study. Female patients aged between 18-45 years were recruited into three separate study groups. These women had a diagnosis of either:

1. Recurrent Implantation Failure (the absence of pregnancy after transfer of at least three good quality embryos and over two or more IVF cycles)
2. Recurrent Miscarriage (three or more unexplained pregnancy losses before 24 completed weeks of pregnancy)

The control group consisted of women who were fertile (all had to be parous, having had had at least one child conceived without difficulty) and without known endometrial pathology. Pregnant women were excluded from the study, as were women who had taken drugs which could have interfered with their biological cycles within the last three months or if they have any active infective disease processes. Any participants with significant psychiatric disorders were also excluded.

3.4.2 Assessing sleep quality

All participants were asked to complete a validated sleep questionnaire and a daytime sleepiness scale. Basic demographic data and information on sleep patterns were also collected on the sleep questionnaire. Sleep quality was determined using the

Pittsburgh Sleep Quality Index (PSQI) questionnaire and daytime sleepiness was assessed using the Epworth Sleepiness Scale (ESS) questionnaire.

3.4.2.1 The Pittsburgh Sleep Quality Index

The Pittsburgh Sleep Quality Index

This self-rated, validated questionnaire (268) assessed sleep quality and disturbances over a one-month period. It consists of 19 questions with seven component scores: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication and daytime dysfunction. Each component score is rated from zero, indicating no difficulty, to three, indicating severe difficulty, and is aggregate to a global score with a maximum difficulty score of 21. A score above five indicates poor sleep quality.

3.4.2.2 Epworth sleepiness scale (ESS)

The ESS measures a subject's usual level of daytime sleepiness (269). It asks the study patient to rate their sleep propensity in eight different situations. The score reflects average sleep propensity (ASP), a measurement of a person's general level of daytime sleepiness. The reference range of 'normal' ESS scores is zero to 10 (as the range defined by the 2.5 and 97.5 percentiles). The scores may be subdivided as follows: 0-5 Lower Normal Daytime Sleepiness, 6-10 Higher Normal Daytime Sleepiness, 11-12 Mild Excessive Daytime Sleepiness, 13-15 Moderate Excessive Daytime Sleepiness, 16-24 Severe Excessive Daytime Sleepiness. A score above 16 indicates a high level of daytime sleepiness and a score of between 9 and 16 suggests inadequate sleep (270).

3.4.2.3 Monitoring sleep–wake patterns

The sleep–wake patterns in a subgroup of women from the two cohorts were monitored using a wrist--worn Actiwatch device (Phillips Healthcare, Respironics, The Netherlands) for seven consecutive days and nights. Actiwatches are small, actigraphy-based data loggers that record a digitally integrated measure of gross motor activity. The device also records white light exposure and activity levels and provides a reliable indicator of sleep–wake state in healthy populations (271). Exposure of the participants to white light was taken into account as white and blue light exposure has been reported to be a key determinant of the sleep–wake cycles and melatonin release (95, 272), and all white light contains some blue light. Unlike polysomnography (PSG), which is the gold standard neurophysiological method for detection of sleep, actigraphy is non-invasive and can be used more cost-effectively for long-term monitoring. A previous study compared these two methods and found that actigraphy

correctly predicted sleep and wake periods 86.6% of the time in adults, compared with polysomnography (95). For the purposes of this study, the primary outcome measure was total sleep time (hours and minutes) and several key sleep and activity variables were also calculated using sleep analysis software (Phillips Healthcare, Respironics, The Netherlands):

1. Total sleep time (hours:minutes): time from sleep onset to morning waking
2. Bed time (clock time)
3. Time of awakening (clock time)
4. Sleep efficiency (% of total sleep time): % time from sleep onset to sleep offset that is recorded as sleep.
5. Time in bed (hours:minutes)
6. Sleep onset (hours:minutes): time taken for subject to fall asleep after bedtime.
7. Wake after sleep onset (WASO) (number of episodes): number of occasions after sleep onset that subject is partially or completely awoken.
8. Awake time after sleep onset (hours:minutes): total length of time of combined WASO.
9. Activity (movement count): number of movement episodes
10. Light exposure (lux/minute): exposure to sunlight

The assessment of activity data will also be performed in the frequency domain. This is because the 'signal' of interest is characterised by frequency and not time or space. That is to say, it would be of interest if women with RIF are particularly active in a certain pattern, i.e. if they exhibit tendencies to be very active then very restful. Summating this data will not reveal differences such as these. It is a technique that can mathematically illicit notable differences such as this that looking at totals from the dataset will overlook (e.g. total time spent active).

3.4.2.4 Power calculation

Power calculation was based on the primary outcomes (length of time asleep between women with RM, RIF and controls, or total sleep questionnaire score) (273). For the Actiwatch data, an a priori sample size of 9 per group was calculated based on an average sleep time for a UK adult (taken from the National Sleep Council UK 2013 data) of 395 minutes (± 15 minutes). We therefore considered a mean of group 1 (controls) to be 395 minutes with a difference of group 2 (RIF or RM) to be $\geq$20 minutes change in average sleep time (with 5% significance and 90% power). If we were to combine cases as a single group and use a 2:1 for controls, this would yield $\geq$ 95% power.

For both the questionnaires, the original scoring system was tested on normal subjects and this data was used in power calculations. A PSQI mean score of 2.67±1.7 was used and we powered the study to detect a difference in scores between cases and controls (1:1) of 2.08 (that is just below the threshold that would take the women into the poor sleep quality). This resulted in n=14 (N=28). Similarly, an ESS of 5.9±2.2 was used and again we powered the study to detect a difference of 2.85. This resulted in n=13 (N=26, both with 5% significance and 90% power (see Figure 3-3). The Power calculation was checked and verified by a medical statistician (a statistician employed by the hospital to aid with 'Research and Development', within the department but who had no involvement elsewhere in the study) who repeated the power calculation using Stata (version 14.1), and calculated only slightly different numbers due to rounding errors (in total 26 for sleep, 32 for PSQI, and 28 for ESS).

Figure 3-3 Power calculation

From Rosner B. Fundamentals of Biostatistics. 7th ed. Boston, MA: Brooks/Cole; 2011.

Where:

$\Delta = \mu 2 - \mu 1$= absolute difference between the two means

$\sigma 1$, $\sigma 2$ = variance of mean 1 and 2

n1 = sample size for group 1

n2 = sample size for group 2

α = probability of type I error (0.10)

β = probability of type II error (0.2)

z = critical Z value for a given α or β

k = ratio of sample size for group 2 to group 1 (2:1)

$$k = \frac{n_2}{n_1} = 2$$

$$n_1 = \frac{(\sigma_1^2 + \sigma_2^2/K)(z_{1-\alpha/2} + z_{1-\beta})^2}{\Delta^2}$$

$$n_1 = \frac{(15^2 + 15^2/2)(1.96 + 1.28)^2}{20^2}$$

$$n_1 = 9$$

$$n_2 = K * n_1 = 18$$

3.4.3 Statistics

Data handling was performed using PRISM Version 6.0a (2012) (GraphPad Software, Inc. USA) and SPSS Version 21 (2012) (IBM, United Kingdom). Group differences of categorical variables were evaluated using the Chi-squared test and continuous data with independent samples t-test. Statistical significance was set at $P<0.05$. Data values are represented as mean ± standard deviation (SD) or percentage (%).

For the Actiwatch data a series of principles were applied to compare the groups and elicit differences. Firstly, a cosine wave was fitted to the data so that as much noise as possible was removed e.g. spikes equivalent to an exercise session. Using one cosine to fit the data essentially averages the data as the phase is proportional to wake up time and the frequency is proportional to the length of a day. The only subject specific value is the amplitude (an average of the data). Training of an Artificial Neural Network was then performed to detect separation of the groups. Secondly, as the groups contained complex time series data with oscillatory components a Fourier transform was performed to convert the signal from the time domain to the frequency domain. A Fourier fit (of the Cosinor rhythmometry) was used to compare the results of this fit and differences between the groups. Each subject data was split into days and transformed to the frequency domain. The average day for each subject was averaged in the frequency domain to produce an average for each group before a Support Vector Machine (SVM) was applied. A SVM is a discriminative classifier formally defined by a separating hyperplane. This means using labelled training data (*supervised learning*), the algorithm outputs an optimal hyperplane which categorises new examples.

Thirdly the Viterbi algorithm was used to estimate which state transitions the series of Actiwatch data went through. To apply this method to the time series activity data, three states were assumed, one for each group. The data was split into days and concatenated into one sequence of days, for all subjects and all groups. The order of days in the sequence was randomised to ensure a random number of transitions in the estimation of the parameters.

Finally, Multivariate Analysis Of Covariance (MANOVA) was used to adjust for confounders with the Fourier fit (Cosinor rhythmometry) to see whether the results of this fit were different in each group (RIF/RM/control).

3.4.4 Ethical approval

All patients gave informed written consent prior to taking part in this study and ethical approval was granted by the local ethics committee, Regional Ethics Committee

number 12/SC/0568 and Registered with the Research and Development department in the hospital.

3.5 Results

88 participants were recruited and completed the Epworth Sleepiness Scale (ESS) questionnaire and the Pittsburgh Sleep Quality Index (PQSI) questionnaire (n=88). 48 women wore an Actiwatch and 41 of these women completed a contemporaneous week-long sleep diary (non-compliance rate 14.6%). Four women failed to answer all the questions in the PSQI and therefore their final PSQI scores were excluded.

3.5.1 There was no difference in the self-reported sleep parameters

The average sleep diary data did not differ between control women and those suffering RM nor RIF, see Table 3-1. The mean PSQI score of the women was 5.32 ±2.67 with no difference between women with RIF (4.70±2.23) nor RM (5.66±2.66) and controls (5.38±2.94) (n=84). The breakdown in the individual components of sleep parameters, as measured by the PSQI is shown in Table 3-2.

The mean Epworth score of women was 6.18±3.72 with no difference between women with RIF (6.43±2.84, n=21) (p=0.42) nor RM (6.58±4.14, n=33) (p=0.34) and controls (5.65±3.80, n=34) (n=88) (see Figure 3-4). 75 (72.7%) women had a normal ESS score and there was no difference in the number of women in either the RM (n=28, 37.3%, p=0.96) or RIF groups (n=18, 24.0% p=0.97) compared with controls (n=29, 38.7%) (Figure 3-5A). 24 women (27.3%) had inadequate sleep as measured by the ESS, and of these 10 women had RM (41.7%) and 5 women were in the RIF group (20.8%) with no differences between with group and controls (p=1.0 and p=0.76 respectively) (Figure 3-5B). Only one woman (3.5%) women suffered daytime sleepiness, and she was an RM patient (Figure 3-5C). The number of women who fell into the levels of daytime sleepiness as measured by the ESS may be seen in Table 3-3.

Figure 3-4: ESS of the different patient groups

A box and whisker plot to demonstrate the range (minimum to maximum) of the ESS scores (whiskers) and the interquartile range (the box). The median is shown as the line within the box (RM and control) or equivalent to the upper quartile (RIF). It can be seen that the median ESS is lower for both disease groups (RM and RIF) than controls. RM: recurrent miscarriage RIF: Recurrent implantation failure

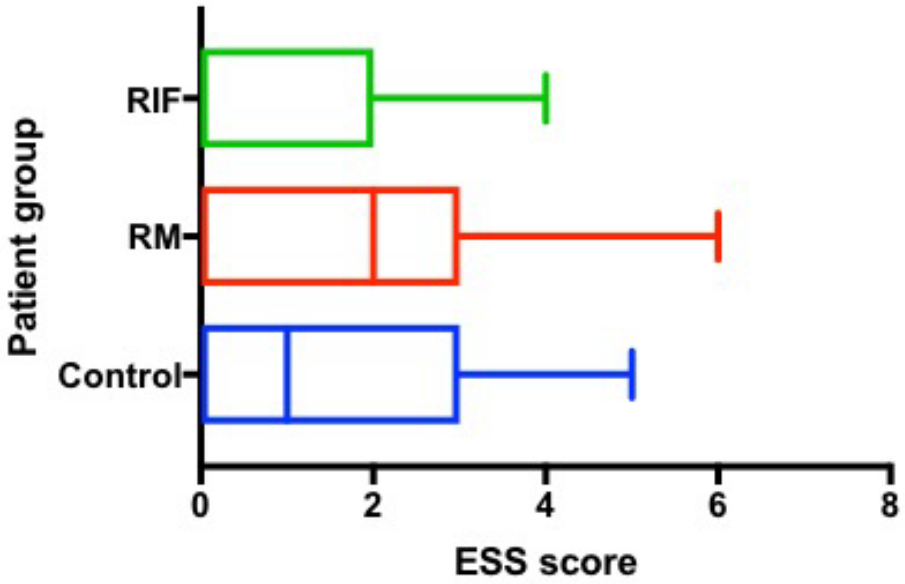
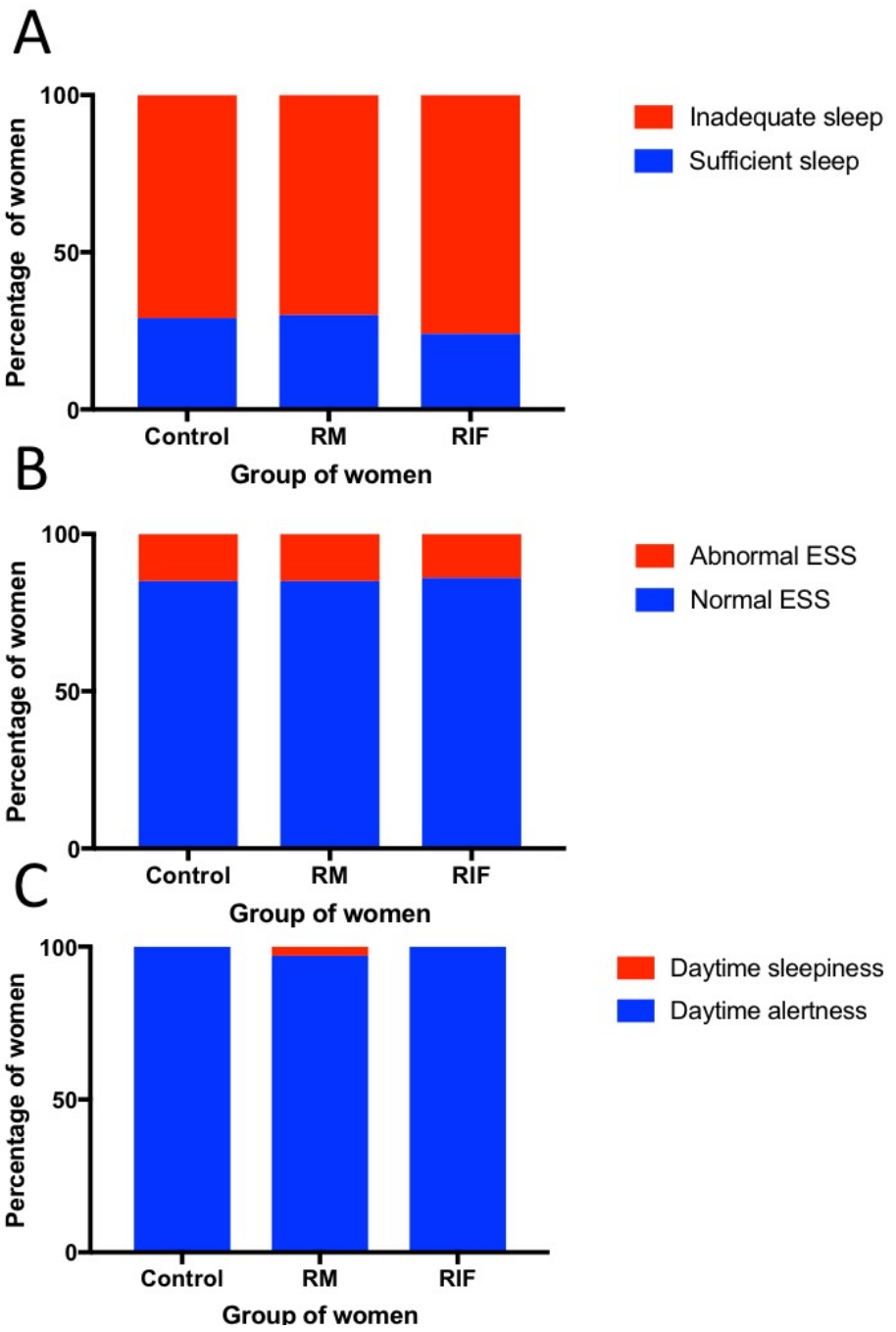


Figure 3-5: ESS group classifications

The classification of sleep by the ESS between the three groups: RM, RIF and controls. (A) The percentage of women not getting adequate amounts of sleep (B) The percentage of women with an abnormal ESS (greater than ten) (C) the percentage of women with severe excessive levels of daytime sleepiness (ESS greater than 16). Actual numbers may be seen in Table 3-2.



3.5.2 The women with RIF sleep for less time than controls, but not because their sleeping habits vary

Of the 48 women who wore the Actiwatch 23 (47.9%) suffered RM, 11 (22.9%) RIF and there were 14 (29.2%) controls. The average time in bed was 8:36±0:05 with no difference between controls and RIF (8:24±1:06, p=0.24) nor RM (8:28±1:20, p=0.19) and controls (8:57±0:58). The average bedtime of all women was with no difference between controls (23:01±0:40) and RM (23:13±0:49, p=0.51) nor RIF (22:55, p=0.78). There was also no difference in the same comparison of get up time between controls (7:50±0:56) and RM (7:31±1:18, p=0.47) nor RIF (7:20±0:43, p=0.18).

The average time spent asleep per night was 7:32±1:06 with no difference between controls (8:27±0:57) and RM patients (7:51±1.13, p=0.80) (see Table 3-4). However, RIF women slept for on average 7:35±0:57, 53 minutes less than controls (p=0.03). The relative risk (RR) of suffering from RIF if she slept for fewer than 7 hours and 35 minutes (the average length of sleep in the RIF group) was 1.2. The average time to sleep onset was 00:08±00.20 with no difference between cases (00:25±00:21) and RM (00:18±00:09, p=0.17) nor RIF (00:36±00:31, p=0.32).

3.5.3 The quality of sleep did not differ between women with RIF, RM or controls

The number of wake after sleep onset (WASO) episodes was 23.29±27.25 with no difference between controls (52.23±39.45) and RM (45.30±22.33, p=0.51) nor RIF (39.37±15.59, p=0.32). The total average length of time awake after sleep onset was 00:38±00:13 with no difference between controls (00:39±00:13) and RM (00:39±00:14, p=0.90 nor RIF (00:35±00:98, p=0.46). Mean sleep efficiency amongst the group was 82.46±11.05 with no difference between controls (82.96±8.02) and RM (84.80±5.40, p=0.42) nor RIF (77.12±19.31, p=0.31). The distribution of all the parameters is shown in Table 3-4.

3.5.4 The women with RIF sleep practise fewer sleep-conserving measures than controls

Although there is a significant difference in total sleep time between controls and women with RIF, this is neither due to a significant overall average later bed time nor earlier getting up time. When the average values are compared 92.9% (13/14) of control women go to bed before midnight, whereas only 81.2% (9/11) of women with RIF go to bed before midnight. However, this results in bedtime in the control women

being only slightly earlier than the RIF women (22:48 ±1:02 compared with 22:54±0:53) (p=0.85). The difference in wake time was almost half an hour later in the control women (7:45±0.54), compared with those suffering RIF (7:18±0:41) (p=0.18) but this was again non-significant. Only 27.3% (3/11) of the RIF women napped over the course of the week. 42.9% (6/14) of the control women nap during the day and these naps are longer on average (19:54±13:59) than those of the RIF women who have an average nap time of 9:14±2:34 (p=0.01).

3.5.5 The PSQI was a good predictor of sleep efficiency

There was a negative correlation (-0.38, p=0.02) between the self-reported PSQI sleep efficiency and the actigraphy-recorded sleep-efficiency across all groups, suggesting that the PSQI was a good predictor of the actual sleep efficiency. This was not true of sleep latency (p=0.31) nor total sleep time (p=0.70).

3.5.6 The women with RIF or RM were no less active than controls

Activity data did not differ between groups with the average activity count per minute being 351.97±97.93 during normal daytime activity, 51.38±74.97 during rest periods and 15.40±10.12 during sleep (on average, the women moved 1.62% as much during sleep as when awake and active, and 5.40% as much during resting periods as opposed to during sleep). Total activity duration was 926.52±371.71 minutes per day during active periods and 513.38±74.97 during rest. The distribution of these results within the three patient groups as shown in Table 3-5.

3.5.7 RIF women are exposed to more light than control women or women suffering RM

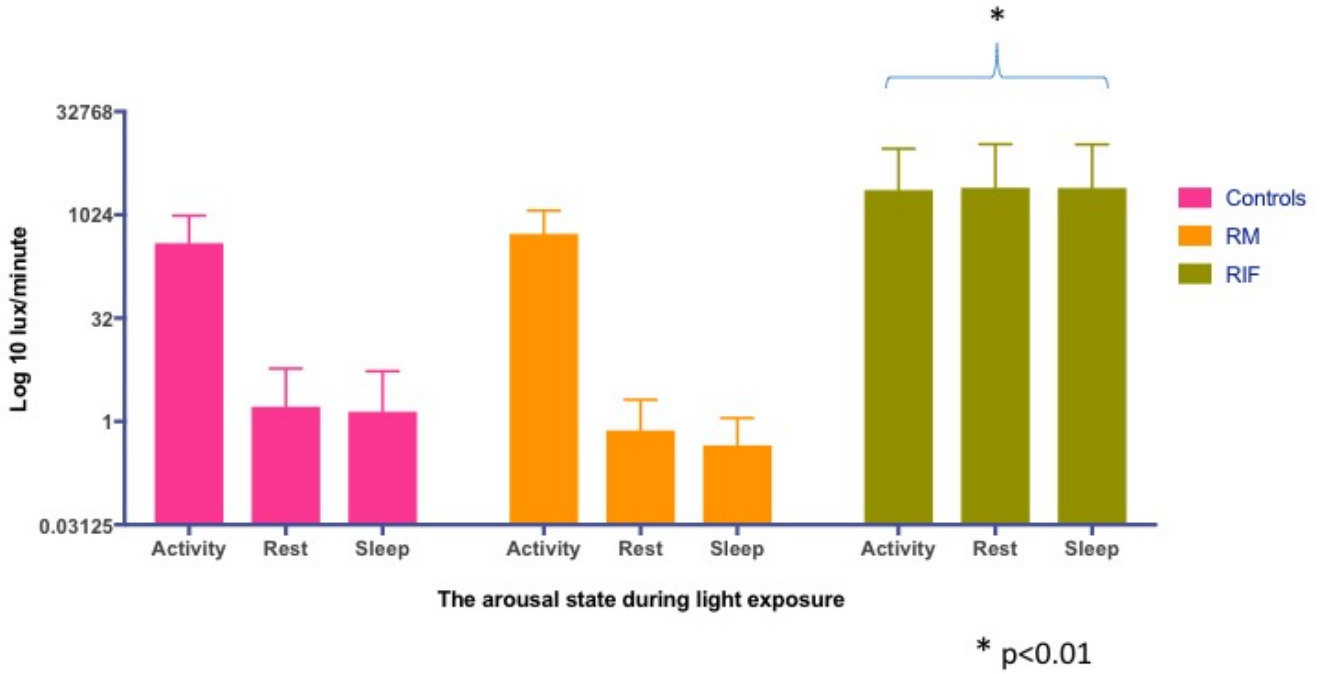
Light exposure was highest during activity with average exposure being 914.11±3405.33, with the average total time above the illuminance threshold (TALT) being 01:04±00:01. During resting, light exposure was 63.68% of the exposure during activity at 582.06±4025.62 lux/minute. During sleep, light exposure fell slightly further to 578.97±4006.04, 63.33% of the light exposure during activity. When the groups were compared, there was no difference in the light exposure during activity, rest or sleep between RM and controls, and between RIF and controls. Total light total exposure (light exposure during activity, rest and sleep combined) between the control women or those RM was no different (133.00±131.50 and 180.30±179.70 respectively, p=0.84). However, women with RIF had significantly more light exposure (2472.00 ± 58.04) overall than controls (p=<0.01) (see Figure 3-6). This was also demonstrable by a negative correlation between total sleep time and light exposure for the women in

the RIF group (r=-0.68, p=0.02) but not the RM (0.29, p= 0.18) or control groups (p=-0.15, p=0.60).

Further manual inspection of each patient's dataset revealed that one patient in the RIF group was an outlier in terms of light exposure. She appeared to be exposed to constant light (i.e. was sleeping with the lights on). When this patient was excluded from the analysis the total difference in light exposure was greater in the RIF women than the control women but the result was no longer significant (74.34±73.69 compared with 133.00±131.5, p=0.72). The same was true when comparing light exposure between RIF and control women during the different arousal states. Light exposure during activity was 221.72±184.67 in RIF compared with 395.96±603.14 in controls (p=0.27), during rest 0.74±0.58 compared with 1.64±4.25 (p=0.31) and during sleep 0.51± 0.53 compared with 1.39± 4.01 (p=0.27).

Figure 3-6: Light exposure during different sleep-wake states

The arousal states as measured by actigraphy were divided into (1) active (2) resting (ie awake but not being active and moving during the day) and (3) sleep categories. The light exposure during each category was averaged for each of the three groups and is demonstrated by the bars shown (mean log lux/minute with error bars representing standard deviation). There was no significance difference between the mean light exposure of the individual categories between control women (pink bars and RM women (yellow bars) nor RIF women (green bars). However, when the total light exposure was summated (total light exposure during active, resting and sleep states), the women with RIF were exposed to significantly more light than controls ($p<0.01$).



3.5.8 It may be possible to classify women according to their activity patterns into disease or control

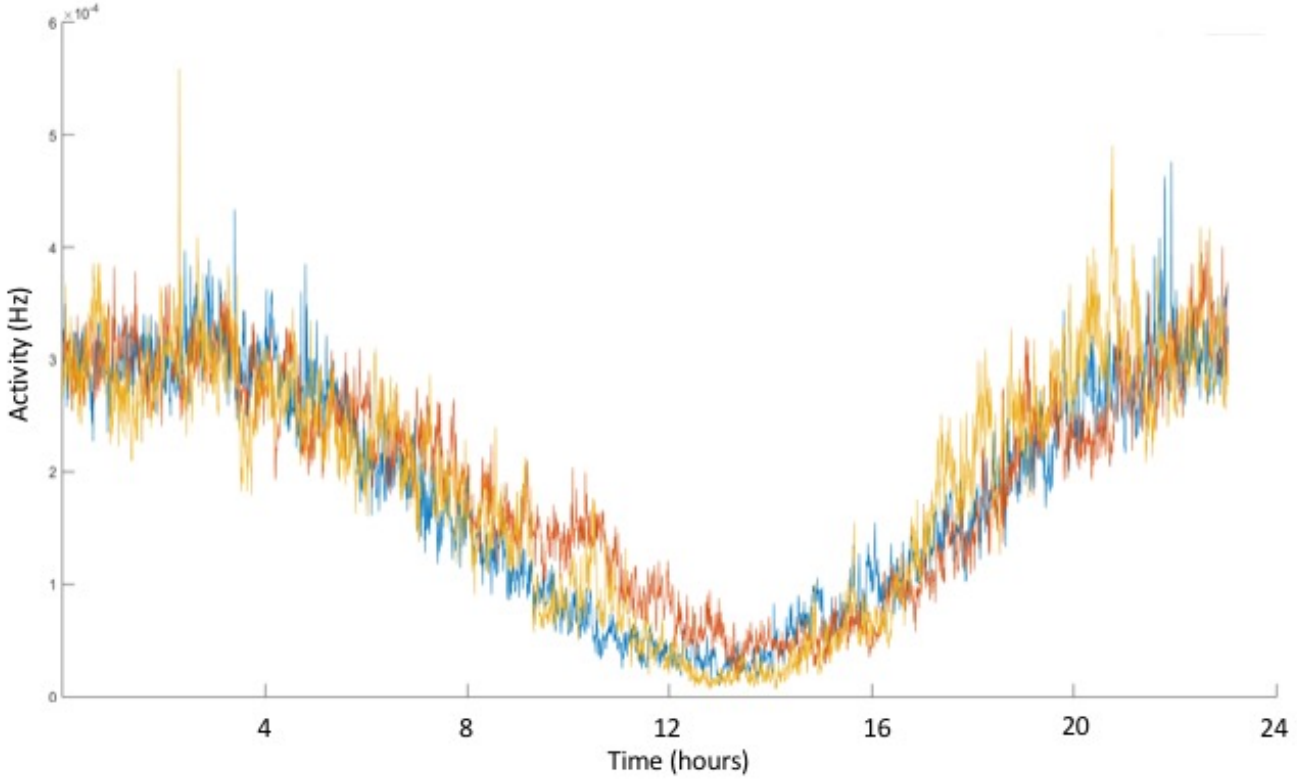
When cosine wave fitting was applied to the activity data, this resulted in a good fit. Training a neural network classifier with the outputs of this cosine fitting resulted in a classification rate of 42.9% (suggesting that the data is similar for controls compared with RIF, and controls compared with RM). Each subject data was split into days and transformed to the frequency domain. For each subject, each day was then averaged into the frequency domain to produce an, 'average day'. For each patient group, the average day for each subject was again averaged in the frequency domain to produce an overall average for each group (see Figure 3-7).

MANOVA was used with the Fourier1 fit (Cosinor rhythmometry) to compare the results of this fit and differences between the groups. With Fourier 1 output, d = 0, p =0.34 (controls vs RM) and p=0.93 (controls vs RIF). The d value suggests that there is no dimensional separation of the means of the input. A value of one would suggest that the means form a line, and a value of 2 would suggest they are all found within a plane. The large p value suggests that the means are equivalent across the three groups, therefore there is no statistical variation between the three data sets when modelled as a single sinusoid. A very similar result was found with a Fourier 2 fit (2 sinusoids) and when increasing integers were applied, up to and including Fourier 8. Table 3-6 shows the difference between the groups increasing with adding more sinusoids to the approximation.

Canonical variables as a method of data reduction were used to allow a graphical representation of the ability to separate the groups using the Fourier 8 fit. These two variables (the two canonical variables) were used to train a Support Vector Machines (SVM) classifier. SVMs are supervised learning models with associated learning algorithms that analyse data used for classification and regression analysis. The SVM was given a set of training examples (i.e. the actigraphy pattern of a couple of patients in each group) and marked each as belonging to one category (i.e. control, RM, RIF). This model assigned a category (RIF, RM, or control) to the new examples. It was used as a non-probabilistic binary linear classifier. The SVM model is a representation of the examples as points in space which are mapped, so that the examples of the separate categories are divided by a clear gap that is as wide as possible. New examples are then mapped into that same space and predicted to belong to a category based on which side of the gap they fall. The SVM model resulted in better separation of the different groups (control, RIF and RM) (Figure 3-8).

Figure 3-7: Average frequency domain for each group

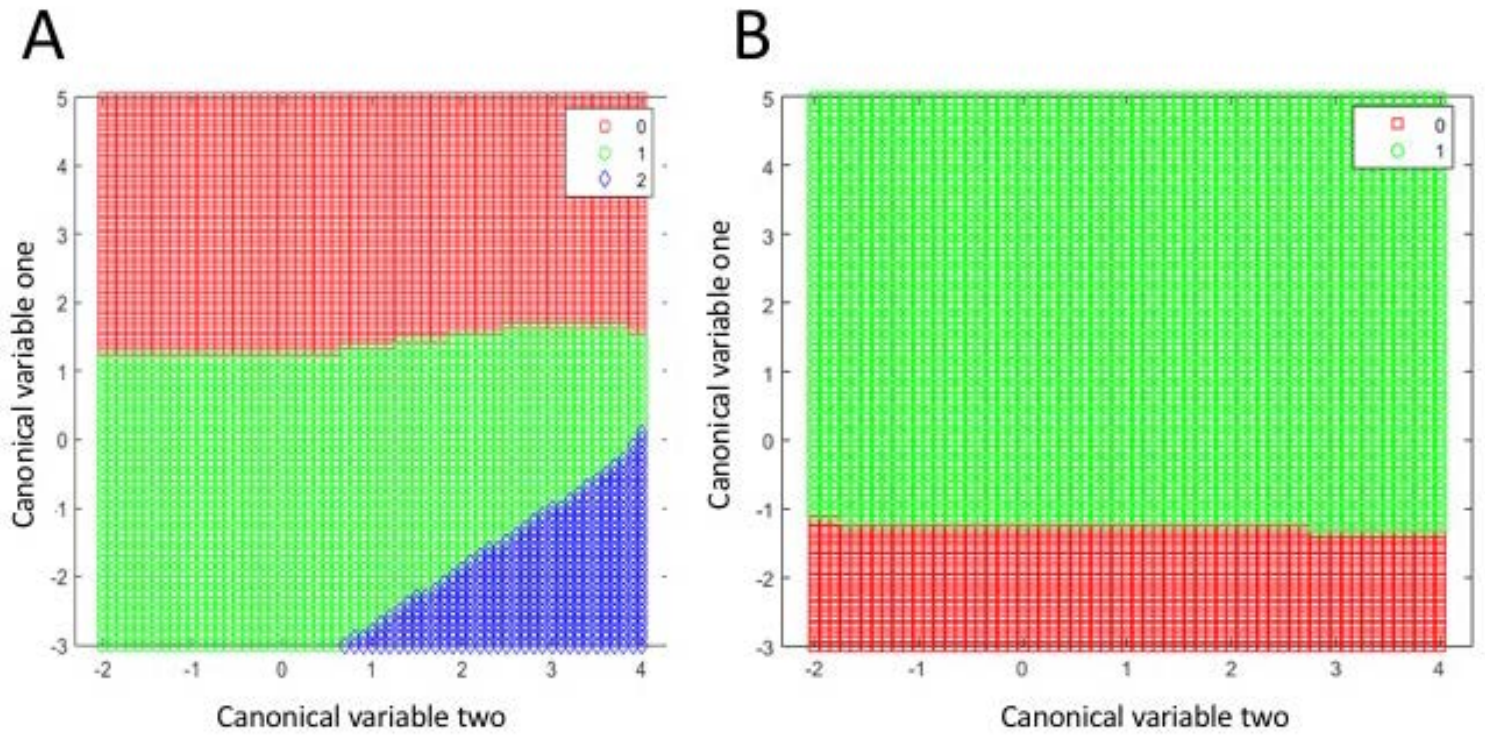
Each patient's data was transformed to the frequency domain and averaged for each patient group. The resultant averages of each group can be seen here for an average day (24 hours, starting at ZT0). For the analysis, the days were summated and an average 24 hour period was calculated from the data collected over the week. The blue line is controls, red RM, yellow RIF). Activity is measured in frequency (Hz, waves of activity per second).



A sample training set was provided and then applied to the whole set to try to distinguish to which group any given woman 'belonged' (control, RM or RIF) using her activity profile alone. This method gave a good estimation of the group according to activity pattern but only some separation was possible because there was a large proportion of each population at the boundary of the three groups. This mathematical model (SVM trained by two canonical variables) was able to differentiate between the women and separate them in to groups (control/RM/RIF) on the basis of their individual activity profile, with a 55.32% success rate. When separated in to two groups, fertile control compared with reproductive pathology (i.e. RM and RIF combined as a group), there was a classification rate of 76.60%.

Figure 3-8: SVM separation of control women from RM and RIF.

Separation of the groups of women by their activity profile was performed using the using the two canonical variables to train an SVM classifier. The results of this are shown here. These figures plot the two canonical variables against each other. The SVM has created a decision boundary between the groups. This is represented by the change in colours of the areas on the charts. Figure (A) is comparing the three groups 0=Controls, 1=RM, 2=RIF. The three groups are divided by two decision boundaries with a 55.32% success rate Figure (B) combines the two disease groups (RIF and RM) and compares these to controls. The decision boundary here between 0=Controls and 1=Pathology (RM/RIF combined) differentiates between cases and controls with a 76.6% successful classification rate. The scales are arbitrary.



3.6 Discussion

This study demonstrated that the self-reported sleep habits and actigraphy-measured sleep patterns in women suffering RM and RIF or without reproductive pathology are grossly similar. However, total sleep time is less in women with RIF compared with healthy fertile controls, on average by 00:53 (07:35±00:57 compared with 08:27±00:57, p=0.03). This was measured by subjective questionnaires and objective measures of sleep parameters using actigraphy. The ways of maximising sleep time i.e. going to bed earlier, getting up later and napping more frequently and for longer, are practiced more commonly by the control women than those with RIF. When looked at in isolation, only one of these factors is significantly different between the control women and those with RIF. Only 27.3% (3/11) of the RIF women napped over the course of the week. 42.9% (6/14) of the control women nap during the day and these naps are longer on average (19:54±13:59) than those of the RIF women who have an average nap time of 9:14±2:34 (p=0.01). It would appear that by having more frequent and longer naps that control women have longer sleep time than RIF women.

Overall light exposure was also greater in women with RIF than controls (mean difference 2339.00±143.70). This result should not be overstated as it was no longer a significant finding when one patient, who exhibited unusual sleeping behaviour (i.e. slept with the light on) was removed from the study group. However, this is a notable finding because it is known that duration of exposure to light is more effective in inducing changes to the circadian clock of humans than increasing light intensity (274). For example, light exposure in the early night induces phase delays of the circadian rhythm of melatonin in humans (275). Equally exposure to increasing durations of bright light is thought to 'reset' the circadian pacemaker in a dose-dependent, non-linear manner. In addition, bright light resets the human circadian pacemaker independent of the timing of the sleep-wake cycle (276). So whilst this result may be important in this patient further studies should specifically look at duration and brightness of light during the sleep time of women with reproductive pathology.

The questionnaires used in this study are reliable and valid in the assessment of sleep problems. However, they are better at detecting self-reported sleep problems and depression-related symptoms than actigraphic measures (277). Whilst aspects of sleep architecture may be related closely to the PSQI/ESS, the latter measures do not detail sleep architecture, for example length of slow-wave-sleep. The quality of sleep is related to total sleep time, insomnia and other disturbances of sleep (278). Time awake in bed and arousals are however more important than quantities of each sleep stage (278, 279). This may explain why there was no difference in the sleep-reported

measures (sleep disturbance, sleep latency, day dysfunction due to sleepiness, sleep efficiency and overall sleep quality) when there was a difference in the actual sleep time. In addition, deepest sleep occurs during the first half of the night as the cellular reconstructive components of sleep occur. The second half is generally a lighter form of sleep, including REM sleep, which is when dreams occur. So, if the subjects were getting an adequate amount of deep 'reconstructive sleep', this may not be affecting the PSQI or ESS scores.

The association observed in this study might be subject to temporal bias, whereby sleep loss might be a manifestation or a symptom of the disease. Women with infertility are more likely to suffer from stress and anxiety and may have sleep difficulties as a result (280-282). This is more likely when, as in RIF and RM, the disease under investigation has a long preclinical phase. However, the same was not seen in RM, a condition with a similarly long prodromal clinical phase and associated with psychological distress. Stress and anxiety are both associated with RIF and RM and so it seems the results are counterintuitive as to why RIF women had a shorter duration of sleep than RM. It might just be a case that that numbers are small, however there were overall differences seen between all three groups in the two variable model (Figure 3-8). It could be possible that the differences in sleep seen between RIF and RM are related to the treatment, or lack of treatment, that the women have for these conditions. The former involves the 'burden' of IVF treatment, whereas RM occurs after natural conception. The women with RIF were not undergoing IVF treatment for the duration of the study, but IVF is an emotionally and physically demanding process and may take an additional toll on these patients.

Given that the light exposure overall was greater in the RIF group of women as opposed to RM or controls, it strengthens the argument that the differences in sleep are specific to the RIF group. It also follows that light exposure could be a factor in the shortened sleep duration. The hormone melatonin produced by the pineal gland is released during the night and provides the body's internal biological signal of 'darkness'. Exposure to light suppresses melatonin production, thus shortening the length of time of exposure to melatonin and may result in shorter sleep duration (283). Early work in to circadian biology suggested that the menstrual cycle in female mammals is under the influence of the suprachiasmatic nucleus (SCN), located in the same part of the brain that controls the biological clock (128, 284). Leading on from this, animal studies have shown that fertility may be improved or reduced according to differences in the light-dark cycle (285). Perhaps the non-discriminative endometrium has different implications on clock biology in under fecundity (RIF and infertility) than it does to pregnancy loss (RM). Studies in animals have shown that that the circadian clock system displays intensity-dependent responses to light stimuli in addition to its

phase-dependent responses to light (286). Detrimental impact on fertility and reproduction due to chrono disruption is well described, although never specifically examined in the area of recurrent pregnancy loss or recurrent implantation failure (287). RM and RIF may be a continuum of a pregnancy loss phenotype. RM may be a condition of over fecundity, with a non discriminative endometrium (see Figure 1-12). Whereas in RIF the endometrium may be an overly discriminatory barrier to implantation. This spectrum could be a reason for the differences seen here. This study suggests that chronobiology provides another insight in to these two conditions. Perhaps the 'discerning endometrium' is clock dependent.

Detrimental effects on reproduction due to chrono disruption is well described, although never specifically examined in the area of recurrent pregnancy loss or recurrent implantation failure (287). Both RIF and RM may be both part of the same, 'implantation spectrum', with RIF as a continuum of a pregnancy loss phenotype to which RM belongs (see Figure 1-12). Our study suggests that chronobiology provides another perspective to these two conditions. Perhaps the 'discerning endometrium' is clock dependent.

Our data demonstrated that there is a poor correlation of PSQI with objective sleep measures, which has also been reported in the literature(277). There may be differences in the sleep parameters of the groups, which our instruments are not sufficiently sensitive to detect. For example, whilst actigraphy monitors arousals and mid-sleep awakenings (WASO), it better monitors total sleep time; and actigraphy does not detect brief WASO without movement, nor does it distinguish the sleep stages. For these reasons, Polysomnography (PSG) is the gold standard for monitoring sleep as it comprehensively records the biophysiological changes that occur during sleep. It is also timely, costly and not widely available and usually expects subjects to sleep in a non-home environment (a sleep laboratory which in itself can affect sleeping habits. The methods used here did not interfere with patient's sleep other than perhaps heightening awareness of their own sleep 'hygiene'.

In recent years, research has overturned the dogma that sleep loss has no health effects, apart from daytime sleepiness. Sleep deprivation (fewer than 7 hours per night) may have wide-ranging effects on the cardiovascular, endocrine, immune, and nervous systems (288). Many studies find graded associations; the greater the degree of sleep deprivation, the greater the apparent adverse effect (289). Sleep deprivation and changes from a normal circadian rhythm are risk factors for disease by causing alterations to psychological and social functioning, as well as short and long-term biological changes (182). Even one night of total sleep deprivation can decrease cognitive functioning (290, 291), impair memory (292), reduce endocrine and

metabolic functioning (293), and cause cardiovascular dysfunction (294). Many adults report receiving insufficient sleep (295, 296), but not all of these will suffer negative consequences. Sleep dose–response experiments have found that the effects of chronic sleep restriction are cumulative, are sleep-dose sensitive (the less sleep that someone has, the faster the rate at which problems develop), and do not result in profound subjective sleepiness (297). The mechanisms underlying the features of neurobehavioral and physiologic alterations during chronic sleep deprivation are unknown. Individual variation appears to be as large as those in response to total sleep deprivation and as stable over time, again suggesting genetic vulnerability to the effects of chronic sleep restriction or differences in the nature of compensatory brain responses to the growing sleep loss.

Circadian dysrhythmia, sleep duration, continuity or quality, may result in infertility. HPA activation is identified as both an independent pathway towards infertility and as a triggering factor for sleep dysregulation, which in turn, may independently affect reproductive capacity (see Figure 3-1). Sleep dysregulation may result in infertility by several methods. Firstly, by causing HPA activation, thus impacting on sleep. However, the self-reported ESS/PSQI did not detect differences in the way the RIF women nor RM women felt about the impact of their sleep. Secondly lack of sleep could cause suppression or augmentation of reproductive hormones. TSH and prolactin display circadian patterns under normal sleep conditions (298) and LH and FSH appear to have altered secretion in women with presumed disturbances in circadian rhythm (299). A disruption to the timing of hormone secretion could have deleterious effects on fertility. This would be supported by the fact that In addition, melatonin, a hormone commonly related to circadian function, has also exhibited altered secretion in shift workers (299). Thirdly via compromised or altered immunity. For example, it is known that normal aging is associated with decreased quantity and quality of sleep and increased secretion of inflammatory cytokines, including interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) (300). It could be that inappropriate secretion of inflammatory cytokines that reduced quantity of sleep in women with RIF may contribute to the pathology. This could cause a derangement in the important inflammatory state thought to vital for successful implantation, or it could be the endometrium is responding poorly as a result of a prematurely aging phenotype.

The mathematical modelling used a SVM to differentiate between the women and separate them in to groups (control/RM/RIF) on the basis of their individual activity profile, with a 55.3% success rate. This demonstrates that activity profiling alone is not sufficiently discriminative. When separated in to two groups, fertile control compared with reproductive pathology (i.e. RM and RIF combined as a group), there

was a classification rate of 76.6%. However, this should be interpreted with caution because the clinical significance of grouping the two independent pathologies has not been shown to be useful in the other datasets. Moreover, the methods used means that there will be an element of statistical 'noise' which may be misinterpreted as useful data.

Finally, it may be that disrupted choronobiology, affects biological rhythms and causes disease and that women suffering poor reproductive outcomes have malfunctioning 'clocks'. For example, with circadian rhythm sleep disorder, delayed sleep phase type, (or Delayed Sleep Phase disorder (DSPD)), the individual's preferred sleep time is delayed three to six hours, compared to conventional times (301). Patients with DSPD exhibit sleep of normal quality and duration for their age and maintain a stable (but delayed) relationship of entrainment to the 24-hour (302). If a number of women in the RIF group suffered from this condition, it may be that if allowed to choose their preferred sleep schedule, they go to bed at a 'non-conventional clock time', and get up much later (for example, go to bed at 1am and would chose to get up 9am, as opposed to a more typical pattern of going to bed at 11pm and arising at 7am). However, this group will be woken at the same socially acceptable morning time. Their total sleep duration is truncated. Similarly, Advanced Sleep Phase Disorder (ASPD) is characterised by a pattern of early evening sleepiness and early morning awakening. This propensity to earlier sleep can interfere with social and work schedules. If for logistical the RIF women are going to bed several hours later than their ideal bedtime (for example 11pm as they have a partner who goes to bed at this time) and yet still awakening at 5am, then their overall sleep time will be shorter than they require. However, there was no difference in the bedtime and awakening time of the women seen in the data and this would not explain the difference in light exposure.

Although the exact aetiology of these sleep phase disorders is unknown, it has been suggested that genetic predispositions, a longer than average endogenous circadian period or alterations in entrainment pathways can result in an altered circadian phase (303). The DSPD phenotype has been associated with polymorphisms of the circadian genes, *Circadian Locomotor Output Cycles Kaput* (*Clock*) (304), *period circadian protein (per)* homolog *3* (*per3*) (305) and *cryptochrome 1 cry1* (306) whilst ASPD has been associated with *per2* (110) and *casein kinase* δ (*ck* δ) 1 (307). The allele frequency of *cry1* implicated in DSPD is thought to exist in up to 0.6% of the population (306). This suggests that gene alterations may be affecting sleep behaviour in a considerable portion of the human population. The importance of genetic influence on a broader range of sleep-disorder phenotypes is also likely.

Regardless of cause, effective treatment for sleep phase disorders and indeed any sleep-related problems require a multimodal approach. These should aim to realign circadian rhythms with the desired sleep and wake schedule. Non-pharmacological should always be first line and approaches may include adherence to good sleep hygiene and timed light exposure. Where this is unsuccessful, treatment with melatonin or hypnotics may provide additional benefit (301).

3.6.1 Summary

It is likely that any link between sleep, reproduction and light is multifactorial. Knowledge from animal studies has improved our understanding of entrainment of human circadian rhythms and revealed important insights into circadian rhythm sleep disorders, light regimens and patients with circadian rhythm sleep disorders (308). However, sleep could be part of a wider problem than is traditionally thought, and its effect on reproduction has not yet been well investigated in humans. Sleep hygiene is not widely noted by clinicians or thought to be of great importance in the traditional practice of medicine. In addition, very little is known about individual differences in circadian sensitivity, nor do we understand how alterations such as relative expression or polymorphisms in "clock genes" play a role in reproductive functioning. Furthermore, many of the current proposed treatments (light exposure and rigid sleep/activity regimes) for such disorders are impractical.

A larger scale study, ideally utilising PSG, is warranted to further investigate the underlying cause of the reduced sleep in the women with RIF. Such studies are time-consuming and expensive to conduct. However, additional well controlled laboratory-based studies where phenotyping and genotyping are conducted in tandem are necessary. Without this information we will not be able to fully understand the effects of human circadian rhythm. This is important as we may be able to translate this knowledge into treatments. If the findings presented here are borne out in future studies, the relationship between implantation and quantity of sleep may have several physiologic and pathophysiologic implications.

Table 3-1 Sleep diary results

	Group	Mean	SD	P value control/RM	P value control/RIF
Awakening time (A)	RM	07:01	00:53	0.61	
(clock time)	Control	07:11	00:49		0.75
	RIF	07:04	00:37		
Out of bed time (O)	RM	07:27	00:56	0.36	
(clock time)	Control	07:46	01:01		0.53
	RIF	07:32	00:36		
Bed time (B)	RM	22:43	00:52	>0.99	
(clock time)	Control	22:43	00:41		0.29
	RIF	23:00	00:26		
Time fell asleep (S)	RM	23:23	00:47	0.98	
(clock time)	Control	23:23	00:47		0.35
	RIF	23:41	00:35		
Timing of naps	RM	16:36	03:38	0.71	
(clock time)	Control	15:53	03:03		0.26
	RIF	13:16	02:29		
Duration of naps	RM	00:15	11:02	0.69	
(minutes)	Control	00:12	09:58		0.86
	RIF	00:11	13:02		
Sleep time (night)	RM	07:37	48:46	0.86	
(hours:minutes)	Control	07:40	35:03		0.27
	RIF	07:24	26:56		
Morning latency	RM	00:15	11:02	0.61	

	Group	Mean	SD	P value control/RM	P value control/RIF
(O-A)	Control	00:35	42:31		0.77
	RIF	00:31	18:26		
Night time latency	RM	00:58	00:30	0.42	
(S-B)	Control	00:49	00:26		13:15
	RIF	00:41	00:32		

Table 3-2 PSQI and ESS component and total scores

	Group	N	Mean	SD	p value RM/ Control	p value RIF/ Control	p value cases/ control
PSQI component score							
Duration of Sleep	RM	32	0.50	0.76	0.55		0.95
	control	33	0.39	0.66		0.42	
	RIF	20	0.25	0.55			
Sleep Disturbance	RM	32	1.22	0.49	0.38		0.27
	control	33	1.33	0.54		0.35	
	RIF	20	1.20	0.41			
Sleep Latency	RM	32	1.31	0.93	0.69		0.95
	control	33	1.21	1.05		0.67	
	RIF	21	1.10	0.83			
Day Dysfunction due to Sleepiness	RM	32	0.72	0.77	0.79		0.61
	control	33	0.67	0.78		0.50	
	RIF	20	0.80	0.52			
Sleep Efficiency	RM	32	0.69	0.97	0.82		0.56
	control	33	0.64	0.86		0.09	
	RIF	21	0.29	0.46			
Overall Sleep Quality	RM	32	1.16	0.81	0.73		0.93
	control	33	1.09	0.72		0.49	
	RIF	20	0.95	0.69			
Total PSQI Score							
	RM	32	5.66	2.66	0.69		0.89
	control	32	5.38	2.94		0.38	
	RIF	20	4.70	2.23			
Total ESS Score							

	Group	N	Mean	SD	p value RM/ Control	p value RIF/ Control	p value cases/ control
	RM	33	6.58	4.14	0.34		0.29
	control	34	5.65	3.80		0.42	
	RIF	21	6.43	2.84			

Table 3-3 ESS sleepiness categorisations

Level of daytime sleepiness n (%)

GROUP	Lower Normal	Higher Normal	Mild Excessive	Moderate Excessive	Severe Excessive
Control	20 (58.8)	9 (26.5)	4 (11.8)	1 (2.9)	0 (0)
RM	14 (42.40	14 (42.4)	2 (6.1)	2 (6.1)	1(3)
RIF	9 (42.9)	9 (42.9)	3 (14.3)	0 (0)	0 (0)

Table 3-4 Actigraphy parameters for the different groups

		Mean	SD	P value control/RM	Mean difference control/ RM	P value control/RIF	Mean difference control/RIF
Time in bed (hours:min)	RM	08:24	01:06	0.19	00:33:26		
	Controls	08:57	00:58			0.24	00:30
	RIF	08:28	01:20				
Time to sleep onset (hours:mins)	RM	00:36	00:31	0.32	-10.52		
	Controls	00:25	00:21			0.17	00:26
	RIF	00:18	00:09				
Sleep Efficiency (%)	RM	77.12	19.31	0.31	5.85		
	Controls	82.96	8.02			0.42	1.84
	RIF	84.80	5.40				
Wake after sleep onset (WASO) (number of episodes)	RM	39.37	15.59	0.32	12.86		
	Controls	52.23	39.45			0.51	6.93
	RIF	45.30	22.33				
Length of time awake after sleep onset (hours:mins)	RM	35.24	9.80	0.46	3.54		
	Controls	38.79	12.86			0.90	0.62
	RIF	38.17	14.30				

Table 3-5 Actigraphy activity data and light exposure

Rest (non-active)

Average per day	Patient group	Mean	SD	P value RM/controls	Mean Difference	P value RIF/controls	Mean difference
Rest Duration	RM	502.36	78.81	0.12	40.72		
(minutes)	Control	543.08	66.88			0.12	44.48
	RIF	498.60	72.01				
Total activity count	RM	8544.19	4165.39	0.14	3308.59		
	Control	11852.78	9111.11			0.49	2139.27
	RIF	9713.51	4853.04				
Activity count/minute	RM	16.95	8.98	0.39	2.70		
	Control	19.65	9.41			0.82	0.86
	RIF	18.79	8.96				
Wake time	RM	55.63	23.43	0.19	14.79		
(minutes)	Control	70.43	43.51			0.38	12.84
	RIF	57.58	22.01				
Percentage wake time	RM	11.06	4.97	0.50	1.17		
(%)	Control	12.23	5.11			0.70	0.77
	RIF	11.46	4.55				

Rest (non-active)

Average per day	Patient group	Mean	SD	P value RM/controls	Mean Difference	P value RIF/controls	Mean difference
Sleep time	RM	446.71	74.46	0.26	25.95		
(minutes)	Control	472.66	50.75			0.19	31.64
	RIF	441.02	65.99				
Percentage asleep	RM	88.94	4.97	0.50	-1.17		
(%)	Control	87.77	5.11				
	RIF	88.54	4.55			0.70	-0.77
Exposure to white light	RM	482.79	952.06	0.35	524.67		
(lux-minutes)	Control	1007.46	2373.65				
	RIF	852513.38	2826077.64			0.27	-851505.91
White light exposure	RM	0.74	1.33	0.35	0.90		
(lux)	Control	1.64	4.25			0.27	-2534.60
	RIF	2536.24	8409.31				
Maximum white light exposure	RM	33.80	53.11	0.94	1.38		
	Control	35.18	51.35			0.27	-3450.53
(lux)	RIF	3485.71	11421.60				

Awake

Average per day	Patient group	Mean	SD	P value RM/controls	Mean Difference	P value RIF/controls	Mean difference
Activity duration	RM	894.18	233.64	0.62	71.09		
(minutes)	Control	965.27	611.19			0.92	20.44
	RIF	944.83	197.00				
Total activity count	RM	293723.03	92086.48	0.80	7610.83		
	Control	301333.86	74861.49			0.49	-28785.77
	RIF	330119.64	128374.67				
Activity count /minute	RM	349.17	100.31	0.96	1.78		
	Control	350.95	88.42			0.84	-8.17
	RIF	359.13	112.68				
Wake time	RM	721.24	78.35	0.47	-28.89		
(minutes)	Control	692.35	161.02			0.23	-80.89
	RIF	773.24	167.44				
Percentage wake time	RM	85.87	5.74	0.22	-6.12		
(%)	Control	79.75	22.31			0.50	-4.95
	RIF	84.70	9.01				
Sleep time	RM	172.35	207.33	0.46	103.67		

Awake

Average per day	Patient group	Mean	SD	P value RM/controls	Mean Difference	P value RIF/controls	Mean difference
(minutes)	Control	276.02	619.31			0.59	104.95
	RIF	171.06	151.95				
Percentage sleep	RM	14.13	5.74	0.93	0.20		
(%)	Control	14.33	8.97			0.79	-0.97
	RIF	15.30	9.01				
Exposure to white light	RM	444313.38	539547.42	0.58	101318.01		
(lux-minutes)	Control	342995.37	511206.51			0.30	-2174361.75
	RIF	2517357.12	7691777.00				
Average white light	RM	539.80	638.51	0.50	-143.85		
(lux)	Control	395.96	603.14			0.31	-1960.24
	RIF	2356.20	7081.44				
Maximum white light	RM	28220.22	23222.20	0.18	-10138.00		
(lux)	Control	18082.22	19798.29			0.57	-4672.01
	RIF	22754.23	20070.05				
Total time above illuminance threshold of white light	RM	59.75	70.53	0.94	-1.95		
	Control	57.80	85.33			0.67	-21.60

Awake

Average per day	Patient group	Mean	SD	P value RM/controls	Mean Difference	P value RIF/controls	Mean difference
(minutes)	RIF	79.40	157.83				

Sleep

Average per day	Patient group	Mean	SD	P value RM/controls	Mean Difference	P value RIF/controls	Mean difference
Sleep duration	RM	471.32	73.33	0.13	36.03		
(minutes)	Control	507.35	57.29			0.03*	52.72
	RIF	454.63	56.56				
Total activity count	RM	6635.57	3959.79	0.16	3036.38		
	Control	9671.95	8949.29			0.61	1664.58
	RIF	8007.37	6750.00				
Average activity counts/minute	RM	14.08	8.86	0.44	2.55		
	Control	16.63	10.53			1.00	0.01
	RIF	16.62	12.52				
Efficiency (%)	RM	85.18	5.36	0.18	-2.82		
	Control	82.37	7.07			0.80	-0.72

Sleep

Average per day	Patient group	Mean	SD	P value RM/controls	Mean Difference	P value RIF/controls	Mean difference
	RIF	83.08	6.66				
Wake time	RM	43.95	22.02	0.25	12.43		
(minutes)	Control	56.38	42.28			0.35	13.58
	RIF	42.80	23.99				
Percentage awake	RM	9.39	5.14	0.62	0.92		
(%)	Control	10.31	5.82			0.67	0.97
	RIF	9.34	5.09				
Sleep time	RM	427.36	70.99	0.27	23.61		
(minutes)	Control	450.97	45.23			0.06	39.15
	RIF	411.82	52.88				
Percent sleep	RM	90.61	5.14	0.62	-0.92		
(%)	Control	89.69	5.82			0.67	-0.97
	RIF	90.66	5.09				
Exposure to white light	RM	275.81	440.78	0.26	464.42		
(lux-minutes)	Control	740.23	1862.92			0.27	-720327.20
	RIF	721067.42	2390583.2				

Sleep

Average per day	Patient group	Mean	SD	P value RM/controls	Mean Difference	P value RIF/controls	Mean difference
			2				
Average white light exposure	RM	0.45	0.67	0.28	0.94		
(lux)	Control	1.39	4.01			0.27	-2522.33
	RIF	2523.72	8368.40				
Maximum white light exposure	RM	17.10	19.51	0.67	5.01		
	Control	22.12	49.48			0.27	-3465.73
(lux)	RIF	3487.85	11420.42				

Table 3-6 MANOVA (Cosinor rhythmometry) with Fourier fitting to the activity data

Number of sinusoids	P value RM/controls	P value RIF/controls
1	0.34	0.93
2	0.20	0.43
3	0.33	0.43
4	0.68	0.49
5	0.48	0.37
6	0.46	0.42
7	0.30	0.67
8	0.51	0.74

Chapter 4 Biological rhythms and the uterine environment

4.1 Implantation

An endometrium, which allows implantation of the embryo, is fundamental to pregnancy success. The human endometrium undergoes hormone-driven cyclical changes in order to support a potential pregnancy. In the pre-ovulatory phase, oestrogen primes the endometrium and stimulates proliferation of uterine epithelial cells and expression of oestrogen and progesterone receptors. After ovulation, the corpus luteum produces progesterone and decidualises the endometrium; thereby transforming the endometrium to a secretory organ whilst bringing about vascular remodelling and attracting immunomodulatory cells.

In humans, decidualisation of the stromal compartment occurs in the mid-luteal phase of the menstrual cycle, independent of pregnancy (see Figure 1-7), whereas in rodents this response only happens in the event of pregnancy. Not only does this mean that an intricate timing system must come into play, but as opposed to in some animals, biochemical analysis of timed endometrial biopsy samples taken in a non-conception cycle in humans can be informative of subsequent pregnancy outcome. The failure to achieve pregnancy when this synchrony is disrupted could be a contributory factor in failed implantation. The underlying mechanism behind this is not fully understood but there are clock-controlled genes within the peripheral reproductive system which could be gate-keepers (see 1.6). It is also clear that the peri-implantation environment extends beyond the timing of implantation, by providing appropriate nutrition to the developing embryo and influencing its long term future by developmental epigenetic programming (309, 310).

4.1.1 Developing an endometrial model

Embryo implantation is dependent on a complex interaction between the developing embryo and the maternal uterine cells. The underlying association between abnormal implantation and reproductive failure is evident, and thought to be at least in part, due in part to the underlying tissue of the endometrium. Practical and ethical considerations make embryonic implantation challenging to study *in vivo* in humans and no good representative animal model has been identified. However, in recent years, substitute *in-vitro* models have been shown to provide a promising way forward in our understanding of the implantation processes (174). Whilst *in vitro* models have

their limitations, using these systems will improve our understanding of the molecular mechanisms at the implantation site.

The highly dynamic nature of the human endometrium is well demonstrated by its response to the rise and fall in ovarian hormones (see 1.7.2). It proliferates, differentiates, sheds and regenerates many hundred times during reproductive years. The endometrium consists of a single layer of columnar epithelium sited upon underlying stromal cells, a layer of connective tissue that varies in thickness according to endocrine influences. In a woman of reproductive age, two layers of endometrium can be distinguished. The uppermost, 'functional layer', is comprised of adult stem cells and migratory resident cells. These are thought to contribute to the coordinated influx of specialised immune cells, controlled inflammation and angiogenesis, needed to bring about cyclical endometrial changes. The importance of migratory and invasive endometrial stromal cells (ESCs) is increasingly recognised as contributing factors to tissue remodelling, trophoblast invasion and endometrial regeneration, as well as acting as regulators at the implantation site (311).

The aim of this study was to investigate the biological cycles of implantation as controlled by the endometrium. To this end, in vivo tissue samples of endometrium, endometrial uterine secretions and an endometrial stromal cell (ESC) were used to interrogate biological rhythm-markers and investigations of potential discrepancies between the endometrium of women with reproductive pathology and healthy women were conducted. Continuing on from this, attempts were made to manipulate HESC (human endometrial stromal cells), with an aim to investigate the possibility of affecting reproductive outcomes.

4.2 Introduction

Monthly human fecundity rates in fertile couples are approximately 20%, which is considerably lower than many species (312). This relative inefficiency of human reproduction has raised questions regarding the natural barriers to human fertility and spurred interest in to the nature of embryo-uterine signalling and the factors determining the health of the embryo and uterine receptivity. The focus of study is often on the embryo, and rarely on endometrial tissue or the peri-implantation environment. Successful implantation requires molecular events resulting in a receptive endometrium, a normal and functional embryo at the blastocyst stage, and a synchronised dialogue between maternal and embryonic tissues. The complexity of embryo implantation and placentation is demonstrated by the number of immunomodulatory compounds, cytokines and growth factors, with demonstrated roles in these processes. Disturbances in the normal expression of these

immunomodulatory compounds may result in an absolute or partial failure of implantation (313, 314). In support of this concept asynchronous glands are seen in the endometrium of some women with recurrent reproductive failure (RIF) (315).

Interest in interrogating the constituent compounds of endometrial secretions has grown because it has been shown to offer a minimally invasive means of studying endometrial function (316). In addition, its close proximity to the endometrium is expected to accurately and rapidly reflect any changes of the juxtaposed tissue. Endometrial secretions obtained from women in fresh IVF cycles during embryo transfer have a predictive, though not sufficiently discriminatory profile for implantation (317). Whilst certain proteins have been postulated as important regulators of endometrial receptivity (318), there remains a discrepancy between established implantation factors (for example leukaemia inhibitory factor (LIF) (319) and proposed, 'molecular fingerprints' of implantation (317).

These inconsistencies remain, possibly because of the confounding effects of clinical heterogeneity in non-specific study populations, comparative differences in study design, analysis techniques and the methodology employed in previous studies. Boomsma et al 2009 (317) and Olivennes 2003 (320) performed studies on stimulated and Ledee-Bataille 2014 (321) and Hannan 2011(322) unstimulated IVF cycles; Fitzgerald 2016 (323), and Heng 2011 (324) performed studies on general fertility populations with fertile controls; Ulbrich 2009 (325) Gray 2001 (313) Spencer 2006 (314) Filant 2013 (326), were all based on animal models. It is plausible that the intricate differences of uterine receptivity between non-specific populations and that during a stimulated IVF cycle are heavily confounded. In addition, studies evaluating the proteome of uterine secretions while informative, are limited to proteins commonly found and have identified non-consensual protein repertoires (327). Whilst it has been possible to categorise cellular functions key for implantation, specific more sensitive protein analyses are still necessary to detail the complex network of interactions involved with implantation. Immunomodulatory proteins including cytokines, chemokines and growth factors play diverse roles in coordinating successful embryo implantation (317, 328, 329).

Many aspects of reproductive function are strongly circadian and the timing of events in female reproductive physiology depends on the hierarchical control of the hypothalamo-pituitary-gonadal axis. In mammals the central circadian 'clock', the suprachiasmatic nuclei (SCN) of the hypothalamus is entrained (synchronised) to the 24 hour day (330). This synchronises circadian oscillators in peripheral tissues (331). However, a cellular clock persists without the central control as an auto regulatory transcriptional feedback loop. This molecular clock is composed of 'clock genes' which

code for basic-helix-loop-helix (bHLH)-PAS transcription factors. Two such genes, *bmal1* (brain and muscle arylhydrocarbon receptor nuclear translocator-like protein) and *clock* (circadian locomotor output cycles kaput), form a heterodimer and bind to E-box (CACGTC) promoters of various clock controlled genes such as *period 1, 2* (*per1, per2*) and *cryptochrome 1, 2*(*cry1, 2*) to induce their transcription (332). Upon translation, the Per and Cry proteins inhibit *clock-bmal1*-transcription in a negative feedback manner (333).

Female reproductive structures contain circadian clocks with clock genes are expressed throughout the hypothalamic-pituitary-gonadal axis (147). Changes in circadian rhythms have been linked to reproductive pathology in animal knockout studies as female mice carrying mutations of clock or *bmal1* display disrupted oestrous cycles under both a light and dark cycles and during continuous darkness (132, 334). In humans, women with single-nucleotide polymorphisms in *bmal1* may have more miscarriages and fewer pregnancies (128). Although the expression of genes within the human endometrium is poorly understood, clock genes are expressed in endometrial stromal cells (159).

The cellular clock operates within the immune system, and this explains how responses to inflammatory stimuli show circadian variation, including timings of leukocyte trafficking and inflammatory signalling (335). Levels of inflammatory markers show robust circadian rhythmicity (336) and alterations of the clock is capable of producing both anti and pro inflammatory consequences (337-339). The importance of circadian regulation of immune function has been highlighted by experiments in which circadian rhythms are disrupted, leading to increased susceptibility to infections and increased inflammatory disease states. This re-affirms the possibility of the circadian clock as a therapeutic target for inflammatory disorders such as RIF.

Melatonin is involved in the phasing of circadian rhythms and its cyclical release has pleiotropic effects. It is thought to be involved in reproductive functioning, and this effect could be mediated via the endometrium in humans. Melatonin is thought to regulate female reproduction due to its receptor-mediated and receptor-independent antioxidant actions (340). There has been some work done on the relevance of melatonin in oocyte and sperm quality (83-87). It has also been considered as an additive in embryo culture media (88) and its effects on luteal function have demonstrated increased levels of progesterone after melatonin supplementation (89, 90). The positive implications of higher melatonin levels on the human menstrual cycle, fertility and pregnancy are therefore well documented but with varying levels of evidence (91-93). Nevertheless, it appears that melatonin serves a purpose in the

human reproductive system, with many of its observed effects not yet being linked to circadian functioning.

Night light exposure because of shift work, has been shown to cause decreases in melatonin levels (299) with an increased risk for certain poor reproductive outcomes (246). The role of melatonin on circadian rhythm clock components is not well understood and few studies have investigated the role of melatonin on circadian rhythm clock components (341). In the current study, we explore the hypothesis that melatonin may resynchronise the deregulated core clock circuitry in patients with RIF.

Women suffering RIF, by definition have an apparently normal uterus. Despite clear efforts in embryology and transferring embryos which display optimal morphological features, the treatment for RIF continues to be elusive and remains mystifying to both clinicians and scientists alike especially when there is no good clinical tool to predict these women's treatment outcome. Therefore, tools that are minimally invasive and predictive of women who achieve implantation and those who have recurrent implantation failures are urgently needed to guide further clinical decisions and patient management. In order to achieve reliable prediction of RIF, better understanding of the *in-vivo* immunomodulatory milieu is needed. Endometrial secretion profiles may serve to discriminate women with higher RIF propensity and delineate plausible mechanisms underlying RIF. Subsequently, therapies designed to modify this environment may provide effective treatment for this group of women for which at present, no robust evidence-based nor effective treatment exists.

4.2.1 Objectives

1. To describe the immunomodulatory profile in endometrial secretions of women with recurrent implantation failure compared with those of fertile controls in unstimulated, normal menstrual cycles.
2. To establish whether clock genes are expressed in a rhythmic manner in the same way in the endometrium of fertile women and those with RIF and whether decidualisation of the cells has an impact on this expression.
3. To determine whether clock gene expression is associated with the cytokine profile of endometrial secretions.
4. If there is a difference in the clock gene and/or cytokine expression in women with RIF compared with control women and whether this can this be altered by melatonin.

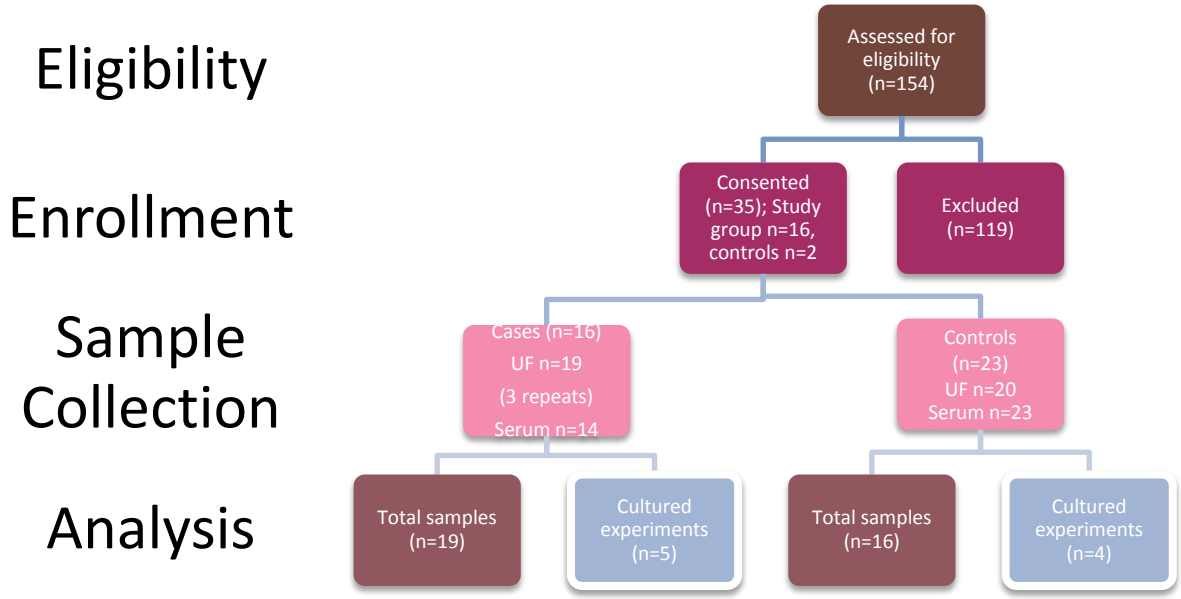
4.3 Methods

4.3.1 Design and setting

A prospective case-control study conducted in an IVF centre in the United Kingdom, according to a predetermined protocol and reported in accordance with the STROBE Statement (342). For flow of participants through the study see Figure 4-1.

Figure 4-1 Strobe flowchart

To demonstrate the flow of participants through the study



4.3.2 Participants

The study was conducted under local ethical (REC number 12/SC/0548) and R&D approval (RHM O&G 0197). Patients were recruited from either elective gynaecological theatre day lists or in Vitro-Fertilisation (IVF) clinics at Princess Anne Hospital, Southampton, UK in accordance with local guidelines. Informed consent was gained from all participants and baseline demographics and fertility characteristics were collected. Women were excluded from both groups if they were using hormones in the three months prior to sample collection.

Eligible participants were women aged between 25-45 years, attending for gynaecological procedures or fertility clinics from August 2012 to December 2013. Baseline demographics and fertility characteristics were collected for all patients (N=36). There were two independent groups. The first were women who had suffered with recurrent implantation failure (women with failure of clinical pregnancy following the transfer of three or more good-quality fresh or frozen embryos transferred over two or more IVF or ICSI cycles) (n=16). The second group, who served as controls, were women who were attending for elective procedures for non-endometrial pathology (n=15) and has had at least one full term pregnancy ending in live birth without a history of recurrent implantation failure or recurrent miscarriage (n=20).

4.3.3 Endometrial fluid collection

Endometrial fluid samples were taken from all patients using an empty embryo transfer catheter (Cook™, Guardia, embryo transfer catheter, USA) as previously described (317). Samples were snap frozen in the collection tubing of the catheter and stored at -80˚C until use. The catheter tubing was flushed with saline and the fluid volume measured before processing. Endometrial secretions were aspirated from 36 women (16 cases, 20 controls). Immediately following uterine fluid collection, whole blood was collected from each patient in a covered test tube and allowed to clot at room temperature for 30 minutes. The sample was centrifuged at 1800g for 10 minutes at 6°C and the resulting supernatant, the serum, was immediately transferred into a clean polypropylene tube, apportioned into 0.5 ml aliquots and stored at −80˚C until use.

4.3.4 Immunoassays

Multi-analyte profiling immunoassays were performed on endometrial fluid and serum samples. High-throughput technology (comparable to ELISA) was performed on the multiplex platform, 'Luminex technology xMAP', to assay each sample (Ebioscience, San Diego, USA). Samples were diluted by a factor of 10 and each sample run in

duplicate. 45 cytokines, chemokines and growth factors (herein referred to as immunomodulatory compounds) were measured. Standard curves were optimised (Bioplex manager and manual verification) and five-parameter logistic regression modelling performed on the Bioplex system (BioRad, California, USA).

4.3.5 Tissue collection

Endometrial biopsies were taken from all patients by suction curette (Pipelle device, Laboratoire CCD, Paris, France) and either collected into general culture media and stored on ice until washing with a small amount of serum free DMEM or washed in sterile saline, divided into uniform size (10mm lengths) and snap frozen and stored at -80°C in separate aliquots or used fresh for primary cell culture.

4.3.6 Serum collection

Whole blood samples were collected within an hour of endometrial tissue/fluid sampling by standard phlebotomy techniques and allowed to clot at room temperature. Cells were removed by centrifugation and the supernatant stored at -80°C until use.

4.3.7 Tissue culture

4.3.7.1 Isolation and preparation of cells

Endometrial stromal cells were isolated from endometrial tissues as described previously (180, 343). In short, endometrial tissue was enzymatically digested in 10 ml 417 U/ml collagenase type IA (Sigma, UK) for an hour (37°C under atmospheric oxygen levels and 5% CO2). To stop collagenase action, the digested tissue was placed in DMEM/F12 medium (Gibco, UK) supplemented with 10% heat-inactivated foetal bovine serum (FCS). The cells were cultured in standard medium (digest medium supplemented with 10% FCS (Gibco) and after three hours media was replaced to out select the glands. Purified HESCs were expanded in maintenance medium of DMEM/F12 containing 10% charcoal-treated foetal bovine serum (FBS; Gibco, UK), L-glutamine (1%; Gibco, UK), 1% Penicillin Streptomycin (Gibco, UK), 1% Amphotericin B (Sigma, UK). Confluent monolayers were decidualised in DMEM/F-12 containing 2% FBS with 0.5mM 8-bromoadenosine-cAMP (8-br-cAMP; Sigma- Aldrich) with or without 1026 M medroxyprogesterone acetate (MPA; Sigma-Aldrich) to induce a differentiated phenotype. For synchronization of cells, dexamethasone (Sigma-Aldrich) was used at 100 M for 30 minutes (159). All experiments were carried out before the fifth cell passage. Cells were grown to 60% confluence and then treated with 100 μM melatonin (Enzo Life Sciences, PA; dissolved in a few drops of 100% ethanol and brought to

desired concentration with DMEM) for the 24 hours prior to, and the duration of experimentation (341). A subset of nine patient's endometrial stromal cells were used for the clock gene experiments (case n=5, controls n=4). Cells and media were sampled and analysed every four hours for 36 hours. The time of first sampling was termed time point zero and each time point was referred to by hours elapsed since initial sampling, the final sampling being 36 hours. This should not be confused with zeitgeber time (ZT) in free running *in vivo* experiments.

4.3.7.2 Freezing cells

Cells were harvested for cryopreservation with 10% dimethyl sulfoxide (British Drug House, UK) before storing at a density of 100 000 cells/ml at -80ºC. Quick-thawing of the cells was performed, pooling if needed. The appearance of the cells was checked and the media changed at 24 hours.

4.3.7.3 Characterising endometrial cells

Characterisation of cells was performed by three separate methods and at each passage of cells in order to confidently define the cell population as ESC in the established endometrial cultures.

1. Immunocytochemisty (ICC)
2. Immunofluorescence (IF)
3. Fluorescent automated cell sorting (FACS)

4.3.7.3.1 Immunocytochemistry and Immunofluorescence

For reagents and suppliers details see Appendix. Discrimination between expected cell types (ESC from contaminant cells) was performed using antibodies to vimentin (a stromal cell marker). As the most likely contaminant cell would be epithelial endometrial cells, cytokeratin (an epithelial marker) antibodies were also used (344).

4.3.7.3.1.1 Immunocytochemistry (ICC)

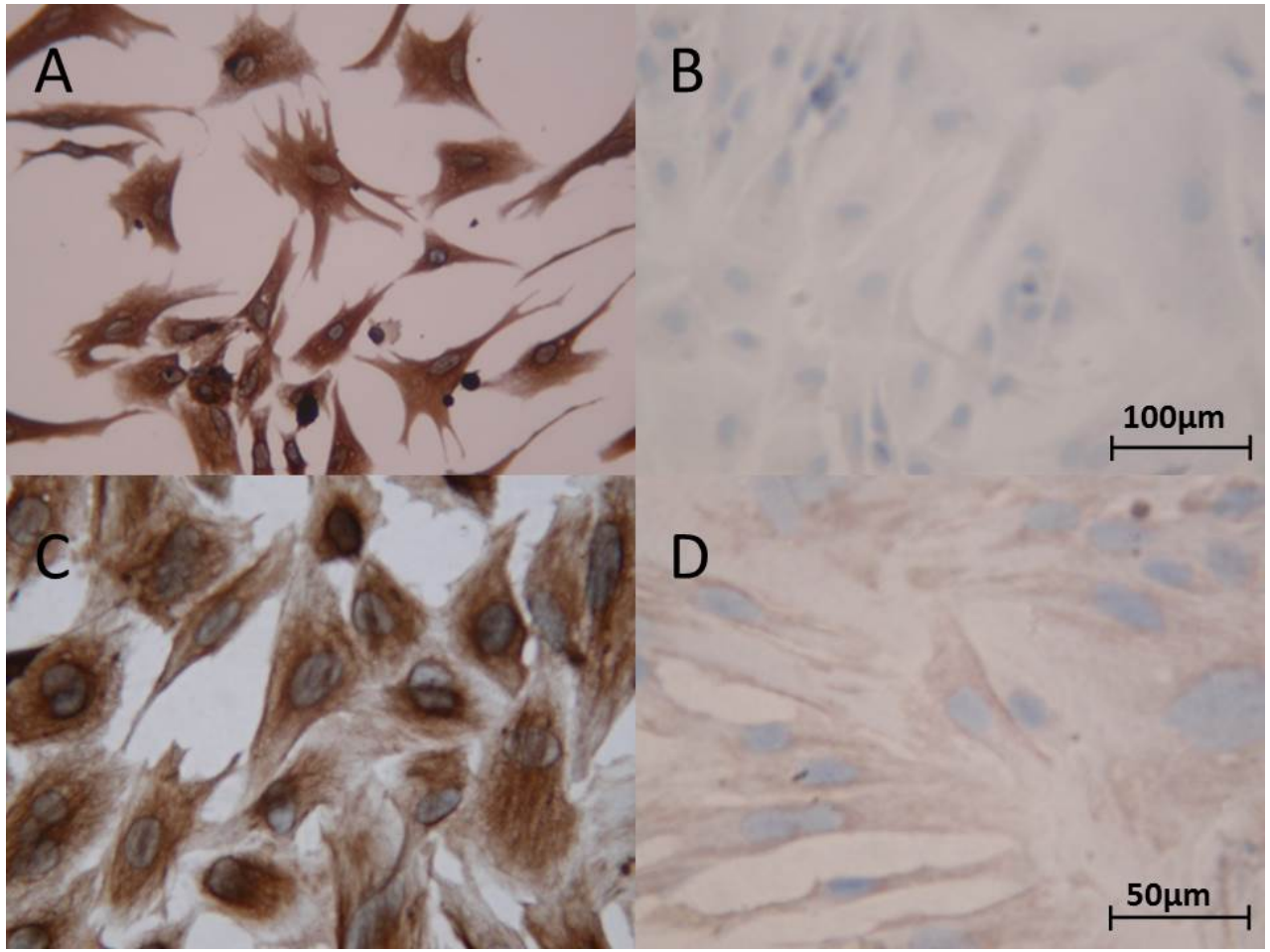
ICC was performed in chamber slides in which cells had been plated at standard density and cultured until confluent. Cells were fixed with methanol before peroxidase blocking. Incubation with primary antibodies (vimentin (VM) (1:1000, VM, mouse mAb Clone V9) and cytokeratin-7 (CK-7) (1:22, mouse mAb Clone OV-TL)) was performed. Positive controls were created without antibody, and negative controls (without cells). Secondary antibody incubation (biotinylated rabbit anti-mouse antibody) followed repeat blocking. The Avidin Biotin Complex method was used (Vectastain® ABC-Elite-Universal:HRP Mouse/Rabbit IgG Kit), prior to application of the

chromagen (DAB, Vector® DAB (3,3-diaminobenzidine). Cells were counterstained with Mayer's haematoxylin.

ESC stained positively with VIM (Figure 4-2, A and C) and negatively with CK-7, an epithelial marker(Figure 4-4, B and D). There was no difference between the appearance of the patient groups, nor number of passages. All cells experimented with resulted in a positive staining and were of similar morphology.

Figure 4-2: ICC staining of ESC

ICC staining of the ESC to ensure the purity of the cells. Slides A and C show staining with vimentin (positive stain, brown), Slides B and D show staining with cytokeratin-7 (negative stain) (A-B x20, C-Dx40). It can be seen that the majority of the cells stain positive for the ESC marker but negative for other cell types.



4.3.7.3.1.2 Immunofluorescence (IF)

For IF studies, cells were plated at standard density and cultured until confluent in 96 well plates. Primary antibodies were goat polyclonal against Vimentin (VIM) (1:50; sc-7558V, Santa Cruz, USA) and rabbit polyclonal against Cytokeratin (CK) (1:50; sc-15367, Santa Cruz, USA). Cells were fixed with 4% paraformaldehyde and permeabilised (1% Triton-100) before blocking prior to incubation with the primary antibody. Re-blocking and secondary antibody incubation was undertaken before the addition of DAPI. Staining was evaluated with a florescent microscope (UIS2, Olympus). ESC stained positive for VM (Figure 4-3) and negative for CK (Figure 4-4), with similar staining in both RIF and control patients and in each passage used for subsequent experiments. There also did not appear to be a difference in staining intensity depending on time of sample or time in the menstrual cycle. All cells resulted in a positive staining and all cells were of similar morphology.

Figure 4-3: VM staining of ESC

Immunoflourescent staining of the ESC to ensure the purity of the cells. Picture A shows the cells stained with DAPI, B shows the cells with a red filter, and C. shows slides A&B overlain. It can be seen that there is staining of ESC only.

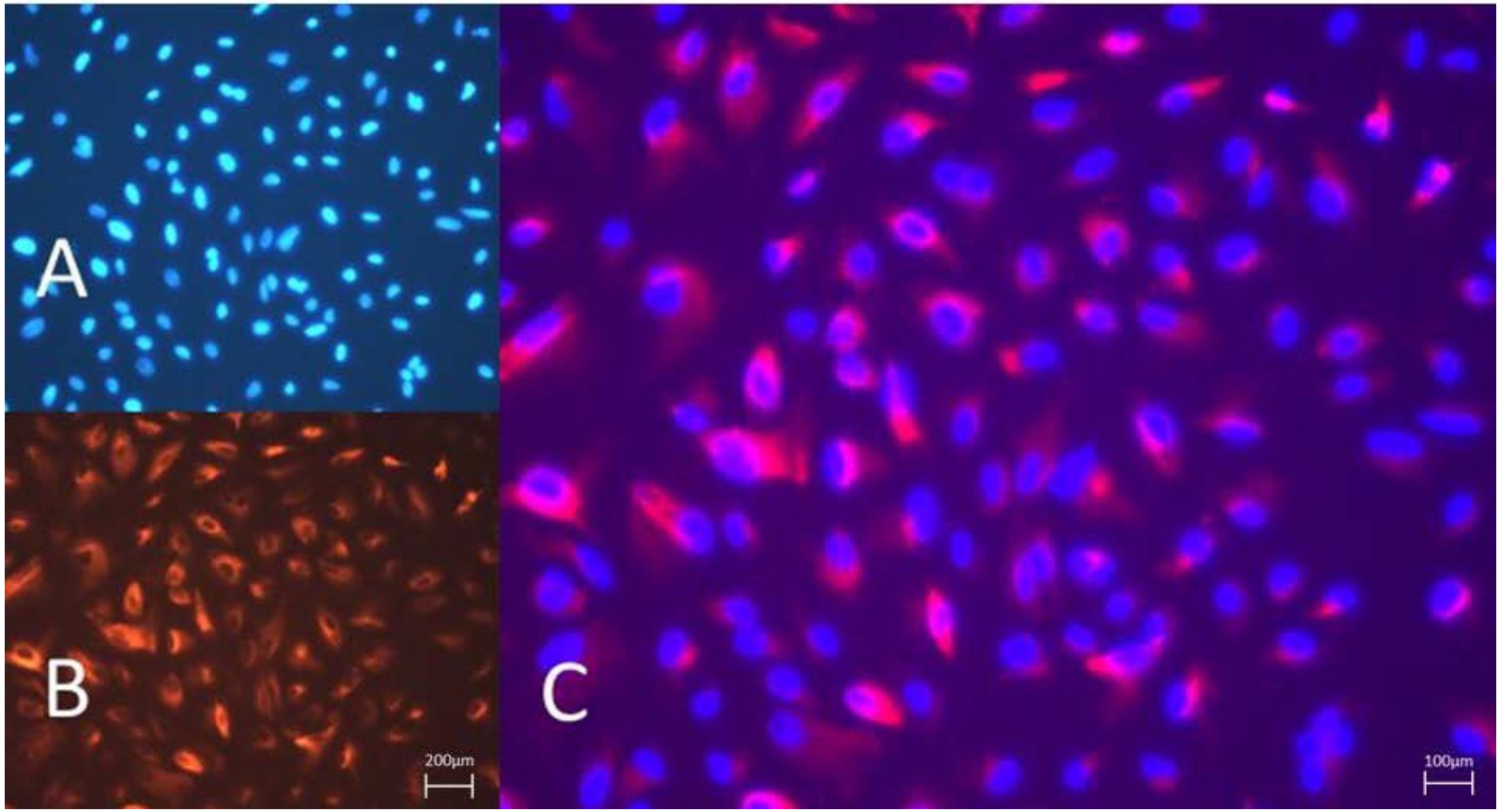
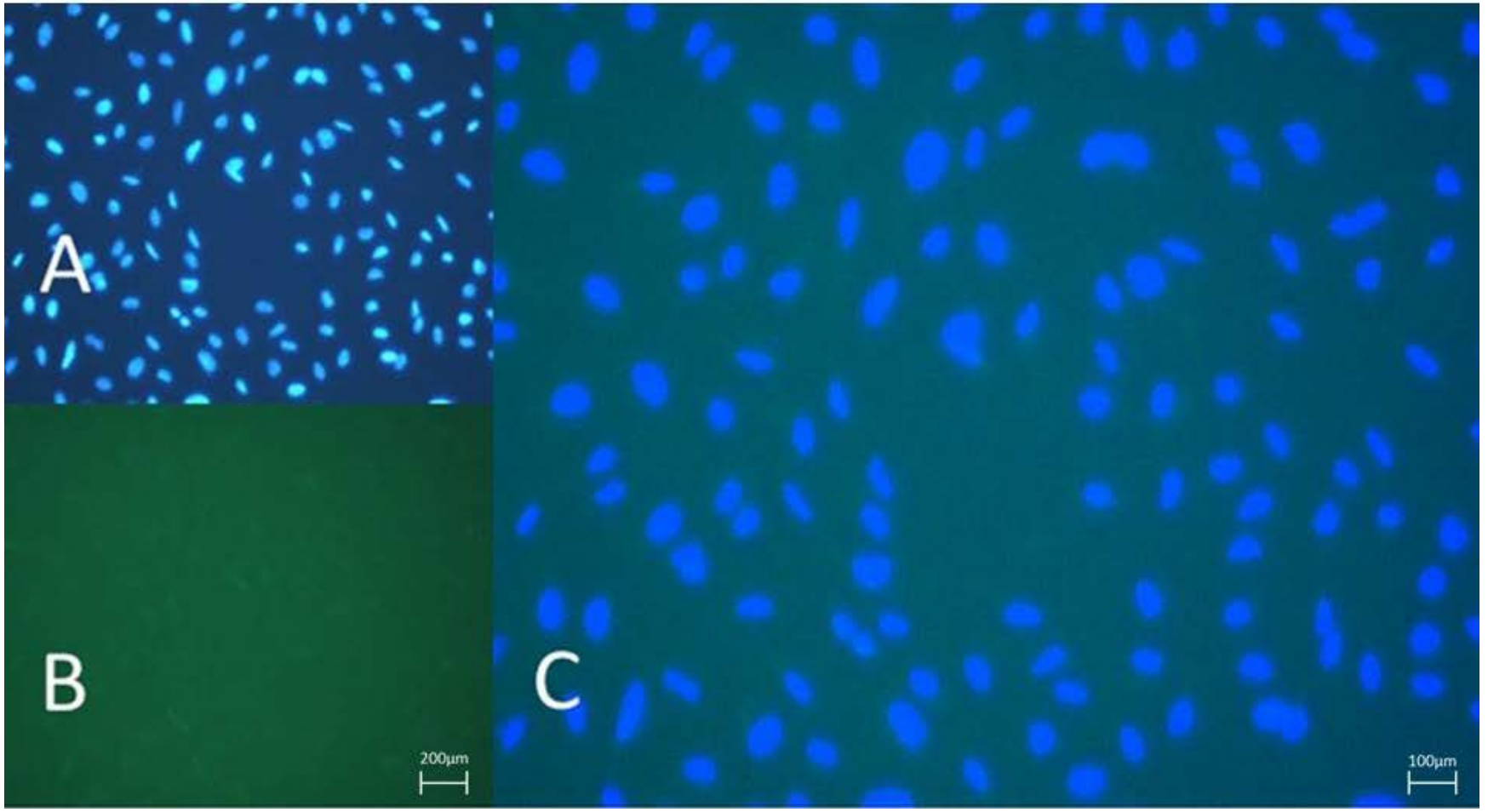


Figure 4-4: CK of ESC

Immunoflourescent staining of the ESC to ensure the purity of the cells. Picture A shows the cells stained with DAPI, B shows the cells with a green filter, and C. shows slides A&B overlain. It can be seen that there is staining of ESC only.



4.3.7.3.2 Fluorescent Automated Cell Sorting (FACS)

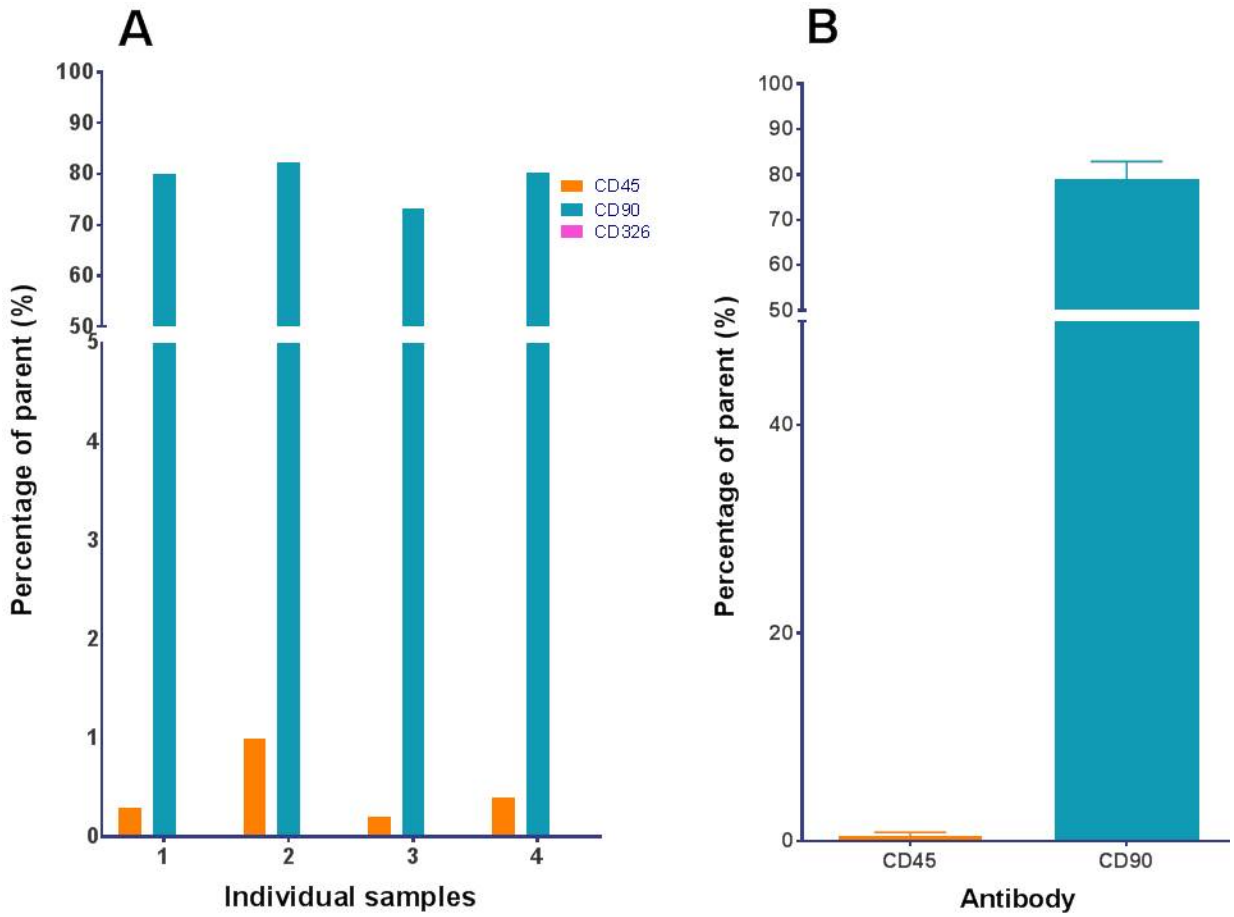
The principle of FACS is that a single cell stream is passed through a light beam. Detectors in line with the light beam (forward scatter; cell size) and perpendicular to it (side scatter; cell granularity) detect the amount of disruption to the beam. One or more fluorescently marked antibodies are tagged to the cells and are simultaneously detected by the machine. This method was used to provide an output of cell number based on the size, granularity and fluorescent marking of the cells. Epithelial cells of the uterus express EpCAM (345) (346), stromal cells CD90 (347) and CD45 is a well-recognised leucocyte marker (348).

HESC of four different patient samples (two RIF, two controls, both decidualised and non decidualised) were digested to single-cell suspensions using collagenase. Antibodies were selected to label epithelial cells (anti-EpCAM PerCP Cy5.5 (BD Biosciences)), leucocytes (anti-CD45 PECF594 BD (Biosciences)) or stromal-type fibroblast (1:10, anti-CD90 APC (BD Biosciences). ESC were chelated by EDTA before the addition of human IgG. Isotype controls were similarly fluorescently labelled (IgG2a PercPCy5.5, IgG1 APC and IgG2a PECF594). ECS were then incubated with the antibodies on ice before performing flow cytometry (polystyrene tubes).

Across the different populations of cells studies uniform expression of CD90 was demonstrated. This suggests that the majority of endometrial cells are stromal in origin. Figure 4-5 demonstrates that the majority of the parent cells (total number of cells analysed) were positively labelled with CD90, and therefore are ESC in origin. There were no cells which were positively labelled with CD326, and very few with CD45.

Figure 4-5 Relative expression of antibodies (FACS)

Fluorescent Automated Cell Sorting of ESC was performed, again to check for cell purity. Four separate patients were used and (A) demonstrates the different cell subtypes in each patient and it can be seen the majority and CD 90, the ESC marker. (B) Shows overall cell counts and the cell type in all samples combined was extremely pure.



4.3.8 RNA extraction and cDNA synthesis

Total RNA was extracted using the TRIzol (Thermo Fisher Scientific, USA). The A260/280 ratio of each sample was measured using mass spectrometry (NanoDrop; Thermo Fisher Scientific USA) and the total RNA concentration for each sample was calculated. Integrity of mRNA was checked by running RNA on denaturing agarose gel stained with ethidium bromide (EtBr) to ensure the appearance of 26S and 18S bands. Total RNA was reverse transcribed to produce cDNA (Precision nanoScript RT kit; Primerdesign Ltd, UK) with Oligo-dT primers. The mRNA expression of the six core clock genes *(bmal1, clock, cry1, cry2, per1, per2)* were measured by qRT-PCR using primers and probes shown in Table 4-1, and normalized against two housekeeping genes *enox2* and *prdm4 (349)*. All samples were measured in duplicate using a LightCycler 480 Instrument (Roche Diagnostics, Germany). The optimised cycle parameters were 95°C for 2min, followed by 40 cycles of 95°C for 15 seconds, 60°C for 60s.

Quantification cycle (Cq) values were transformed into relative quantification data using the delta-delta-CT method (350). These were then converted into relative quantities (RQs) by calculating the average Cq value for replicates and using the average Cq for the given gene and the amplification efficiency. These were normalized to two housekeeping genes previously shown to be stable in endometrial tissues using geNorm software (qbasePLUS, Version: 2.6.1, Southampton UK) (349, 351).

4.3.9 Histology and immunohistochemistry (IHC)

10µm sections of endometrium were prepared on a Leica cryo microtome and placed on saline-coated slides. For paraffin sections, endometrium was fixed in 4% PFA overnight, dehydrated in a graded series of ethanol, and embedded in paraffin for further assessments. 5µm sections were prepared and placed on saline-coated slides. Sections were stained with haematoxylin and eosin (H&E) for basic histological analysis.

For IHC studies the endometrium sections were fixed in acetone and permeabilised in 3% H_2O_2, prior to the blocking of non-specificity. In the paraffin sections, the endometrium was rehydrated, permeabilised in 3% H_2O_2 and antigen retrieved with hot citrate buffer. Blocking was performed prior to incubation with the primary antibody and then incubated with secondary antibody (goat anti-rabbit (HRP), ab6721, Abcam, Cambridge, UK). Sections were developed with DAB (Dako). The slides were dehydrated and mounted with DPX for microscopic analysis. Primary antibody for rabbit polyclonal antibodies against BMAL1 (1:200; ab93806, Abcam, Cambridge, UK) and rabbit

polyclonal antibodies against CLOCK (1:1000; ab134165, Abcam, Cambridge, UK) were used. The slides incubated without primary antibody or using IgG primary antibody (sc-2027, Santa Cruz, USA) were served as the negative control. Slides were mounted with Histomount (Shandon, USA) and evaluated with a differential interference contrast microscope (DMR, Leica) with a colour digital camera (DFC420c, Leica).

4.3.10 Statistics and data analysis

Data handling was performed using PRISM Version 6.0a (2012) (GraphPad Software, Inc. USA) and SPSS Version 21 (2012) (IBM, United Kingdom). Group differences of categorical variables were evaluated using the Chi-squared test and continuous data with independent samples t-test. Bivariate correlations were drawn by Pearson product-moment correlation coefficient test and Analysis Of Covariance (ANOVA) used to adjust for confounders. Statistical significance was set at $P<0.05$. Data values are represented as mean ± standard deviation (SD) or percentage (%). Where data was missing, that patient was excluded from that particular analysis. Circadian time, unless specified are measured in units of hours. Period was measured as a proportion of 24 hours in Circadian Units (CU).

Non-linear regression was used to compare the relationship of immunomodulatory markers over time with the clock gene expression. PLS modelling was performed using Unscrambler X Version 10.1 (CAMO software, Norway). This method of PLS indicate what percentage of the variance in an original variable is explained by a factor.

Non-linear curve fitting was performed using PRISM Version 6.0a (2012) (GraphPad Software, Inc. USA). The least squares model of logistic regression was applied where:

$$Y = Amplitude * \exp(K * X) * \sin((2 * pi * X/Wavelength) + PhaseShift) + baseline + Amplitude2 * \exp(-H * X)$$

Cosinor circadian analysis was performed using the Biodare website (https://biodare2.ed.ac.uk, 2016 BioDare2, University of Edinburgh). The MFourierFit (MFF) method was used for curve-fitting because phase of entrainment was of particular interest and MFF enforces the same waveform for each cycle and so gives the most accurate period (352). The MFF model function consists of a main cosine component of phase (φ), amplitude (A) and period (τ). The 'phase by fit method', was used with one cosine function having period matching the estimated data period is fitted to the data. This fitting procedure finds phase and amplitude parameters of the cosine that follows the data the most closely, and reports those values as phase and amplitude. The main advantage of MFF is that it provides the same best-fit waveform for each cycle, which better reflects the underlying biology of an entrained system (353). In addition, Maximum Entropy Spectral Analysis (MESA) was applied to the data

set. MESA uses a completely different approach based on stochastic modelling and was therefore used as an independent validator (354). The main advantages of MESA are that it does not model data assuming any a priori shape of waveform (so is less sensitive to the trends in the data), and it has much better precision than Fourier transform based methods (355).

4.3.11 Ethical approval

All patients gave informed written consent prior to taking part in this study and ethical approval was granted by the local ethics committee, Regional Ethics Committee number 12/SC/0568 and registered with the Research and Development department in the hospital.

4.3.12 Minimising bias

All samples were blinded from point of sample collection for sample measurement and interpretation. Results were only unblinded for data interpretation.

4.1 Results

In total, 41 women were recruited, 24 controls women and 17 women with RIF. The demographic data for these women may be seen in Table 4-2. Of these women all had endometrial tissue samples taken but there was failure to obtain uterine fluid and paired serum samples in five women. The demographic data for these two groups has been separated in Table 4-2 and Table 4-3. Overall, the women included in the study had age ranged between 21-45 years (mean 35.58 ± 5.41) and had an average BMI of 27.11 ± 4.51 (range 20-39kg/m^2). Most women were of British Caucasian origin (31, 86.1%) and non-smokers (31, 83.8%). The demographics did not differ significantly between groups Table 4-2 and Table 4-3). The two women aged over 40 years were in the control group and neither was used in the *in vitro* studies. No recruited women reported discomfort or side effects of the aspiration or when endometrial samples were obtained.

The study group of women (n=17) had been subfertile for between 2 and 13 years (mean 6.19 years ± 3.40, see Figure 4-6) and undergone between two and six In vitro fertilization (IVF) or Intra Cytoplasmic Sperm Injection (ICSI) cycles (mean 3.18 ± 1.52) and had on average 5.88 (± 3.63) embryos transferred in total. These cycles comprised of IVF in 7 (43.8%) and ICSI in 9 cases (56.3%). Of these, the majority were fresh, as opposed to frozen cycles (average number of fresh cycles 1.94 ± 1.44 (see Table 4-4). The embryo quality data can be seen in Figure 4-7. The average blastocyst transfer rate was 51.4%, (37/72), which is in-keeping with the fertility unit average (54.4%, Complete Fertility Southampton 2016 data). It is thought that blastocyst, as opposed to cleavage stage transfer (day 2-3), is better for both uterine and embryonic synchronicity and better self-selection of viable embryos. In fresh transfers, it is thought that fewer women will achieve live birth after fresh cleavage stage transfer than after fresh blastocyst stage, although the evidence behind this is weak (odds ratio (OR) 1.48, 95% confidence interval (CI) 1.20 to 1.82, 1630 women, I^2 = 45%)(356).

The total volume of endometrial fluid collected ranged from 1-12µl (5.0µl ± 2.58µl), with no difference in volume between the study group (4.44µl ± 2.19µl) and controls (5.45µl ± 2.584µl) (P=0.25). IL-1α, IL-2, IL-4, IL-5, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-21, IL-22, IL-23, IL-27, IL-31, GM-CSF, TNFα, TNFβ, IFNα, IFNβ, PlGF-1 and VEGF-D were removed because >50% of the data were below detection limits.

Figure 4-6 Subfertility characteristics of RIF women

The length of subfertility of the woman in the RIF group may be seen in this bar chart. There is a positively skewed distribution, with the modal length of subfertility being four years, the minimum two years and the maximum 13 years.

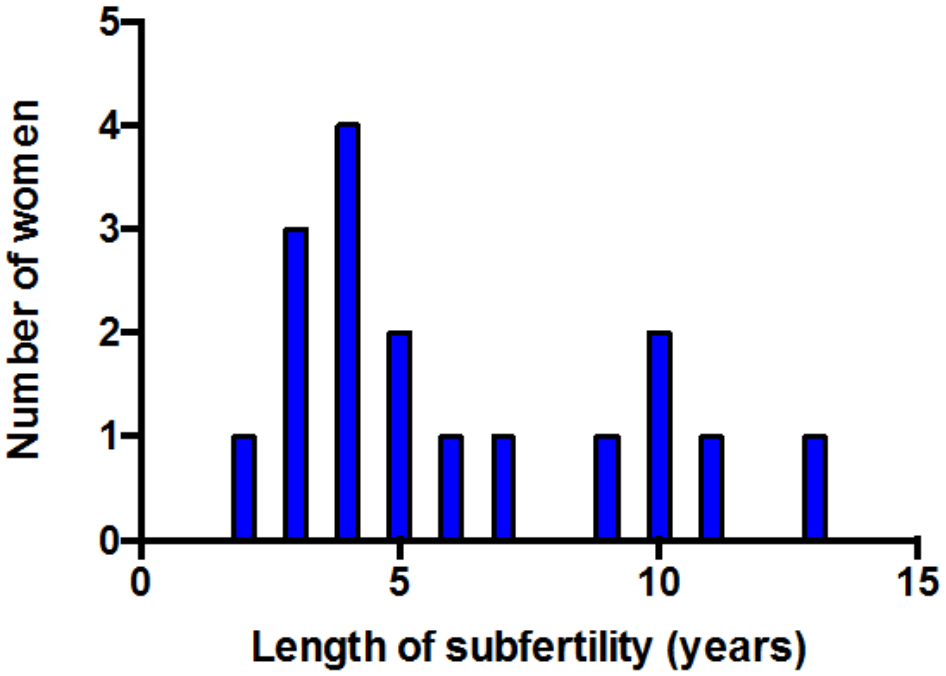
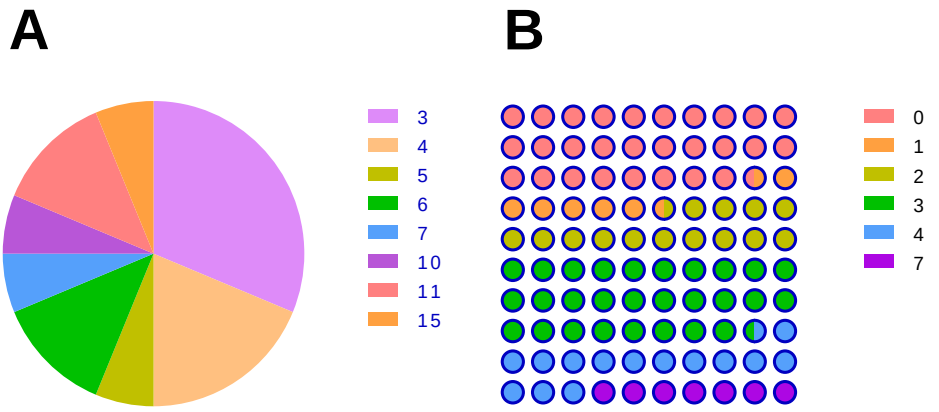


Figure 4-7 Embryo characteristics of RIF women

These two charts demonstrate the embryo characteristics of the women with RIF. (A) Shows the total number of embryos transferred per woman with RIF (the colour-coding legend down the right side), with all women having three or more good quality embryos transferred and most woemn having between three and four transferred. Figure (B) Shows the total number of blastocysts transferred per woman with RIF (the colour-coding legend down the right side). Most women were having blastocysts as opposed to D3 embryos transferred. This should theoretically result in higher pregnancy rates than having D3 embryos transferred.



4.1.1 The immunomodulatory profile of uterine fluid, but not the serum, differs in RIF and controls

The mean concentrations of the immunomodulatory compounds profile of the cytokines measured in the uterine fluid and the serum can be seen in Table 4-5 and in Figure 4-8. The levels of βNGF and LIF were lower in the uterine secretions of the women with RIF compared with controls (28.12±20.35pg/ml vs 9.34±7.45pg/ml, p=0.01 and 263.84±381.078pg/ml vs 60.92±98.19pg/ml, p=0.04 respectively). There was no difference seen in the serum of LIF (controls 19.54±11.64pg/ml vs RIF 32.11±33.78 pg/ml) nor βNGF (controls 288.46±100.68pg/ml vs RIF 323.42±156.23pg/ml, p=0.49).

Multivariate analysis indicated that βNGF, LIF and RANTES, IL-1RA, IL-6, BDNF, PDGF-ββ, SCF and SDF-1α could all be potential candidates in the influence on the model differentiating between cases and controls. This strengthens the potential role of βNGF and LIF as important modulators in the localised uterine environment. Both univariate and multivariate analyses were in agreement.

Five-parameter Partial Least Squares (PLS) logistic regression modelling with factor correlation loading, demonstrated that the immunomodulatory milieu of the uterine fluid could differentiate the RIF women from controls, see Figure 3-8. The RIF patients and controls therefore have a different discriminatory uterine immunomodulatory profile. The correlation between the concentration of the immunomodulatory in the uterine fluid, as compared with the serum can be seen in Table 4-5. There was a positive correlation of MCP1 (r0.56, p=0.04) and IL7 (r0.58, p=0.05) and a negative correlation of βNGF (r-0.85, p=0.03). The ratio of the concentration of the immunomodulatory markers in the uterine fluid, compared with the serum can be seen in Table 4-6.

Figure 4-8 Concentrations of immunomodulatory markers

The concentration of the immunomodulatory markers in the endometrial fluid (A) as opposed to the serum (B). The log mean of the immunomodulatory marker is shown in separate bars for the control women (lilac) and the RIF women (pale blue). The error bars represent the standard deviation. Significant differences in the concentration of the compounds is represented by an asterisk * ($p<0.05$).

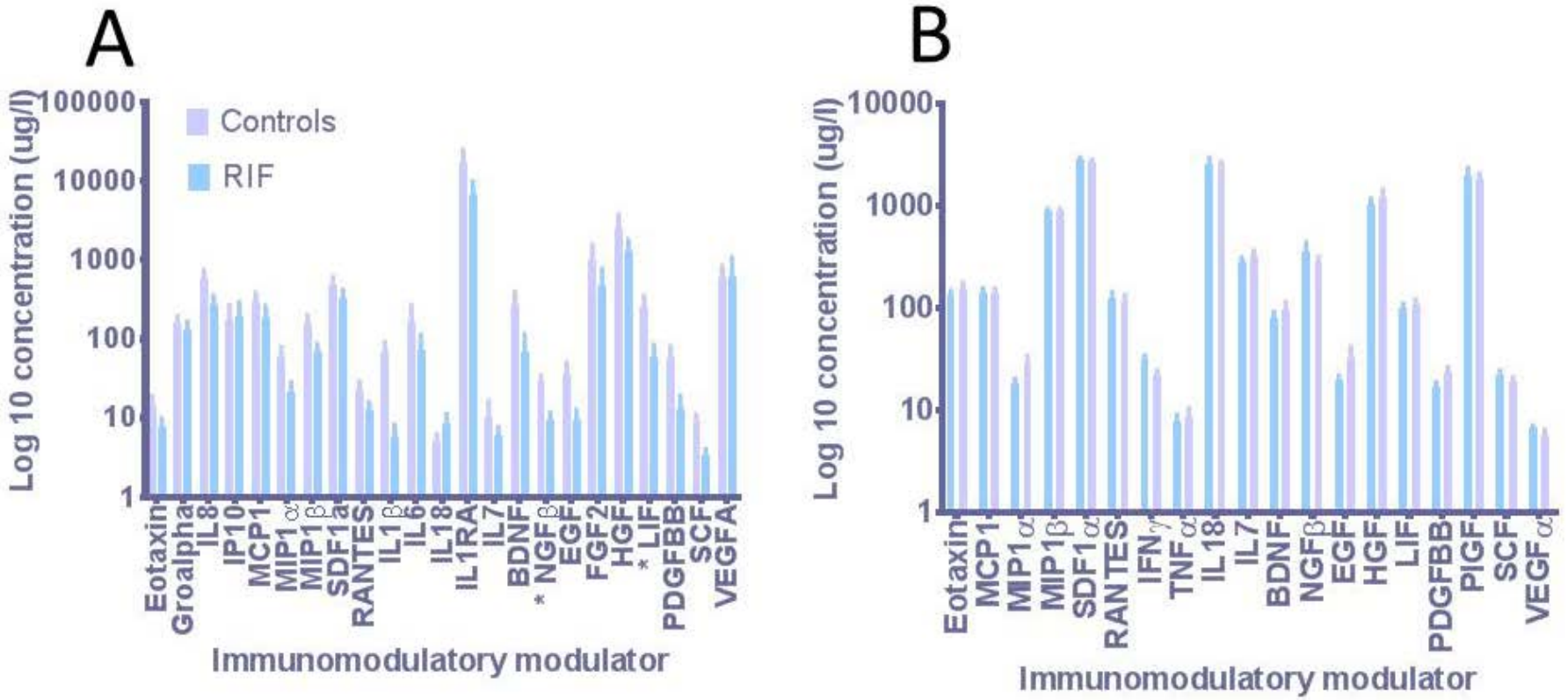
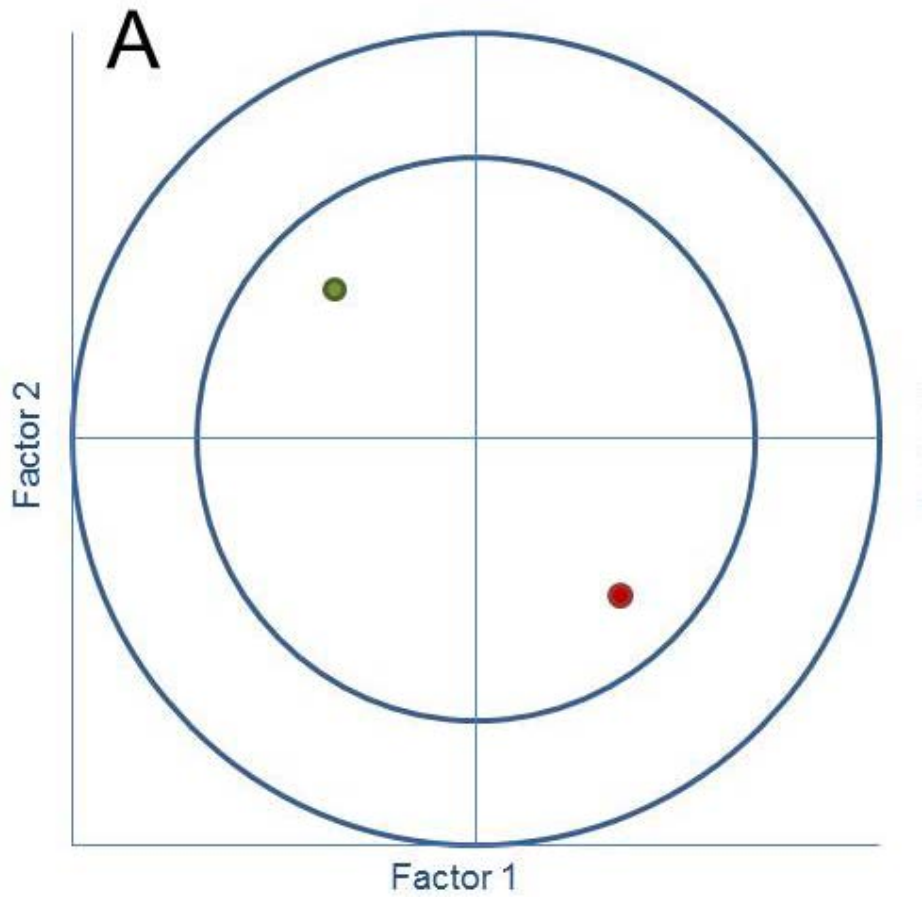


Figure 4-9 Correlation loading of PLS

The PLS model allowed a separation of the control patients from the RIF patients in a decriminatory manner in the uterine fluid. This is an extension of the multiple linear regression model. Factor 1 and factor two are the two, 'variables' used to separate the groups on the basis of the cytkino profile of their uterine fluid. The control group of patients is shown by green dot, RIF groups red.



4.1.2 Clock genes are expressed in the stroma and glandular tissue of human endometrium

Immunohistochemical analysis of the endometrium showed the expression of clock and BMAL1 in the stroma and glandular tissue (one control, one patient with RIF). Both samples were taken in the secretory phase to examine the glandular tissue as well as the stroma. The cytoplasm, epithelial edge and glands displayed strong staining of both CLOCK and BMAL1 in both the case and control patients see Figure 4-10.

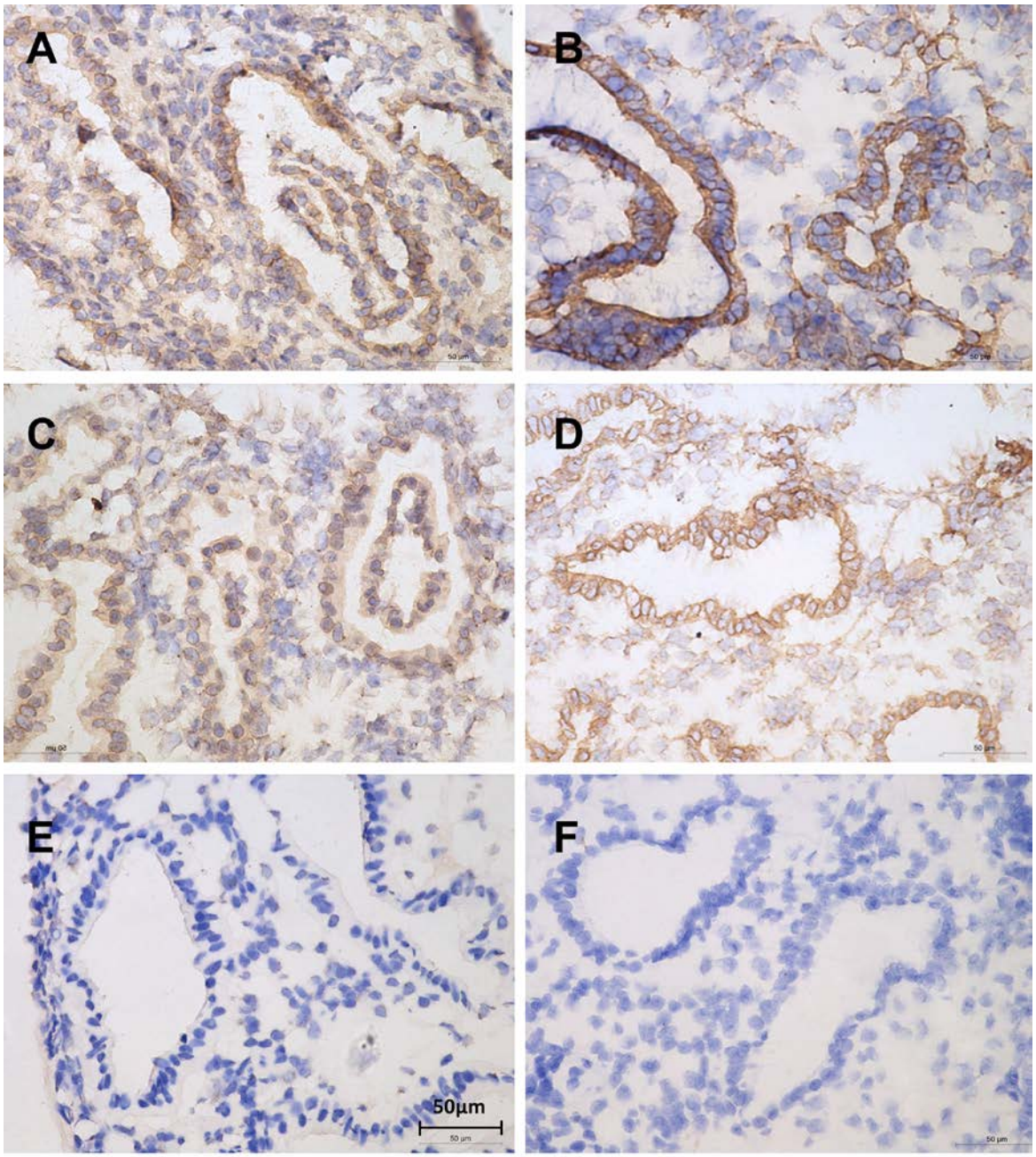
4.1.3 Endometrial clock gene expression varies with time of day *in vivo*

41 women had samples of endometrium collected for the analysis of gene expression at a single time point. The average day of menstrual cycle on day of sampling was day 13.3±8.5 in the morning and day 13.9±7.2 in the afternoon, with no difference between those women with RIF (morning sampling 15.6±10.2, afternoon sampling 16.0±3.3, p=0.30) nor controls (morning 11.8±7.2, afternoon 12.3±9.0, p=0.30). The average time of sampling of endometrial tissue collection was 11:28am±1:59 with no difference between women with RIF (11:29±2:07) nor controls (11:25±1:51) P=0.83.

The endometrial clock gene expression in mornings compared with afternoons may be seen in Figure 4-11 and Figure 4-12. It has been previously noted that decidualisation of endometrial cells plays a role in the expression of clock genes and so the following comparisons were made, proliferative compared with secretory, morning compared with afternoons and then RIF and controls. In the proliferative phase, the mean expression of per2 was significantly higher in morning compared with the afternoon (mean difference 0.19, p=0.04). This was not seen in the secretory phase (mean difference, 0.15 p=0.36). Conversely, in the secretory phase there was a higher level of clock expression in the morning than the afternoon (mean difference 0.03, p=0.04) but not the proliferative phase (mean differences 0.00, p=0.93). When the RIF women were compared with controls, there was no difference in the expression of clock genes in the proliferative or secretory phase, nor between morning and afternoon.

Figure 4-10: IHC staining

It can be seen that the various clock genes are present in the endometrium frozen sections. Each picture represents a different core clock gene stain, the left hand side are RIF patients with controls down the right hand side for comparison. (A) RIF *bmal1* (B) RIF *clock* (C) Control *bmal1* (D) Control *clock* (E) Control IgG (F) Control negative. The positive brown staining of the core clock genes is strongly positive around the glands but also in the stroma (the space in between).



.

Figure 4-11: Gene expression over the course of the day:

Single time point measurements of the core clock genes are shown over the course of the day. These are measurements from different pateitns but it is interesting to see the expression over time. Each core clock gene is shown in sucessive intervals along the X-axis.

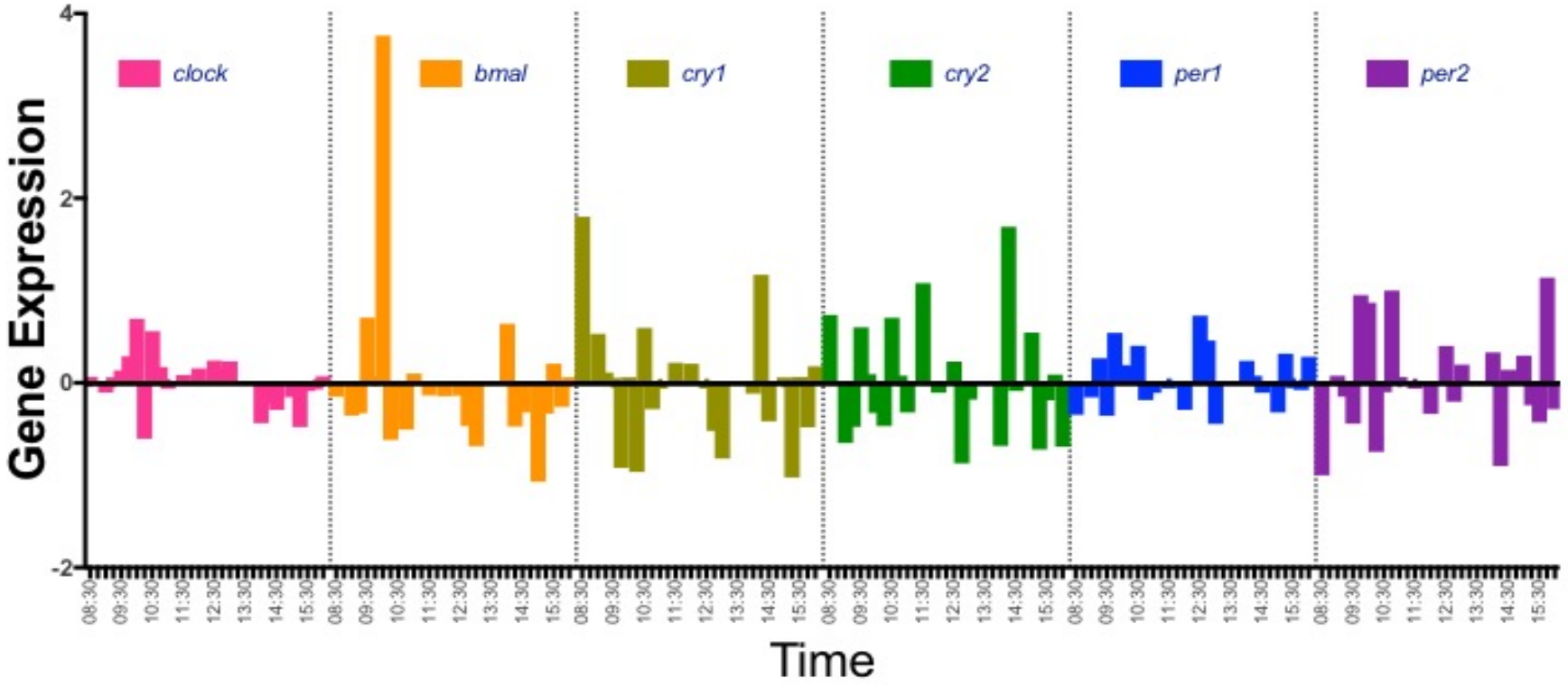
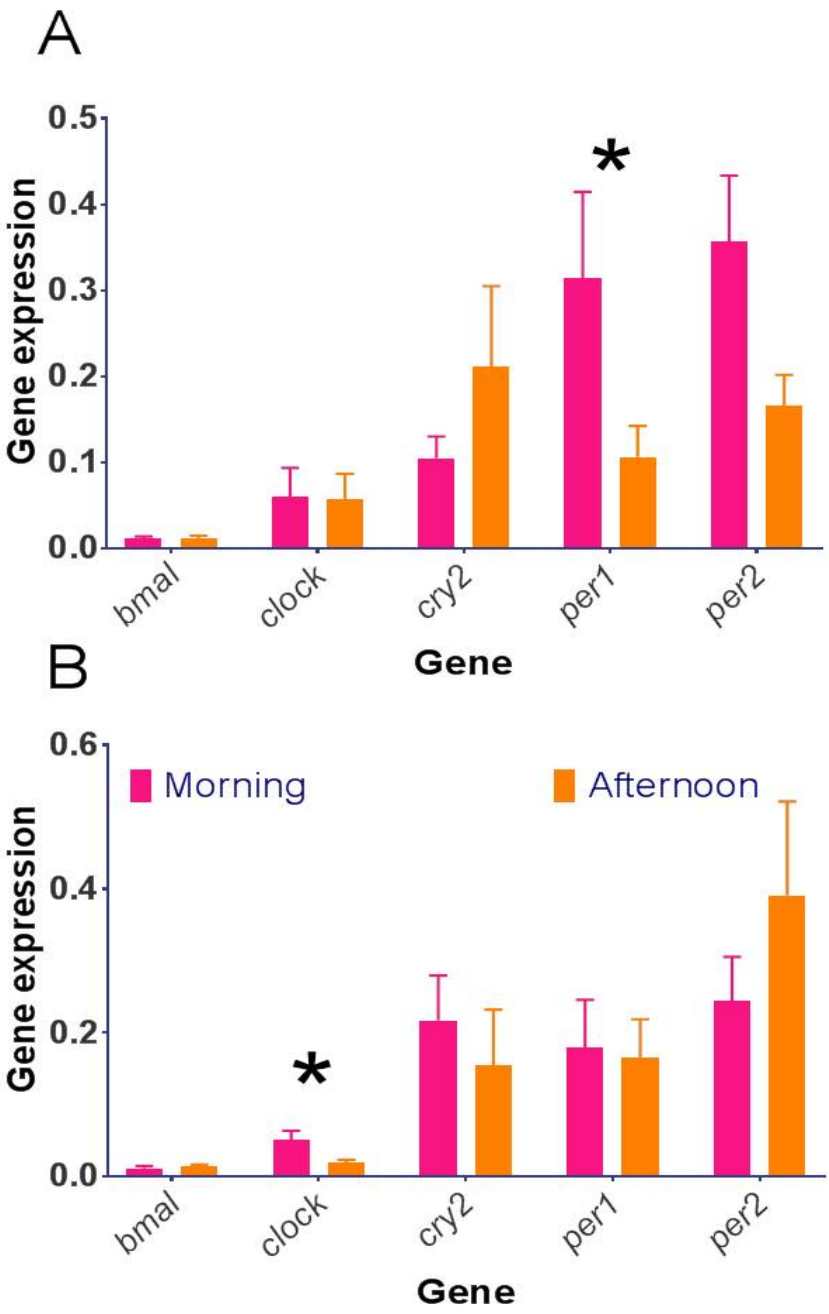


Figure 4-12: Single time point comparisons

The expression of core clock genes in the morning compared with the afternoon. (A) Is limited to proliferative phase endometrium and (B) limited to secretory phase endometrium. Asterix *=p<0.05.



4.1.4 Endometrial clock gene expression has a rhythm of expression which differs in decidualised and non-decidualised stromal cells

Endometrial samples (n=11) were cultured for a total of 36 hours (6 RIF women, 5 controls). A circadian (24 hour) rhythm was seen in the cells, see Figure 4-13. When the cells were decidualised, phase shifting was observed, but the actual period persisted see Table 4-10.

4.1.5 Endometrial clock gene expression has a circadian rhythm

For circadian analyses, visual inspection of the resulting time series was performed. As a result, linear detrending (linear regression performed, and the resultant curve subtracted from the fitted line) was applied (for an example of how this effects the results, see Figure 4-15. This allowed for baseline trends to be removed in order to prevent detrimental effects on period analysis prior to Cosinor analysis. MESA analysis and FFT both demonstrated that there was circadian rhythm exhibited by the expression of the six core clock genes (*bmal1, clock, cry1, cry2, per1, per2*), in both decidualised, and non decidualised cells from control women. This was also true of the cells from women with RIF (non-decidualised and decidualised). The average period across all genes was 24.62±0.36 (range 21.36- 27.10 CU) using MESA. This was supported by the FFT method (mean 25.63±0.38 CU, range 21.72-28.95 CU) see Table 4-10. There was a non-significant difference in means of the circadian period (τ) using MESA or FFT when the two methods were compared on an individual gene basis. For the purpose of further analysis, the MESA results are quoted.

Figure 4-13 : The circadian expression of the core clock genes in endometrium from control women

Each of the core clock genes shows a cyclical pattern of expression over 24 hours in endometrium from control women. The mRNA expression, after normalisation to a housekeeping gene is shown for each of the core clock genes. Error bars represent the stadard deviation. (A) *bmal1* (B) *clock* (C) *cry1* (D) *cry2* (E) *per1* (F) *per2*.

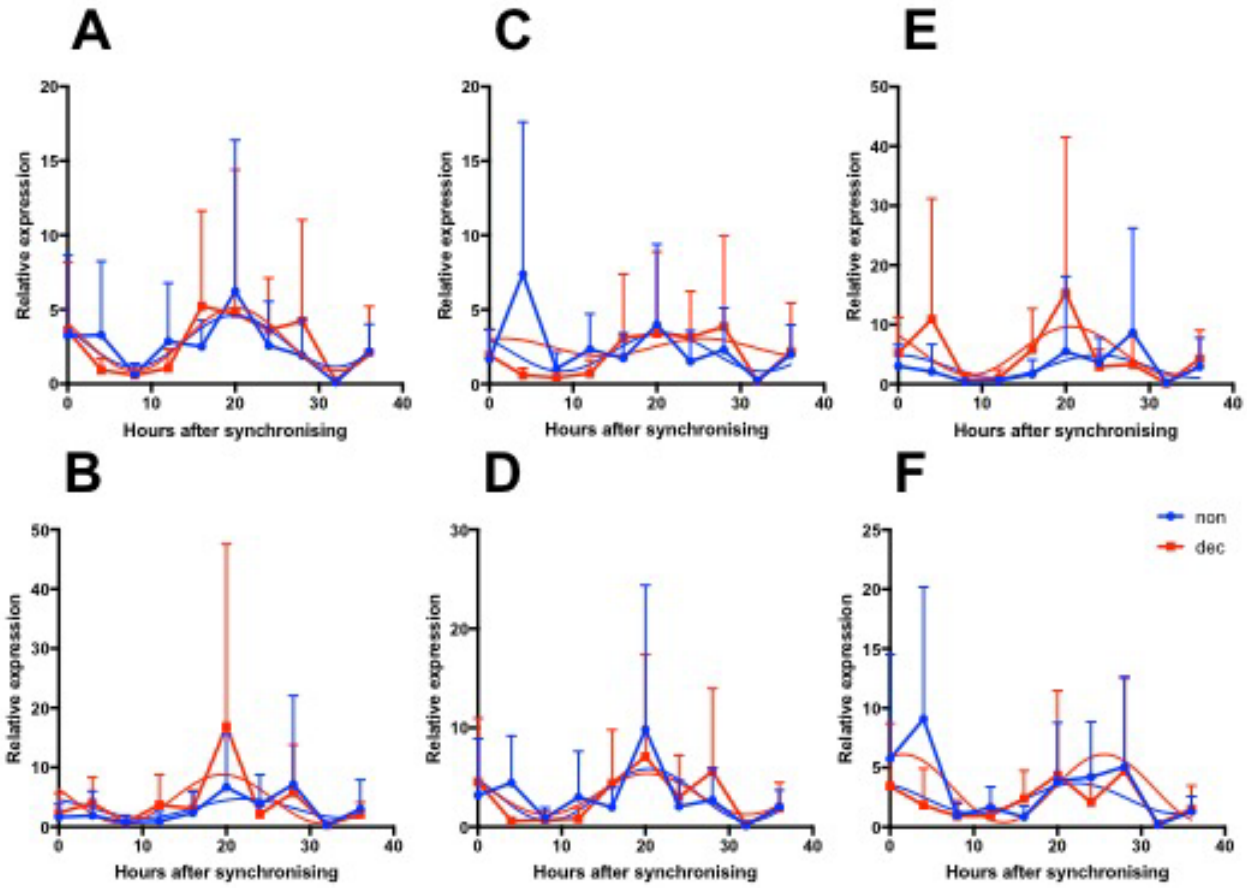


Figure 4-14 The circadian expression of the core clock genes in endometrium from women with RIF

Each of the core clock genes shows a cyclical pattern of expression over 24 hours in endometrium from women suffering from RIF but this is different from that of cases and phase shifted. The mRNA expression, after normalisation to a housekeeping gene is shown for each of the core clock genes. Error bars represent the stadard deviation. (A) bmal1 (B) clock (C) cry1 (D) cry2 (E) per1 (F) per2.

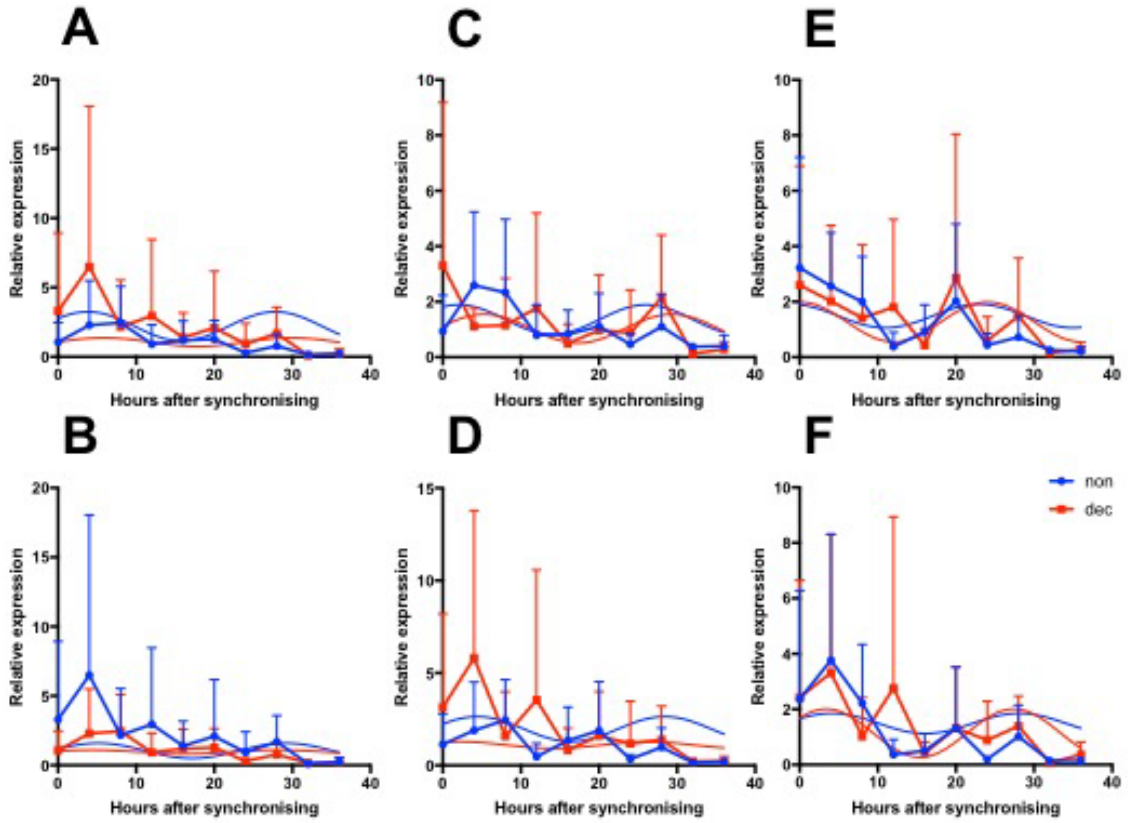
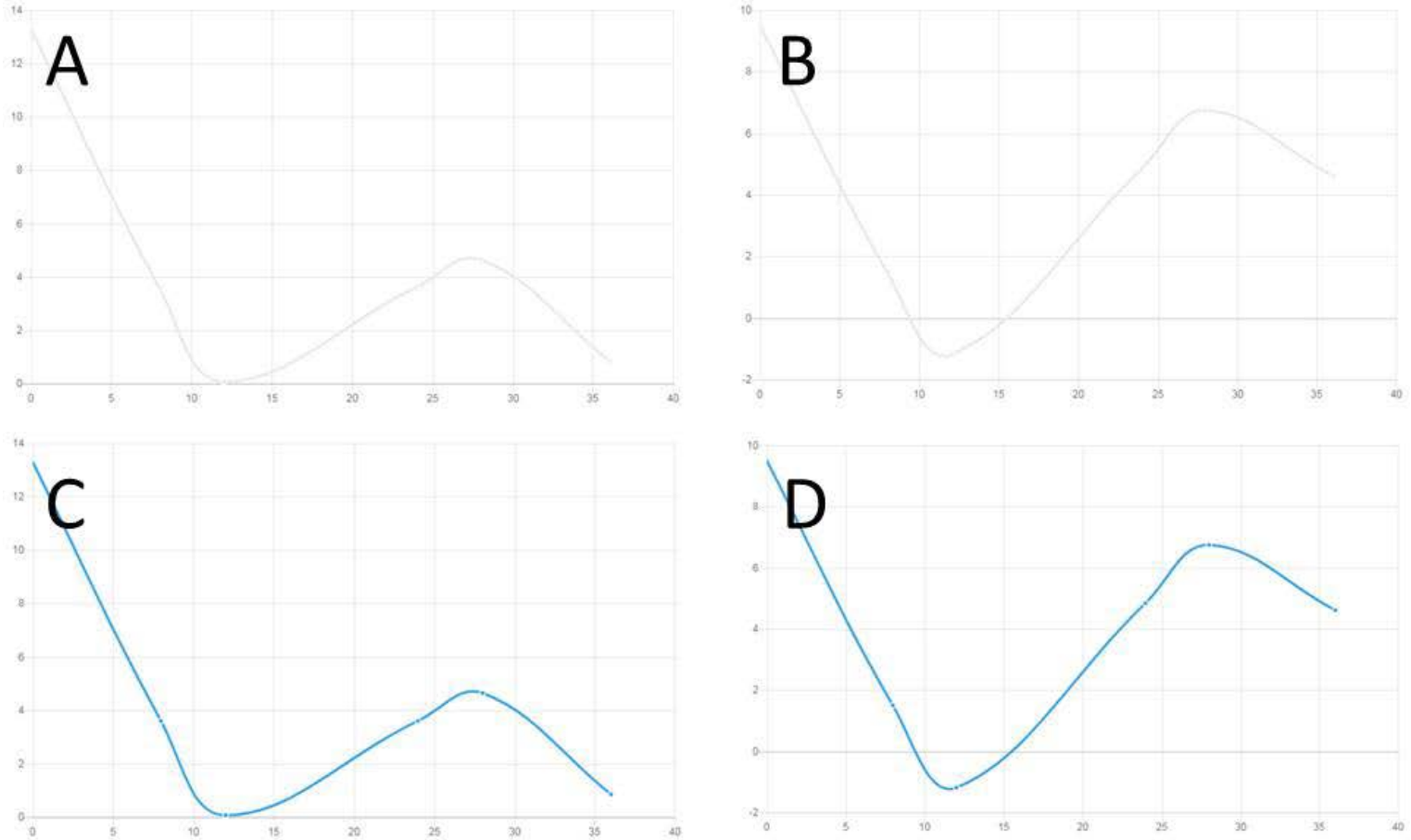


Figure 4-15: Linear detrending

An example of linear detrending of the curves before analysis was performed in *bmal1*. Figures (A, C) show before and (B, D) after linear detrending. The figures on the top row are control non decidualised (A-B) and the bottom row are *bmal1* control decidualised (C-D).



4.1.6 *In vitro* HESC have a robust circadian period which persists after decidualisation

There appeared to be a circadian rhythm of mRNA of the six core clock genes. In rat fibroblasts, a serum shock may induce circadian rhythms in cells already known to oscillate *in vivo* (117). In non decidualised cells from control women there was up regulation of all core clock genes except for *per1,* after what is in effect a cell shock. The main entrainment pathway of the mammalian clock is mediated via short term clock-independent transcriptional induction of *per1*and *per2* in response to a variety of stimuli (e.g. light, dexamethasone, temperature). When cultured cells are exposed to a synchronising treatment, the resulting transcriptional profile shows a temporary increase in *per1* and/or *per2*. This is followed by a period of repression of *clock/bmal1*-regulated transcripts (due to the negative feedback from the increase in *per1/2* protein levels). Only then do steady circadian rhythms commence. As a result, the first cycle following synchronisation may be different compared with subsequent cycles.

Whether this would be apparent in the experiments described here, is not entirely clear as most of the early induction may have already dissipated by the 4 hour time point. However, some impact of this synchronisation may be apparent in the early time points. In the decidualised state, there was down regulation or *bmal1, cry1* and *cry2,* and *per2*. In non decidualised cells from women with RIF there was again upregulation of all core clock genes except for *per1*. In the decidualised cells there was upregulation of all but *cry1* and *per1*. Due to these inconsistencies it was not felt the initial times of data collection should be disregarded and all data was used for the purposes of further analyses.

To establish whether the period of gene expression was stable, the mean period (τ) was compared across the six core clock genes. In cells from fertile controls, the mean period of each gene (*bmal1, clock, cry1, cry2, per1, per2*) in non decidualised cells was compared with the mean period in decidualised cells. There was no significant difference seen between any of the genes (see Table 4-10), and so the period was shown to be stable in the core clock genes. The same comparison was made in RIF women. Again, mean period across the six core clock genes was compared and was not shown to differ significantly (Table 4-10). This stability was therefore true in both decidualised and non decidualised cells, in healthy women and RIF (Table 4-10).

4.1.7 *In vitro* HESC have a stable amplitude after decidualisation

The same comparisons were made for amplitude. The mean amplitude of (*bmal1, clock, cry1, cry2, per1, per2),* was not significantly different in control non decidualised women compared with decidualised cells, nor RIF women non decidualised, compared with decidualised cells. Therefore, decidualisation did not alter the amplitude of the circadian expression Table 4-10.

4.1.8 Endometrial decidualisation shifts the phase of *per*1 in fertile controls. It also shifts the phase of *per2* in RIF women

When comparing non decidualised controls with decidualised controls, the phase of per1 expression was significantly phase-shifted forward (21.15 in decidualised instead of 0.04 in non decidualised, p=<0.01) (Table 4-10). So if the results are likened to ZT time, decidualisation of healthy women results in the period of expression of *per1* happening later in the day. All other genes except for *per1* did not have significant changes in their phase when comparing the non decidualised and decidualised controls i.e. they demonstrated stable phases. The endometrium from women suffering RIF also expressed a rhythm, but these women demonstrated a different phase again (see Figure 4-14). When the same comparison was made for RIF women, the same forward shifting of *per1* was seen (non decidualised 19.05, decidualised 0.29, p<0.01). However, *per2* was also shifted forward in the RIF patients (non decidualised 0.04, decidualised 23.09, p<0.01). These phase shifts can be seen in Table 4-10.

4.1.9 In vitro non decidualised and decidualised endometrium, RIF women have different phase patterns compared with controls

In non decidualised endometrium of women in the control group compared with that of RIF women, the phases of all core clock genes except for *bmal1* were significantly different, and further forward in time, see Table 4-10 and supplementary data. After decidualisation, there was an altered clock gene expression phase pattern seen between the same genes (*clock, cry1, cry2, per1, per2*) in control and RIF women.

4.1.10 The amplitude of clock gene expression is different in endometrium of controls women compared to those with RIF

In the non decidualised endometrium of RIF women compared with controls, the amplitudes of *clock* (2.24 vs 0.25, p=0.05) and *per1* (3.14 vs 0.76, p=0.04) were both

higher. The discrepancy in amplitude in *clock* and *per1* was no longer present between RIF women and controls after decidualisation (see Table 4-10 and Figure 4-13 and Figure 4-14).

4.1.11 Endometrial stromal cells of women with RIF have a unique time-dependent rhythm-sensitive *in vitro* secretomic signature compared with controls

To examine the circadian secretome of the culture model, the concentration of immunomodulatory compounds was measured in the cell media. This was done at the corresponding cell sampling time of the gene expression experiments (i.e. time point 0, and every 4 hours until 36 hours) for half the cell cultures experiments (n=4, two RIF patients and two controls). The immunomodulatory compound profile at each time point was compared with the gene expression at the same time point, and four hours previously (i.e. the 'current' hour, minus 4).

The comparison of the core circadian gene expression to the immunomodulatory compound concentration four hours later was compared and significant correlations are shown in. (Figure 4-18). At synchronous timing, whilst the same immunomodulators were present in the media, the relationships did not exist. The differential expression of the correlated immunomodulators was compared before and after decidualisation in the controls compared to those with RIF (Figure 4-19, for the additional patient's data, see supplementary data). Not all of these correlations were statistically significant and therefore these were removed from the model, see Figure 4-18 and Figure 4-19. After removing the non-significant correlations, when looking at the controls women only, there were five significantly correlated immunomodulatory markers/genes before decidualisation and five after decidualisation (Figure 4-18). In the RIF patients there were three significantly correlated immunomodulatory markers/genes before decidualisation and nine after decidualisation.

4.1.12 Melatonin alters the circadian rhythm of HESC in culture

Melatonin was added to the HESC model and the resultant effect on the circadian rhythm of the core clock genes may be seen in Figure 4-16 and Figure 4-17. The degree of phase shift that occurred with the addition of melatonin to HESC from control women was minimal. This phase shift also appeared to be less marked than that which occurred in HESC from women with RIF (Figure 4-14). In HESC from control women, the degree of phase shift was greatest in *per2*. In HESC from women with RIF,

the addition of melatonin appeared to shift the phase of the core clock genes to be more in-keeping with those of the control women's ESC than the untreated HESC with the exception of *per2* (see Figure 4-17).

..

Figure 4-16 Endometrium from control women with the addition of melatonin

Each of the core clock genes shows a cyclical pattern of expression over 24 hours in endometrium from control women after treatment with melatonin (compare with Figure 4-13). This was little altered by the addition of melatonin. The mRNA expression, after normalisation to a housekeeping gene is shown for each of the core clock genes. Non decidualised cells are pale blue, decidualised are dark blue (A) bmal1 (B) clock (C) cry1 (D) cry2 (E) per1 (F) per2.

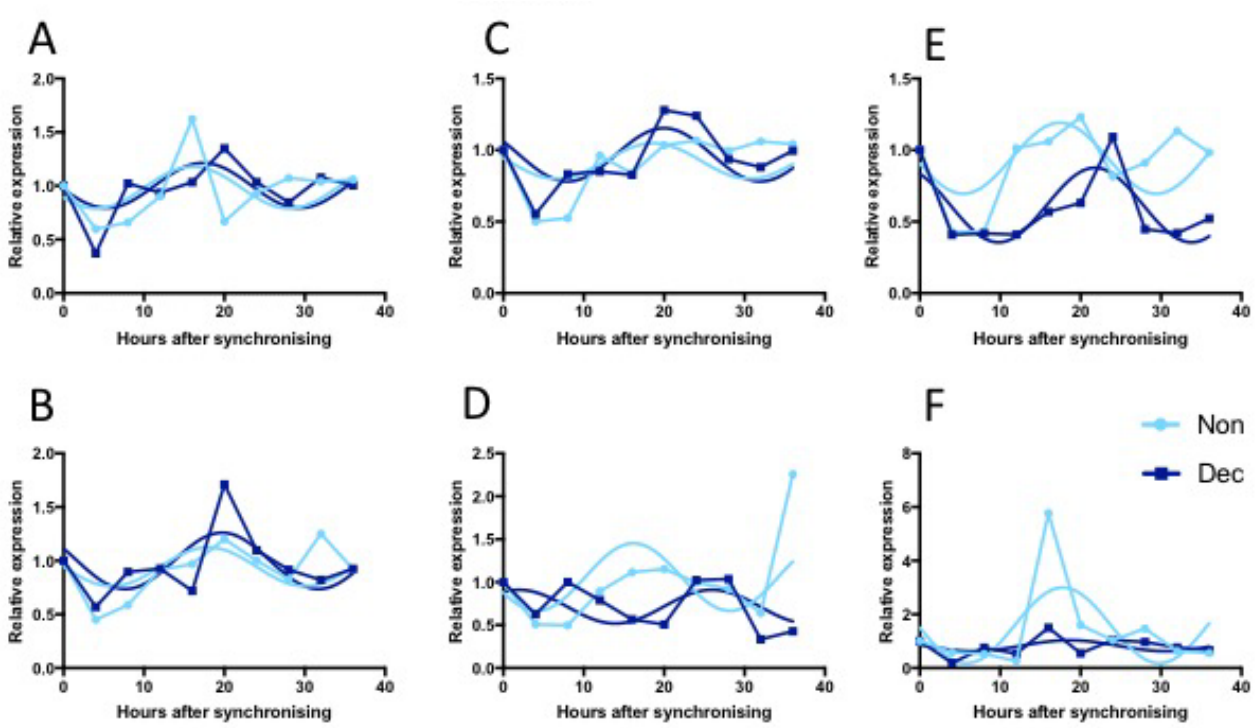


Figure 4-17 Endometrium from women with RIF with the addition of melatonin

Each of the core clock genes shows a cyclical pattern of expression over 24 hours in endometrium from control women after treatment with melatonin (compare with Figure 4-14). The cyclical pattern was little altered by the addition of melatonin. The mRNA expression, after normalisation to a housekeeping gene is shown for each of the core clock genes. Non decidualised cells are pink, decidualised are maroon. (A) *bmal1* (B) *clock* (C) *cry1* (D) *cry2* (E) *per1* (F) *per2.*

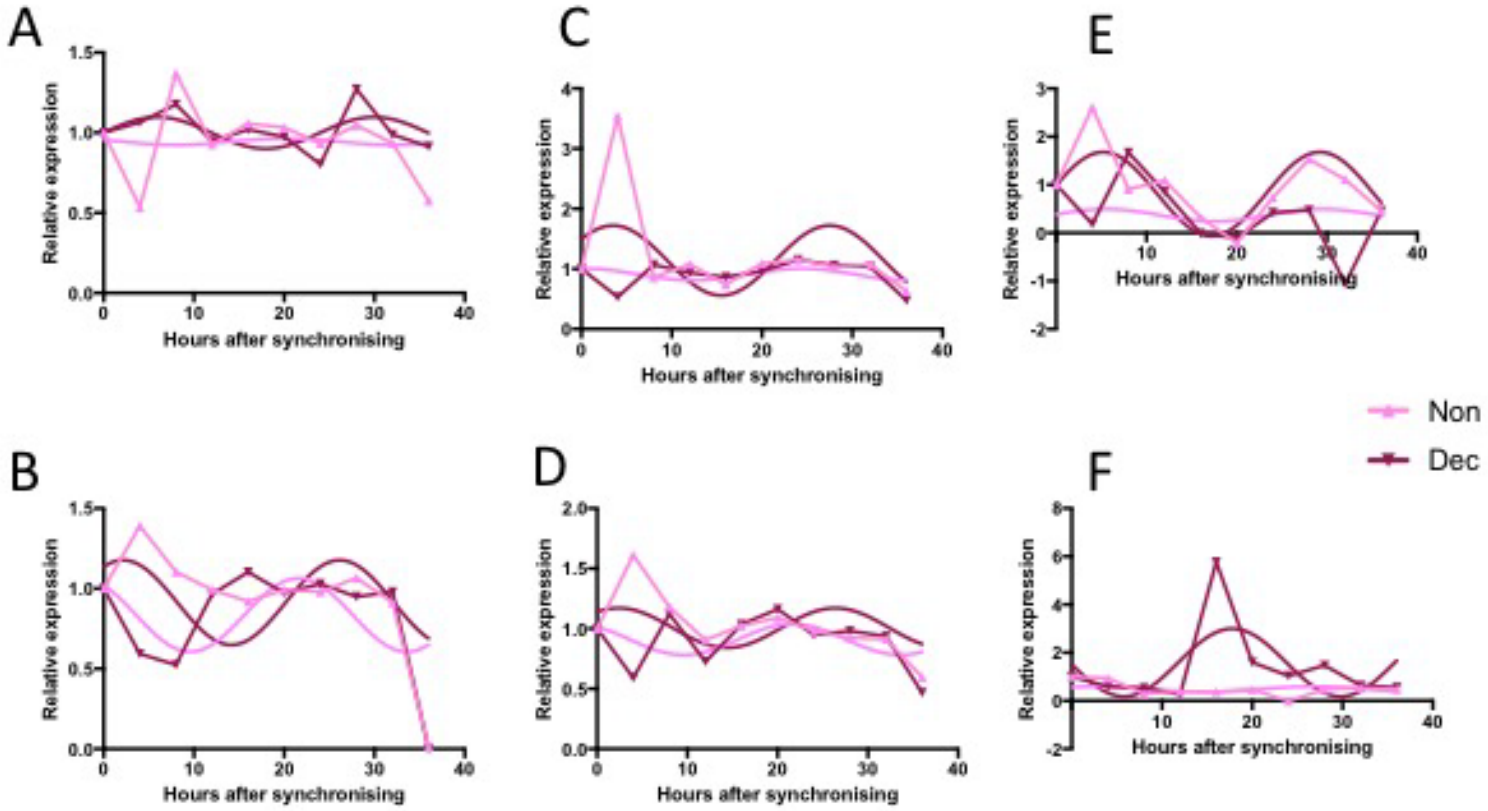


Figure 4-18 Significantly correlated immunomodulators and comparative core clock genes

Significantly correlated markers are indicated by the coloured boxes (core clock genes on Y-axis, immunomodulators on X-axis), with paler coloured (pink) representing more significant values. White indicates non-significant correlations. Each graph represents a different patient with both decidualised and non decidualised states being shown on separate lines.

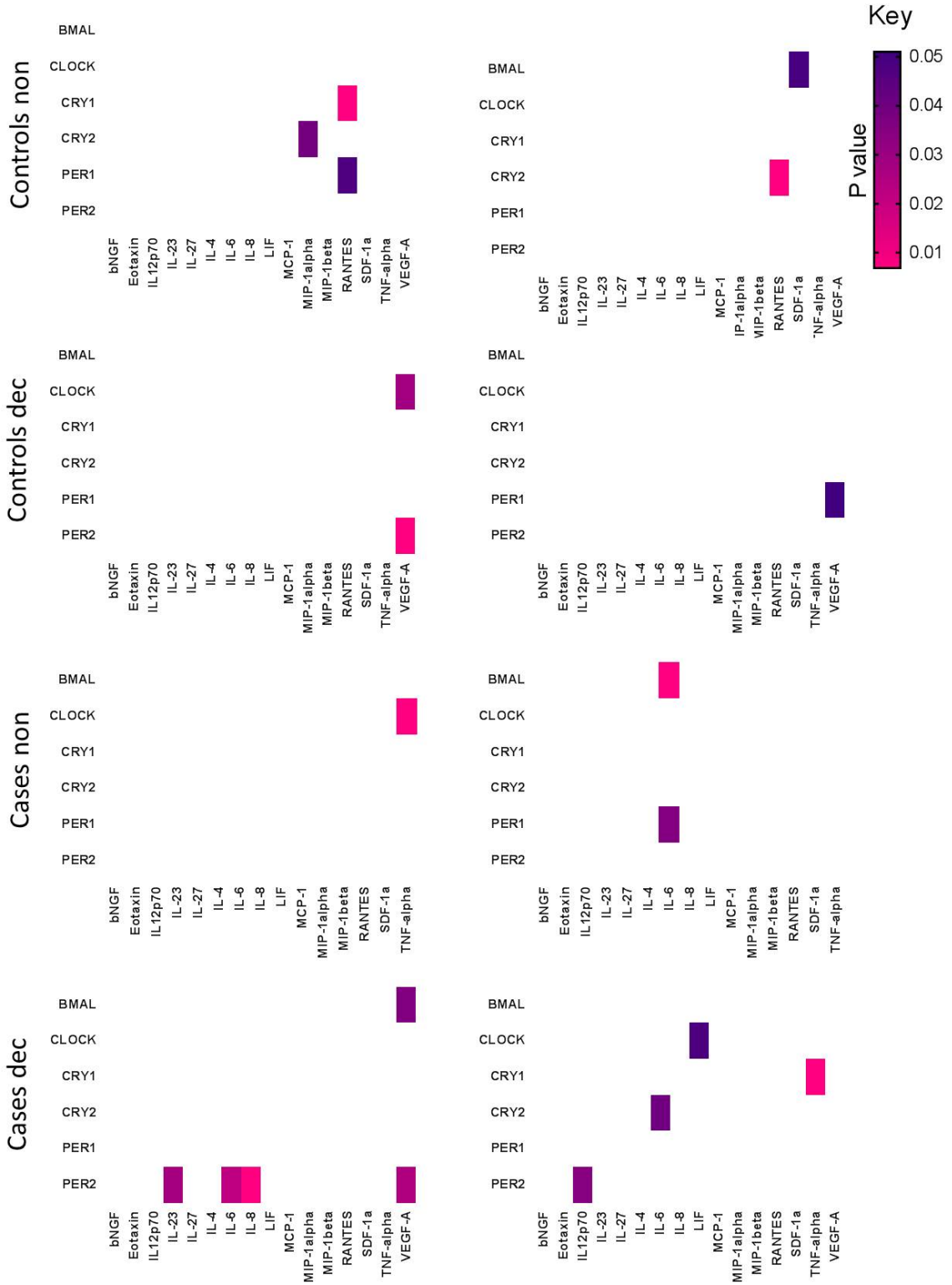
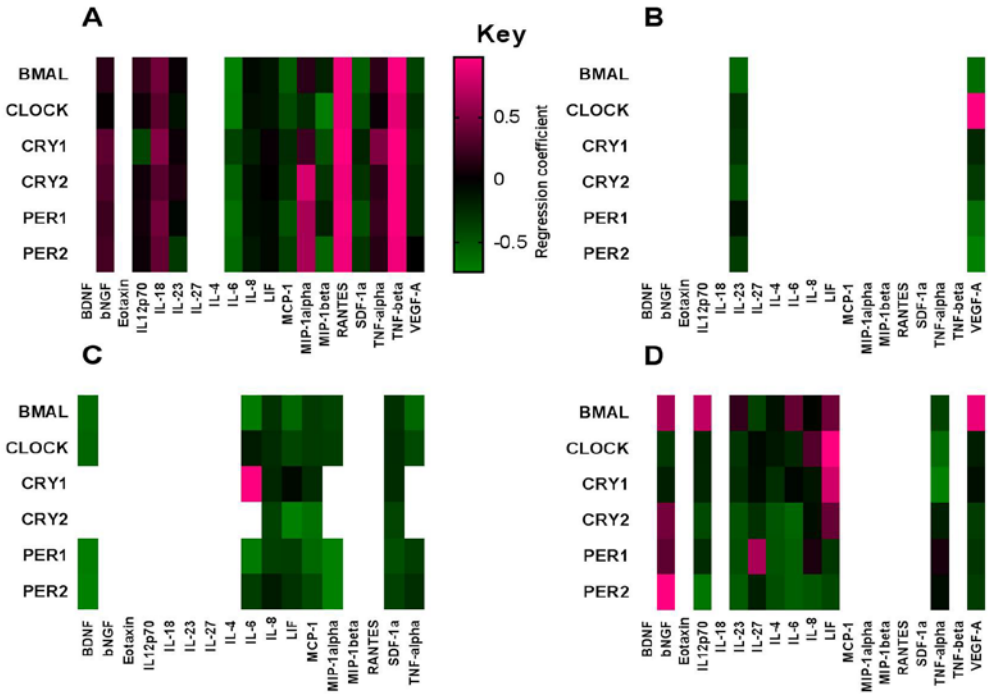


Figure 4-19 Heat map demonstrating immunomodulatory markers correlated with clock genes

The regression coeffecient of the correlated markers and core clock genes are indicated by the coloured boxes (core clock genes on Y-axis, immunomodulators on X-axis), with pink showing a positive correlation; green a negative, and black no correlation. Each line represents a different patient with both decidualised and non decidualised states being shown in separate columns. (A-B: RIF, C-D: control); A and C before decidualisation, B and D after decidualisation.



4.1.13 Melatonin down regulates the correlation of the *in vitro* circadian inflammatory secretome in the non decidualised endometrium of women with RIF but not in controls

In order to see if this time-dependent *in vitro* circadian secretome could be altered, four cell cultures were exposed to melatonin; two patients with RIF and two controls. This was done in both non decidualised and decidualised phenotypes. The media of the retrospective cell time of sampling was examined for the correlation of immunomodulatory compounds with the six core clock genes at time point zero, and again with a four-hour time lag.

Some of the immunomodulatory compounds in the RIF patients were consistently associated with certain genes, with or without the addition of melatonin. IL12p70, VEGF-α and TNF-α were consistently correlated with *per2*. IL12p70 was also correlated with *bmal1*. *Clock* was consistently correlated with LIF and IL8. In RIF, one of the patients' LIF was also correlated with *cry2* (decidualised) and *cry1* (non decidualised).

The number of significantly correlated immunomodulators in non-decidualised cells was compared with the number of significantly correlated immunomodulators in decidualised cells, with and without the addition of melatonin (see Figure 4-20). In controls, there was no difference in the number of correlations in non decidualised cells compared with decidualised cells before (five to six) after the addition of melatonin (four to seven, p=0.66) (Figure 4-20A). This suggests that decidualisation is not effected by or melatonin-dependent in control cells.

In RIF, without the addition of melatonin, the number of significant correlations in non decidualised cells was three compared with nine in decidualised cells (Figure 4 21B). After the addition of melatonin, the number of associated immunomodulatory clock correlations in the non decidualised cells was five (Figure 4 21C). After the addition of melatonin, the number of associated immunomodulatory clock correlations in the decidualised cells was one (Figure 4 21C). This means that with the addition of melatonin there was a significant change in the number of gene-immunomodulator associations in the non decidualised compared with the decidualised RIF women (non decidualised changed from 3 to 5, and decidualised changed from 9 to one, p=0.02).

After the addition of melatonin to non decidualised RIF cells, the number of associated immunomodulatory clock correlations was the same as in the non decidualised control group (five and five, Figure 4-20A and C). After decidualisation, when melatonin was added to the cells from women with RIF, the number of associated immunomodulatory

clock correlations was decreased (nine to one) (Figure 4-20A and Figure 4-20C). This meant the pattern of behaviours after decidualisation was more similar to that of the control cells after the addition of melatonin.

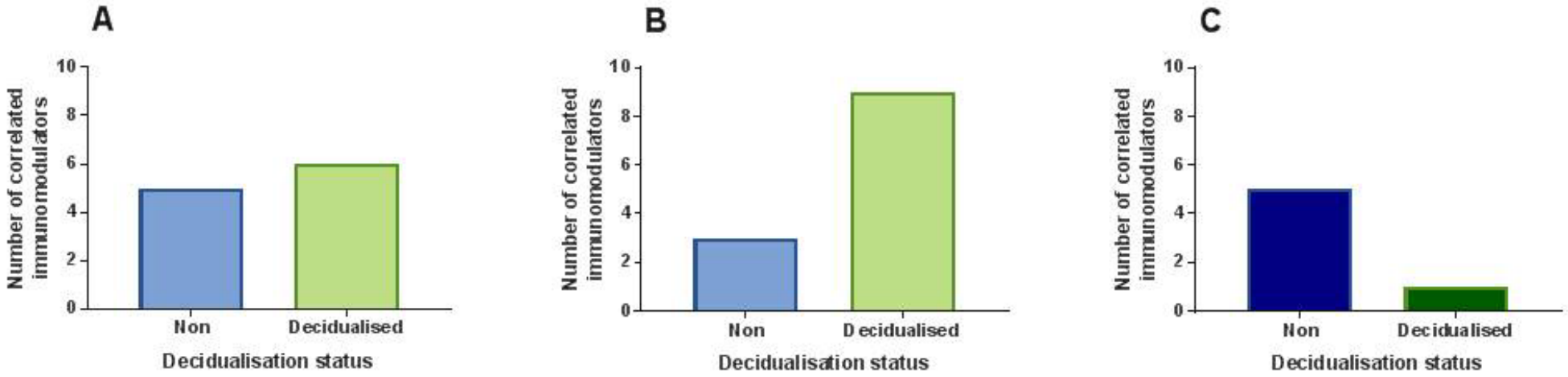
In control cells the endometrium is relatively stable in terms of the number of associated immunomodulatory clock correlation. In RIF, the secretome-clock correlation *in vitro* is relatively more 'active' in the decidualised phase than the non decidualised phase. The addition of melatonin makes it less 'active'.

Figure 4-20 The number differentially expressed immunomodulators /clock genes before and after the addition of melatonin

Control women no melatonin

RIF no melatonin

RIF women with melatonin



4.1 Discussion

The experiments described here show a difference in the expression of clock genes between women with healthy endometrium and RIF *in vivo*. In the proliferative phase, the mean expression of *per2* was significantly higher in morning compared with the afternoon in the proliferative phase (mean difference 0.19, p=0.04), but this was not seen in the secretory phase after decidualisation (mean difference, 0.15 p=0.36). Conversely, there was a significantly higher level of *clock* expression in the morning than the afternoon of the women in the secretory phase but not the proliferative phase (mean differences 0.00, p=0.93, and 0.03, p=0.04 respectively). This is a crude marker of how clock genes have a circadian dependent rhythm in the endometrium. One might expect to see a reciprocal difference in *cry* and *clock*, as a reflection of the negative feedback loop. However, it should be remembered that these samples although timed, are from different women. These women may have a circadian rhythm which is out-of-sync from their evening or morning comparators, some being late risers ('night owls') or early-risers ('larks) and thus making the numbers in this analysis too small to make a true comparison. It would have been ideal to obtain two samples from the same woman both in the morning and evening but for ethical and practical reason this was not possible. Also, the sampling method itself may affect gene expression as described above and these samples would therefore need to have been taken in separate cycles.

We also describe how *in vitro* HESC have a robust circadian period and amplitude which persists after decidualisation. This is not in keeping with the only previous study to our knowledge to examine clock gene expression in human endometrial stromal cells in vitro. Muter et al describe a 'uniform loss' of circadian rhythmicity in decidualising human endometrial stromal cells. However, this study did not examine the circadian rhythm *per se*, they looked at relative gene expression over 26hours and then at individual time points over 8 days without performing circadian analysis. They state that, 'decidualisation elicited modest but consistent changes', with constant clock expression, up regulation of *bmal1* and down-regulation of *cry1, cry2* expression and a, 'rapid and profound inhibition of *per2*'. It would be interesting to know whether or not these rhythms were indeed still circadian or merely being expressed.

The study of circadian rhythms has relied heavily on the principles and tools of circular mathematics and statistics. Any recurrent rhythm may be measured by the same parameters as trigonomic functions e.g. amplitude, period and phase. Simple techniques used to estimate and statistically analyse these variables may be useful, but omit to consider the waveform of the oscillations (and is the shape of the rhythm over the cycle) and commonly, physiological rhythms deviate from such waveforms. Only

2% of circadian studies consider the waveform as opposed to 'phase' (357). It is often necessary to assume that in constant conditions the frequency of a measured rhythm reflects the underlying clock. Waveforms, the additive effect of many rhythmic processes that jointly affect the same measure, may not fit this pattern. Any given waveform may be a combination of clock and non-clock processes, encoded and modulated by the central or peripheral clock but responding differently in peripheral conditions. This may in part explain why Muter et al found a 'loss of cyclicity', which may have been dampening over time.

Secondly, the previous HESC study was small, comparing three endometrial samples from women at an 'implantation clinic', and therefore could well be from a subset of women suffering pathology (although not necessarily RIF as defined in this study). The underlying pathology may also play a role in circadian expression. It follows that if there is a difference in the expression with RIF patients, as demonstrated here, that this may be inconsistent depending on the phenotype of the particular individual. Muter et al 2015 may have chosen 'pathological' patient samples which may have skewed results. Studies involving cancer cell cultures of the endometrium have found different gene expression patterns within cancer cell populations and possible multiple asynchronised circadian rhythms in the same cancerous tissue (154).

Animal studies have demonstrated a persistent circadian cyclicity of genes with decidualisation (133, 155, 160), indeed a study in bovine cells demonstrated that clock was acyclic without progesterone (154). In the human ESC experiments it could be that the quantity is much decreased, as opposed to an actual absence of rhythm. Following decidualisation we found that all core clock genes except for *bmal1* were phase shifted forward with no difference in period or amplitude. After decidualisation there was an altered, albeit different, phase shift pattern seen between the same. However, there were still different phases between RIF and control women. Furthermore, this is related to a delayed alteration in the profile of immunomodulatory compounds involved. Decidualisation appears to impact on this relationship, with control subjects maintaining the same levels of immunodulatory modulators released by endometrial stromal cells regardless of progestogenic changes as exhibited by the peri implantation endometrium. This is a notable finding given that many genes have an altered expression during the window of implantation (358, 359). However, in RIF patients this association persists and perhaps therefore contributes to the maladaptive environment of the endometrium at the time of implantation.

The results presented here suggest that the studied panel of this uterine secretome differs when comparing RIF patients with unstimulated healthy controls. Specifically, there are different levels βNGF and LIF in the uterine secretions of patients with RIF

compared with controls. This suggests that these immunomodulators may be important in the inflammatory milieu of RIF. These may be confined to the uterus as opposed to being a systems-wide phenomenon. A specific and comprehensive panel of immunomodulatory molecules was involved and multivariate analysis supported the initial data. The divergence between women with RIF and those without this disorder relied on the panel of molecules rather than one or two dominant molecules. There are also many reports of reduced levels of LIF in RIF cases in the literature; suggestive of only an attenuated inflammatory response the in endometrial peri-implantation environment. The PLS model, also supports that there are differences in the uterine environment of the two groups of women (RIF and RM) that may be explained by the different immunomodulatory compounds of the uterine fluid. This should be confirmed in a larger data set to increase the validity of this claim.

Recent evidence suggests that endometrial injury in women undergoing IVF cycles significantly improves the rate of implantation, clinical pregnancies and live births. These observations suggest that mechanical injury of the endometrium may enhance uterine receptivity by generating a favourable inflammatory reaction (360). The up-regulation of genes seen in endometrial injury may be deficient in RIF patients (361). Of specific note is that inability to elevate the expression of genes related to endometrial receptivity in a spontaneous manner, will alter the secretome. This supports the theory that decreased endometrial receptivity is linked to the endometrial environment in a potentially reversible manner.

In terms of the immunomodulatory markers which influenced the secretome model there are several which have been previously thought to be important for implantation. For example, the importance of LIF in implantation is consistent with established reports(362),. In humans, LIF is expressed in the endometrial glands during the secretory phase of the menstrual cycle when implantation would occur (363). Animal studies have shown that implantation cannot happen without expression of LIF in the uterus (362, 364). Interestingly, IL-RA was also identified as a significant influence in the secretome of RIF. IL-RA is the receptor agonist of IL-1R that binds non-productively to the cell surface interleukin-1 receptor (IL-1R), the same receptor that binds interleukin 1 (IL-1), thereby blocking its action. IL-1 is a downstream marker of the NF-kappaβ pathway which is dramatically up-regulated in the epithelium during the peri-implantation period (365). This finding also supports the theory that implantation in RIF may be due to a suboptimal inflammatory response. However, TNFα was not a significant modulator and this too is thought to be associated with NF-kappaβ signalling.

However, as a group, these pro-inflammatory mediators have a collaborative role in implantation. During the implantation window, there are interactions between the embryo and the endometrium to allow attachment, adhesion and invasion of the embryo. At the molecular level, this is regulated by alteration in gene expression of cytokines, growth and transcription factors as well as adhesive molecules (361) (366). Whilst this embryo-endometrial cross-talk is currently not fully understood, the endometrial secretome described here may have an important role.

Here, for the first time data is presented which show that endometrial stromal cells of women with RIF have a unique time-dependent rhythm-sensitive *in vitro* secretomic signature compared with controls. Furthermore, melatonin down regulates the correlation of the *in vitro* circadian inflammatory secretome in non decidualised RIF women, but not in controls. Melatonin, a neuro hormone of the pineal gland, is involved in the phasing of circadian rhythms and sleep promotion, it regulates the sleep/wake cycle and acts as an immunostimulator and cytoprotective agent (66). Its cyclical release is a universal feature of all vertebrates (67). The importance of this regulation can be demonstrated by the wide distribution of melatonin receptors (68, 69) and it is likely that these receptors exist on the membranes of all cells whose activity must be synchronised by the melatonin cycle (70). Melatonin acts via G protein-coupled receptors expressed in various areas of the central nervous system and in peripheral tissues (66). However, its actions are pleiotropic, that is it influences multiple, seemingly unrelated phenotypic traits.

The effects of melatonin are also mediated via nuclear receptors and other binding sites or chemical interactions. The cellular processes that are controlled by melatonin independently from receptors may mean that the membrane mediators may not be a requirement for melatonin to influence circadian gene expression in peripheral cells (70) as in the model described here. Circadian melatonin has been identified as being relevant to reproductive physiology; the administration of melatonin reduces the weight of the ovaries (77, 78) and affects reproductive ability in a wide variety of species 71. However, the role, if any, of melatonin during human implantation and pregnancy is unclear. It has been suggested that the diurnal maternal rhythm serves as a signal for the foetus to entrain the circadian rhythms in newborns after delivery as serum melatonin levels during human pregnancy are higher than in a non-pregnant state (74). It may be that (367) it is also a vital regulator at the moment of implantation by facilitating a cascade of down-stream pro-implantation events. In RIF, decidualisation revealed a stronger association with the immunomodulatory profile than the non decidualised group. This was decreased with the addition of melatonin, making the endometrium less dissimilar to the control patients. If this resulted in a damped, ‘quiet endometrium’, akin to Henry Lee’s ‘quiet embryo’(367) it may be that

the circadian synchrony between endometrium and embryo is restored. This more quiescent environment possibly making for more successful implantation.

4.1.1 Limitations

These results may be confounded by a small sample size; a larger scale study in this specific subgroup of women is further needed to confirm these findings. It is uncertain to what extent tissue biopsies or indeed uterine fluid from preceding menstrual cycles reflect the mid-luteal endometrium in the current cycle nor whether it will be predictive for future cycles. Additionally it is unclear to what extent timing of the sample within one particular cycle is representative of the uterine profile as a whole. Studies of endometrial fluid have shown intra- and inter- patient inconsistencies (317), which could be due to the degree of dilution and/or differences in the timing of aspiration in the menstrual cycle (324, 368). Also, a non-pregnant uterus may not completely reflect the behaviour of a gravid uterus as the presence of early embryonic and placental factors, for example, HCG may affect the expression of endometrial markers.

Despite this, endometrial secretion analysis at the time of embryo transfer offers advantages over more traditional techniques which usually entail gathering endometrial tissue and disrupting the underlying endometrium (369). Whilst fluid sampling circumvents this problem endometrial secretions are also susceptible to contamination and it is impossible to obtain a completely atraumatic sample. This means the sample can contain cellular material, mostly epithelial and immune cells but also blood and lysed cells, debris and mucinous cervical secretions; which also contribute to the intrauterine environment in vivo.

4.1.2 Strengths

This study is unusual in that it reports endometrial fluid sampling from recurrent implantation failure patients and compares them with women who have no reproductive difficulties. Many studies using RIF patients from IVF unit, rely on subfertile 'control' which obviously is a flawed technique. Not only, because RIF is a, 'retrospective diagnosis'. In addition, all these women were in 'normal cycles', and not undergoing sampling during IVF treatment – another downside of the interpretation of results of many other studies.

4.1.3 Conclusions

An understanding of the in-vivo immunomodulatory milieu in women with RIF, reconfirmed in larger studies, may provide a method to discriminate women with a higher RIF propensity from those who will go on to have successful outcomes whilst serving to delineate plausible underlying mechanisms. This method offers a non-disruptive approach to study the role of the endometrium in human embryo implantation and may be useful as a clinical indicator of those women in whom multiple treatment cycles have failed. The circadian oscillation of clock genes in the endometrium is evident, and likely to be present and relevant in the endometrium of the peri implantation period. Women suffering RIF have a different uterine secretome compared with healthy controls, and this is directly related to this oscillating sequence of events. A link between circadian genes and immunomodulators may have a role in the implantation process, and be mediated by clock genes. Other internally self-sustaining oscillations, underlying pathology, the influence of endocrine control and circulating melatonin may facilitate any such processes. The present findings contribute to our understanding of the reproductive molecular clock but further investigation into this widely unknown field is warranted.

Table 4-1 Core clock gene Primer sequences

Human clock gene	Forward Primer	Reverse Primer
Circadian locomotor output cycles kaput (clock)	TCA-CAC-GGC-CGT-CTC-AGA	TGG-GTG-GAG-TGC-TCG-TAT-CC
Brain and muscle arylhydrocarbon receptor nuclear translocator-like protein (bmal1)	GGT-CCA-CTG-CAC-AGG-CTA-CA	TCA-TCA-TCT-GGG-AGG-GAA-ACA
Cryptochrome circadian clock 1(cry1)	GGG-AAG-AAG-GAA-TGA-AGG-TAT-TTG-A	CAA-CAG-GGC-AAT-AGC-AGT-GAA-A
Cryptochrome circadian clock 2 (cry2)	GGA-CAG-GAT-CAT-TGA-GCT-GAA-TG	TCC-ATG-CGG-CTG-ATG-ATG
Period circadian clock 1(per1)	TCC-ATT-CGG-GTT-AGC-AAG-CT	TGT-GTG-CCG-CGT-AGT-GAA-A
Period circadian clock 2(per2)	CTT-TGT-GTG-TGT-CCA-CTT-TCG-AA	TGA-TGG-CAA-AAT-CTG-AAC-ACA-AC

Table 4-2 Patient demographics all patients

Demographics		Control group (N=24)	RIF group (N=17)	*P*
Mean age (range)		34.5 (21-45)	36.2 (25-43)	0.35
Mean BMI (SD)		29.3 (5.9)	25.7 (4.6)	0.14
Cycle characteristics				
Day of cycle		14.9 (9.6)	15.9 (8.6)	0.61
Stage of cycle (%)	Proliferative	14 (56)	6 (35)	0.42
	Periovulatory	3 (12)	3 (18)	
	Luteal	8 (32)	8(47)	
Mean time of sample (range)	Time	11:29 (9:00-15:30hrs)	11:25 (9:00-16:00hrs)	0.82
	Morning	17 (68)	11 (65)	
	Afternoon	8 (32)	6 (35)	

Table 4-3 Patient demographics excluding those without uterine fluid samples taken

Patient demographic		Study group n=16	Control group n=20	P value
Age (mean (SD))		35.6 (5.07)	35.6 (5.79)	0.96
Body mass index (BMI) (mean (SD))		25.69 (4.19)	28.25 (4.53)	0.09
Smoking status (number (%))		1 (6.3%)	4 (20.0%)	0.36
Stage of menstrual cycle (number (%))	Proliferative	7	10	0.43
	Secretory	9	10	
Ethnicity	White British	12 (75%)	19 (95%)	0.12
	South East Asian	3 (18.8%)	0 (0%)	
	White Eastern European	1 (6.3%)	1 (5%)	

Table 4-4 Fertility characteristics

(*unknown data excluded from that particular analysis n=2)

Patient	IVF/ ICSI	Total no of embryos transferred	Number of cycles	Average no of embryos transferred per cycle	Number of fresh cycles (%)	Number of blastocysts transferred (%)
1	ICSI	3	2	2	2 (100)	0 (0)
2	IVF	6	4	2	3 (75)	3 (50)
3	ICSI	4	3	1	2 (67)	4 (100)
4	IVF	4	2	2	0 (0)	2 (50)
5	ICSI	3	3	1	2 (67)	0 (0)
6	IVF	3	2	2	1 (50)	0 (0)
7	ICSI	4	2	2	1 (50)	2 (50)
8	IVF	11	6	2	6 (100)	Unknown*
9	ICSI	15	6	3	6 (100)	Unknown*
10	IVF	5	3	2	2 (67)	1 (20)
11	ICSI	3	2	2	1 (50)	3 (100)
12	IVF	11	6	2	3 (50)	7 (64)
13	ICSI	6	3	2	3 (100)	0 (0)
14	IVF	10	3	3	3 (100)	4 (40)
15	ICSI	3	2	2	2 (100)	3 (100)
16	ICSI	7	4	2	3 (75)	3 (43)

Table 4-5 Concentration of immunomodulators in the uterine fluid and serum

Significant correlations *=p<0.05

Immunomodulator	Site	Control women			RIF patients			Correlation between UF:serum			
								Controls		RIF	
		Mean	SD	Ratio	Mean	SD	Ratio	r	p	r	p
Eotaxin	UF	14.21	21.07	9.57	7.72	8.97	19.79	0.19	0.50	0.36	0.25
	serum	135.94	56.85		152.77	108.61					
MCP1	UF	300.63	384.63	0.48	184.47	337.09	0.75	-0.30	0.24	0.56	0.04*
	serum	143.29	66.11		138.25	62.41					
MIP1α	UF	59.02	77.86	0.32	21.84	27.73	1.32	-0.11	0.74	-0.27	0.38
	serum	18.72	8.89		28.88	22.29					
MIP1β	UF	150.04	206.31	6.12	69.80	78.49	12.52	0.53	0.06	-0.19	0.49
	serum	917.87	133.91		874.01	176.29					
SDF1α	UF	487.40	556.10	5.81	337.03	351.79	7.98	0.17	0.95	0.34	0.25
	serum	2829.98	579.78		2689.55	704.77					
RANTES	UF	22.75	29.28	5.97	12.64	13.52	7.90	-0.12	0.65	-0.17	0.59

	serum	135.91	87.46		99.78	44.43					
IL18	UF	5.00	4.61	6.18	8.32	10.65	2.59	0.16	0.64	-0.10	0.80
	serum	30.91	16.72		21.54	10.90					
IL7	UF	10.43	25.22	0.76	6.10	6.92	1.42	-0.07	0.81	0.58	0.05*
	serum	7.93	5.06		8.68	6.96					
BDNF	UF	276.26	442.22	10.05	69.65	169.84	30.71	-0.14	0.69	-0.51	0.16
	serum	2776.62	1565.88		2139.03	1145.49					
βNGF	UF	28.12	20.35	10.26	9.34	7.45	34.61	-0.28	0.43	-0.85	0.03*
	serum	288.46	100.68		323.43	156.23					
EGF	UF	34.92	57.77	2.28	9.26	11.19	10.73	0.37	0.21	0.27	0.56
	serum	79.80	54.03		99.30	73.75					
HGF	UF	2613.69	5024.84	0.14	1290.10	2247.12	0.21	0.36	0.17	0.35	0.23
	serum	370.24	298.13		273.46	173.72					
LIF	UF	263.84	381.08	0.07	60.92	98.19	0.55	-0.18	0.49	-0.04	0.89
	serum	19.44	11.34		33.23	34.89					

PDGFββ	UF	56.52	93.80	18.29	13.12	22.78	96.22	-0.14	0.65	0.09	0.83
	serum	1033.63	592.86		1262.35	932.09					
SCF	UF	8.67	8.33	1.91	3.30	2.23	7.51	-0.04	0.89	0.31	0.55
	serum	16.58	10.01		24.77	9.65					
VEGFα	UF	589.50	1067.13	3.36	625.77	1525.22	2.94	-0.16	0.56	-0.18	0.64
	serum	1980.59	1537.94		1839.41	1037.43					

Table 4-6 The mean correlation of the quantity of the immunomodulatory compound in the uterine fluid and serum

Red is positive correlation, blue is negative correlation. Measured in μg/l (*p<0.05)

Immunomodulatory compound	All women	Controls	RIF
Eotaxin	0.18	0.19	0.36
MCP1	-0.97	-0.30	0.56*
MIP1α	-0.25	-0.27	-0.11
MIP1β	0.08	-0.19	0.53
SDF1α	0.17	0.02	0.34
RANTES	-0.04	-0.01	-0.17
IL18	0.04	0.16	-0.10
IL7	0.06	-0.07	0.58*
BDNF	-0.11	-0.14	-0.51
βNGF	-0.37	-0.28	-0.85*
EGF	0.28	0.37	0.27
HGF	0.38	0.36	0.35
LIF	-0.17	-0.18	-0.04
PDGFββ	-0.15	-0.14	0.90
SCF	-0.13	-0.05	0.31
VEGFα	-0.15	-0.16	-0.18

Table 4-7 Significant uterine fluid immunomodulatory mediators and their possible role in the uterus

Immunomodulatory molecule	Roles (370)	Expression in endometrium/phenotype	Expression in RIF compared with controls
Interleukin-1 RA **(IL-1RA)**	Anti-inflammatory cytokine which Inhibits activities of interleukin 1, alpha (IL1A) and interleukin 1, beta (IL1B), and modulates a variety of interleukin 1 related immune and inflammatory responses. Four alternatively spliced transcript variants encoding distinct isoforms have been reported.	Vital for embryo implantation (371) and may play a crucial role in embryo-maternal interaction by regulating stromal cell expression of IL-1beta and IL-1ra resulting in embryonic implantation (372). The embryo modulates changes in the IL-1R throughout early gestation; modulation of endometrial receptivity to IL-1 by embryo-driven signals may play a role in the receptive phenotype in endometrium (373).	Decreased
Interleukin-6 **(IL-6)**	Pro-inflammatory cytokine and an anti-inflammatory myokine. Important mediator of the acute phase response, stimulates inflammation and has a role in adaptive immunity. Mobilises extracellular substrates and augments substrate delivery.	Vital for embryo implantation (374, 375) and reduced fertility; viable implantation sites decreased in mice.	Decreased
Beta Nerve Growth Factor (βNGF)	A neurotrophin implicated in the pathophysiology of inflammation. Regulation of growth and the differentiation of sympathetic and certain sensory neurons. Mutations in this gene have been associated with hereditary sensory and autonomic neuropathy, type 5 (HSAN5), and dysregulation of this gene's expression is associated with allergic rhinitis.	NGF and TrkA mRNA and protein higher in endometritis than in normal tissue (376). Andrographolide treatment reduced immunoreactivity of NGF in ectopic endometrium (377).	Decreased
Leukaemia Inhibitory Factor (LIF)	Lymphoid factor, promotes long-term maintenance of embryonic stem cells by suppressing spontaneous differentiation. LIF is normally expressed in the trophectoderm of the developing embryo,	Embryonic LIF is vital for human embryo implantation (inhibition of blastocyst LIF decreased its cell survival factor p-AKT and increased apoptosis) (374, 378, 379)	Decreased?

Immunomodulatory molecule	Roles (370)	Expression in endometrium/phenotype	Expression in RIF compared with controls
	with its receptor LIFR expressed throughout the inner cell mass. As embryonic stem cells are derived from the inner cell mass at the blastocyst stage, removing them from the inner cell mass also removes their source of LIF.	Thicker endometrial lining exhibiting higher expression of LIF (380)	
Regulated upon Activation, Normal T-cell Expressed, and Secreted **(RANTES or CCL5)**	Pro-inflammatory chemokine, chemotactic for T cells, eosinophils and basophils. Recruits leukocytes into inflammatory sites in conjunction with other cytokines (IL-2 and IFN) that are released by T cells and causes the release of histamine. Induces proliferation/activation of NK cells to form CHAK (CC-Chemokine-activated killer) cells. An HIV-suppressive factor released from CD8+ T cells.	Increased RANTES expression, in a P4-dependent manner, may be regulated by VIP and may have an active role in the decidualisation process in endometrial stromal cells.(381) Hormone withdrawal (oestrogen and progesterone support) leads to production of RANTES by decidualised stromal cells (382).	Decreased
Brain-Derived Neurotrophic Factor **(BDNF)**	Promotes survival of neuronal populations that are all located either in the central nervous system or directly connected to it. May play a role in the regulation of the stress response.	BDNF is present in human menstrual blood and endometrium - supporting its in female reproductive function (383). BDNF is conserved across several mammalian species in the uterus (glandular and luminal epithelium, vascular smooth muscle, and myometrium (384).	Decreased
Platelet-derived growth factor beta polypeptide, Becaplermin **(PDGF-β)**	Platelet-derived growth factor is a cell surface tyrosine kinase receptor for members of the platelet-derived growth factor family. These growth factors are mitogens for cells of mesenchymal origin.	Trophoblast signals attract endometrial stromal cells, while PDGF-BB has not been identified as trophoblast-derived it is a local growth factor that may serve to fine-tune directed and non-directed migration at the implantation site (385). PDGF-β reduced in recurrent miscarriage (386)	?
Stem cell factor	Cytokine which as the ligand binds the tyrosine-kinase receptor encoded by the KIT	SCF derived from endometrial cells and the implanting embryo exerts paracrine and/or autocrine action on the process of implantation by stimulating trophoblast	Decreased

Immunomodulatory molecule	Roles (370)	Expression in endometrium/phenotype	Expression in RIF compared with controls
(SCF)	locus. A pleiotropic factor that acts in utero in germ cell and neural cell development, and haematopoiesis, and has a role in cell migration. In adults, it functions as a growth factor and for its continued requirement in haematopoiesis.	outgrowth (387). SCF is weakly supportive of clonogenicity of epithelial cells (388).	

Table 4-8 Single time point gene expression

	bmal1	*clock*	*cry2*	*per1*	*per2*
Morning	0.01±0.01	0.56±0.10	0.15±0.16	0.25±0.33	0.31±0.26
Afternoon	0.01±0.01	0.04±0.07	0.19±0.24	0.13±0.11	0.23±0.19
P value	0.63	0.64	0.57	0.08	0.32

Table 4-9 Non binary logistic sine curve fitting of core circadian gene rhythms

Gene		*bmal1*		*clock*		*cry1*		*cry2*		*per1*		*per2*	
Curve parameters		non	dec	non	dec	non	dec	non	dec	non	dec	non	dec
Controls	Amplitude	1.67	1.89	1.31	1.23	2.00	2.00	1.47	-0.52	1.70	2.34	3.15	5.76
	K	0.08	0.03	0.06	0.06	0.10	0.05	0.04	0.00	0.08	0.10	0.11	0.14
	Period	11.37	14.14	11.56	10.43	13.07	12.91	11.20	20.25	11.44	10.25	18.66	9.72
	Phase Shift	22.41	2.41	-2.10	-0.05	10.95	1.71	22.78	72.72	10.66	0.24	12.59	12.62
	Baseline	-933.20	0.43	-0.21	310.00	0.23	232.80	-4.38	0.60	0.34	0.23	-0.27	1885.00
	Amplitude2	935.10	2.11	2.24	-308.70	2.70	-231.30	6.54	-0.08	2.99	2.23	2.53	-1882.00
	H	0.00	0.07	0.04	0.00	0.08	0.00	0.01	10.42	0.12	0.10	0.04	0.00
	Frequency	0.09	0.07	0.09	0.10	0.08	0.08	0.09	0.05	0.09	0.10	0.05	0.10
	Half Life	13.12	33.29	17.35	17.42	9.63	18.39	24.58	>500	11.99	9.83	9.18	7.20
	Constraints (Y = Amplitude*exp(-K*X)*sin((2*pi*X/Wavelength)+PhaseShift)+baseline+Amplitude2*exp(-H*X))												
	Amplitude	1.67	< 2	< 3	< 3	< 2	< 2	NA	NA	NA	NA	< 5	< 8
	K	0.08	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0
	Period	11.37	> 10	NA	NA	> 0	> 0	> 0	> 0	> 0	> 0	< 19	< 15
	H	NA	< 1	< 1	< 1	< 1	< 1	NA	NA	NA	NA	NA	< 0.01
	r2	0.93	0.96	0.96	0.78	0.99	0.89	0.92	0.93	0.98	0.96	0.93	0.85
RIF	Amplitude	1.00	1.00	1.29	2.53	151.80	2.68	1.72	1.58	3.09	-2.15	5.69	1.92

Gene	*bmal1*		*clock*		*cry1*		*cry2*		*per1*		*per2*	
K	0.00	0.00	0.00	0.00	0.53	0.03	0.00	0.00	0.02	0.00	0.04	0.02
Period	22.45	28.30	8.69	36.13	8.80	32.45	21.70	9.64	21.55	8.69	21.24	28.89
Phase Shift	21.10	28.26	55.97	53.75	6.28	28.41	20.80	20.63	14.86	-2.84	6.97	27.80
baseline	2.77	2.92	3.02	3.21	2.19	2.37	3.20	3.00	3.36	2.97	2.92	2.50
Frequency	0.04	0.04	0.12	0.03	0.11	0.03	0.05	0.10	0.05	0.12	0.05	0.03
Half life	>500	>500	>500	>500	1.89	31.87	>500	>500	33.78	>500	15.99	41.71
Constraints (Y = Amplitude*exp(-K*X)*sin((2*pi*X/Wavelength)+PhaseShift)+baseline)												
Amplitude	0 < A < 1	0 < A < 1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
K	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0
Period	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0
r2	0.05	0.06	0.06	0.07	0.16	0.12	0.06	0.05	0.11	0.03	0.15	0.06

Table 4-10 COSNOR (MESA) circadian analysis of core clock genes

Gene	Patient type	Progesterone exposure	Period (CU)	Phase	Amplitude
bmal1	Control	Non decidualised	27.1	16.63	0.72
		Decidualised	26.25	21.37	1.84
	RIF	Non decidualised	23.46	19.66	2.72
		Decidualised	23.13	19.76	2.45
clock	Control	Non decidualised	26.54	4.76*	0.25
		Decidualised	26.16	11.23	0.95
	RIF	Non decidualised	25.12	19.58*	2.24
		Decidualised	26.19	17.74	3.28
cry1	Control	Non decidualised	21.68	7.73*	0.65
		Decidualised	24.9	11.5&	1.13
	RIF	Non decidualised	22.53	21.12*	2.12
		Decidualised	25.39	22.68&	2.30
cry2	Control	Non decidualised	22.53	2.08*	0.33
		Decidualised	21.36	1.86&	0.73
	RIF	Non decidualised	24.64	22.06*	2.77
		Decidualised	23.17	21.83&	3.34
per1	Control	Non decidualised	26.58	0.04^*	0.76*
		Decidualised	24.47	21.15^&+	1.12
	RIF	Non decidualised	25.78	19.05*	3.14*
		Decidualised	24.62	0.29&+	1.89
per2	Control	Non decidualised	26.52	0.06	0.89
		Decidualised	25.66	2.7&	1.70
	RIF	Non decidualised	25.5+	0.04+	3.58
		Decidualised	21.77+	23.09&+	1.30

^p<0.05 control non decidualised vs control decidualised
+p<0.05 control non decidualised vs control decidualised
***p<0.05 control non decidualised vs RIF non decidualised**
&p<0.05 control decidualised vs RIF decidualised

Chapter 5 General Discussion and conclusions

This thesis has provided an overview of the relationship between biological and circadian rhythms with female reproduction. In a systematic review and meta-analysis, the impact of shift work on menstrual disturbance and early reproductive outcomes was explored. When compared with non-shift workers, shift working was associated with greater risk of menstrual disruption and increased rates of subfertility but not an increased rate of miscarriage. However, working purely night shifts was associated with an increased rate of miscarriage. This review provided good evidence for an association between performing shift work and poor early reproductive outcomes which is consistent with later pregnancy findings. There was insufficient evidence to advise as to whether or not clinicians should advise restricting shift work in women of reproductive age or those with reproductive difficulty. This is mainly because it is difficult to draw firm conclusions as to whether adverse outcomes were a direct effect of working the shifts, or mediated by confounders. Whilst the association may be even explained, by confounders (i.e. class, income, smoking, diet and other poor-health behaviours), women's specific reproductive concerns and amelioration of these effects should be considered.

However, circadian control is found throughout the hypothalamic-pituitary axis in women, playing a role in the timing of ovulation and progesterone secretion. Inappropriate exposure to light in order to deliberately deregulate circadian rhythm has been shown to negatively impact upon mammalian implantation and pregnancy success. Human reproductive outcomes are reliant on circadian timings and previous systematic reviews have revealed associations between shift work and increased risks of preterm birth, low birth weight, foetal loss. It therefore seems plausible, or even likely that the link shown here might be causal.

Shift workers are known to suffer more commonly with sleep deprivation than those women who do not work shifts, both at the time of shift work and in the days/nights which follow. In the prospective cohort study which followed in this thesis, it was demonstrated that women who suffered from recurrent implantation failure were more likely to be exposed to prolonged periods of light and to sleep for a shorter duration than healthy fertile controls or women suffering recurrent miscarriage. However, quality of sleep, latency of sleep, time for sleep onset and waking did not appear to be different between the groups. This was taking into account self-reported information from questionnaires and subjective measures of sleep quantity and quality.

When considering the activity profile for these women, i.e. when they are most or least active relative to the hour of the day, it was possible to delineate a pattern of behaviour that was specific to each group of women; fertile controls, those suffering from RM and those suffering RIF. This suggests that there is a property inherent to the circadian activity of these women which is borne out in their early reproductive capacities. This link was not explained by other parameters such as overall activity, timing of light exposure nor demographic factors such as weight or age. Given this finding, in combination with the fact that women with RM and RIF also had unusual activity patterns as represented by shift workers, it seemed likely that there was underlying circadian disruption in these women.

Women with RIF had striking differences compared with controls in the actigraphy study. They were found to sleep for less time which appeared to be related to less 'sleep conserving behaviours'. One patient in particular was also exposed to more light. All human behaviour and physiology is subject to daily rhythms, which are controlled by the circadian clock. This endogenous time keeping system provides a temporal organisation of bodily functions in relation to environmental time and allows for anticipation of daily rhythms. Chronic circadian rhythm disruption might result in an increased risk of long-term health effects, including the inability to reproduce.

In order to understand this process mechanistically, this thesis investigated the disruption of biological rhythms within the endometrium of women with RIF compared with fertile women. The uterine immunomodulatory environment and molecular transcriptome of endometrial circadian clocks was explored in *in vivo* and in an *in vitro* model. Firstly, it was shown that the immunomodulatory profile of uterine fluid, but not the serum, differs in RIF and controls. This is prudent as the inability to elevate the expression of genes related to endometrial receptivity in a spontaneous manner, will alter the secretome. This evidence supports the widely accepted theory, that decreased endometrial receptivity is linked to the endometrial environments' inability to up-regulate the genes important for implantation. It is thought that this is a pro-inflammatory profile that is required for successful embryo invasion, akin to the repertoire of immunomodulators described within this experiment.

The clock gene expression within the endometrium was then studied in more detail and directly related back to the endometrial secretome. It was shown that endometrial clock genes are expressed in the stroma and glandular tissue of human endometrium and this expression varies with time of day *in vivo.* There was a different expression of per2 in morning and afternoon sampling in proliferative phase endometrium but not in the secretory phase, after the addition of progesterone.

Endometrial clock gene expression was then described *in vitro* and was shown to have a robust circadian rhythm of expression, which differs upon decidualisation of ESC. *In vitro* HESC also had a stable amplitude, which persists after decidualisation. The stable phases also persist, but remain different before and after progesterone exposure in controls compared with RIF women. In non decidualised endometrium of women in the control group compared with that of RIF women, the phases of all core clock genes except for *bmal1* were significantly different, and further forward in time. After decidualisation, there was an altered clock gene expression phase pattern seen between the same genes (*clock, cry1, cry2, per1, per2*) in control and RIF women.

Given the discrepancy in the endometrial secretome between fertile women and women with RIF, and the different phases of the core clock genes, these two features were compared. It was shown that ESC of women with RIF have a unique time-dependent rhythm-sensitive *in vitro* secretomic signature compared with controls. This differential could be explained by the clinical data relating to sleep and activity, which in turn may have an impact on these women's endometrial clocks. However, as light supresses melatonin production, and it had been shown that the women with RIF had greater quantities of light exposure, we hypothesised that by replacing the potential deficiency in physiological melatonin, we could reverse this effect.

Melatonin, when added to the cell culture model, appeared to have more effect on the rhythm of mRNA expression of the six core clock genes in HESC from women with RIF than in the HESC from controls. Melatonin possible also down regulated the correlation of the circadian downstream inflammatory secretome in non decidualised RIF cells. After decidualisation, RIF women had a stronger association with the immunomodulatory profile than the non decidualised group. Exposure to melatonin meant the association with pro-inflammatory markers was decreased.

So in summary, a link between clinical data, uterine receptivity and implantation has been shown to exist. Tightly controlled human physiology is governed by clocks throughout the many biological regulators which are important for optimal functioning. Disruption to these intrinsic modulators is likely to be important in the context of reproduction, outside the mechanism of implantation alone. Although the circadian network of biological systems is apparent in all aspects of reproductive functioning; from menstruation to fertility and pregnancy, further work is needed in this evolving field.

.

Chapter 6 Future work

This thesis has gone someway to further investigate the link between biological rhythms, circadian biology and early reproductive outcomes. More information in to this area is required to better our understanding. Outlined here are some further study designs that could be employed to build upon the work contained here.

6.1 The effect on the behaviour of melatonin on ESC

We have demonstrated in an *in vitro* model for human implantation, that the behaviour of ESC in women suffering RIF is modulated by, or modulates, the circadian rhythm of clock genes. This is different in RIF women compared with controls, and in turn appears to be altered by the addition of melatonin. Further work will be needed to determine whether the difference in the ability for ESC at the endometrial-embryonic interface is purely because their secretory profile varies, or because also the phenotype of the cells themselves change. The migratory ability of ESC is required for implantation and is known to be increased at implantation sites. If human ESC migration and that the motility of the stromal cells is also downregulated in RIF, and the addition of melatonin improves their motility, then it seems likely that the behaviour of the endometrium in response to an embryo is altered in RIF. I melatonin makes no difference to the motility of these cells, then it seems more likely that the secretome is the key factor affecting implantation in women with dysregulated clock. This investigation of localised endometrial responses may give insights in to how the clock governs the implantation of the human embryo.

6.1.1.1 Objective

1. To determine if women with RIF have a decreased speed of ESC migration compared with controls
2. To establish the effect of melatonin on the speed of migration of human ESC
3. To distinguish whether this is the same in both RIF women and fertile controls.

6.1.2 Method

Monolayers of decidualised ECS would be generated from endometrial biopsies of women with RIF and fertile controls. Cell-free migration zones would be created and the effect of the addition of melatonin to the migratory activity of the cells observed.

Subsequent analysis of the timing of any migration would be compared. The primary outcome measures would be speed and magnitude of migration.

6.2 3D modelling systems

Implantation involves embryo apposition and adhesion to the endometrial epithelium followed by penetration through the epithelium and invasion of the embryonic trophoblast through the endometrial stroma. The molecular mechanisms controlling implantation in the human are unknown. In recurrent implantation failure this complex process fails, either because it too tightly regulated at the level of the endometrium, or because of asynchrony between endometrium and embryo. Having demonstrated the circadian rhythm of clock genes in the ESC, it would be informative to see if the same were true in epithelial endometrial cells and see if this response if altered in response to trophoblastic invasion.

6.2.1.1 Objective

1. To determine whether clock genes have circadian rhythm in the epithelial cells of the endometrium
2. To determine whether clock genes have circadian rhythm in the epithelial cells of the endometrium which is in synchrony of that of the ESC
3. Whether or not the expression of clock genes alters in a co-culture model involving embryo surrogates

6.2.2 Method

A uterine cell culture model would be created, involving ESC and epithelial cells and spheroid models. This could be as a co-culture, a multi-layer co-culture or a multilayer invasion assay. The embryo 'surrogate' could be trophoblastic spheroids or first trimester explants. Equally, the endometrial tissue could come from an endometrial explant. Following on from this, organoid models could be used to explore in a single cell analysis how each compartment varies with good quality or bad quality embryo and the expression of clock.

6.3 Clinical trials with melatonin

Melatonin, a neuro hormone of the pineal gland, is involved in the phasing of circadian rhythms and sleep promotion. It regulates the circadian rhythm of sleep and acts as an immune stimulator. Circadian melatonin has been identified as being relevant to reproductive physiology; the administration of melatonin reduces the weight affects

reproductive ability in a wide variety of species. However, the role, if any, of melatonin during human implantation and pregnancy is unclear. It may be that it is also a vital regulator at the moment of implantation by facilitating a cascade of down-stream pro-implantation events as demonstrated by the results in this thesis. Melatonin is a widely available drug and is used therapeutically for sleep disturbance. It would be informative to see how the drug, used in a research setting, affected reproductive outcomes.

6.3.1.1 Objective

To discover if melatonin has an effect on clinical pregnancy rates in women undergoing ART who are exposed to melatonin

6.3.2 Method

A randomised controlled trial (RCT) in women undergoing ART. This RCT would compare those women who are given exogenous melatonin to those who are not. The drug could be given systemically (orally) or directly into the uterine cavity at the time of embryo transfer (i.e. applied to the endometrium). The main outcome measure would be clinical pregnancy rate in those exposed to melatonin, compared with those who are not. Plasma melatonin levels would be taken to compare background melatonin levels, although these can also be measured in saliva.

6.4 Activity monitoring

Actigraphy has been used to assess activity patterns in both fertile and early pregnancy pathologies. The sleep and activity patterns are thought to be different. Large-scale data which directly compares measures the activity and sleep-wake cycles in healthy women, and active monitoring of circadian rhythm in women trying for a pregnancy would be informative. Robust data for pregnant and non-pregnant activity-sleep cycles is also lacking.

6.4.1.1 Objectives

1. To compare the activity and sleep patterns of women trying to conceive compared with healthy controls
2. To compare the activity and sleep patterns of women who have conceived compared with healthy controls
3. To compare the activity and sleep patterns of women who are pregnant, and suffering later pregnancy pathologies, compared with healthy controls

6.4.2 Method

A large scale actigraphy and questionnaire based study. This could be multi-centre or even international to compare inter-racial/inter-cultural differences.

6.5 Lifestyle evaluation

This study demonstrated that the self-reported sleep habits and actigraphy-measured activity in women suffering RM and RIF or without reproductive pathology are grossly similar. However, total sleep time is less in women with RIF compared with healthy fertile controls and the activity pattern across RIF and RM is different from controls. Animal studies have shown that fertility may be improved or reduced according to differences in the light-dark cycle and it was hypothesised in the chapter three that the 'discerning endometrium' may be clock dependent. An epidemiological data analysis of activity patterns and lifestyle habits of women would provide further information as to whether lifestyle and activity patterns are a reliable predictor of early reproductive outcomes.

6.5.1.1 Objective

To assess whether lifestyle factors have an impact on early reproductive outcomes

6.5.2 Method

A cohort study using questionnaires and telephone calls, as well as collecting data from institutes holding relevant information. This study could potentially involve quality of life information and interventional measures such as coping strategies. These may help inform and advise as how best to deal with the burden of fertility treatment and reproductive difficulties.

6.5.3 Final comments

These are just brief outlines of some of the further work that could be undertaken to follow on from this thesis. Any and all of these ideas would help inform scientists about the biological clock and reproduction, an area still much unknown and not widely studied. The development and the long term health of the foetus may be well determined *in utero,* and any biological process affected by the circadian clock which are implicated in reproduction may have lasting consequences. In this thesis the role of the clock in the foetus, in the placenta and in neonatal life has not been explored but certainly should be an area where we endeavour to gain more information in the future.

Appendix A Publications

Review

Influence of Shift Work on Early Reproductive Outcomes

A Systematic Review and Meta-analysis

Linden J. Stocker, BM, BS, Nicholas S. Macklon, MD, MBChB, Ying C. Cheong, MD, MBChB, and Susan J. Bewley, MD, BMBS

OBJECTIVE: To determine whether an association exists between shift work and early reproductive outcomes.

DATA SOURCES: MEDLINE, Embase, and Web of Science were searched. Additional sources included Google Scholar, the Cochrane Library, online publications of national colleges, the ClinicalTrials.gov, and references of retrieved papers.

METHODS OF STUDY SELECTION: Included studies compared female shift workers (work outside 8:00 AM to 6:00 PM) with nonshift workers with menstrual disruption (cycles less than 25 days or greater than 31 days), infertility (time-to-pregnancy exceeding 12 months), or early spontaneous pregnancy loss (less than 25 weeks).

TABULATION, INTEGRATION, AND RESULTS: Two reviewers extracted adjusted and raw data. Random effect models were used to pool data weighting for the inverse of variance. Assessments of heterogeneity, bias, and subgroup analyses were performed. Sixteen independent cohorts from 15 studies (123,403 women) were subject to analysis. Shift workers had increased rates of menstrual disruption (16.05% [2,207/13,749] compared with 13.05% [7,561/57,932] [n=71.681, odds ratio {OR} 1.22, 95% confidence interval {CI} 1.15–1.29, I^2 0%]) and infertility (11.3% [529/4,668] compared with 9.9% [2,354/23,811] [OR 1.80, 95% CI 1.01–3.20, I^2 94%]) but not early spontaneous pregnancy loss (11.84% [939/7,931] compared with 12.11% [1,898/15,673] [n=23,604, OR 0.96, 95% CI 0.88–1.05, I^2 0%]). Night shifts were associated with increased early spontaneous pregnancy loss (n=13,018, OR 1.29, 95% CI 1.11–1.50, I^2 0%). Confounder adjustment led to persistent relationships between shift work and menstrual disruption (adjusted OR 1.15, 95% CI 1.01–1.31, I^2 70%) but not infertility (adjusted OR 1.11 95% CI 0.86–1.44, I^2 61%). The association between night shifts and early spontaneous pregnancy loss remained (adjusted OR 1.41 95% CI 1.22–1.63, I^2 0%).

CONCLUSION: This review provides evidence for an association between performing shift work and early reproductive outcomes, consistent with later pregnancy findings. However, there is currently insufficient evidence for clinicians to advise restricting shift work in women of reproductive age.

(Obstet Gynecol 2014;0:1–12)
DOI: 10.1097/AOG.0000000000000321

Women account for almost half of the working population and the majority are of reproductive age.[1,2] Shift work occurs across occupations and social classes[1] with almost 20% of women performing duties outside standard hours.[1] Altered psychological, social, and biological functioning results, posing risk factors for disease including cardiovascular pathology,[3] metabolic disturbances, and cancer.[4]

Whereas the mechanisms behind this are largely unknown, the greatest physiological problem is sleep alteration. Noncircadian activity encourages poor sleep hygiene and sleep deprivation. Internal timekeeping is maintained in 24-hour oscillations despite being out of synchronicity with the external environment[5] unless long-term circadian disruption persists.[6] Exogenous alterations to these tightly entrained processes are produced by altered light[7] and working schedule.[8]

The hypothalamic–pituitary axis is under circadian control and affects timing of ovulation and hormone secretion.[9] Deregulating circadian rhythms by inappropriate light exposure or manipulating the body clock at a molecular level negatively affects implantation and

From the University of Southampton, Faculty of Medicine, Academic Unit of Human Development and Health, Southampton, and Kings College London, Women's Academic Health Centre, St. Thomas' Hospital, London, United Kingdom.

Corresponding author: Linden J. Stocker, Room F86, Level F Princess Anne Hospital, Southampton, SO16 5YA, 02380 796033l UK; e-mail: stocker@soton.ac.uk.

Financial Disclosure
The authors did not report any potential conflicts of interest.

ISSN: 0029-7844/14

Appendix A

www.nature.com/scientificreports

OPEN

Identifying stably expressed housekeeping genes in the endometrium of fertile women, women with recurrent implantation failure and recurrent miscarriages

Received: 8 March 2017
Accepted: 20 June 2017
Published online: 01 November 2017

Linden Stocker[1], Felino Cagampang[2] & Ying Cheong[1,3]

Housekeeping genes (HKG) are presumed to be constitutively expressed throughout tissue types but recent studies have shown they vary with pathophysiology. Often, validation of appropriate HKG is not made. There is no consensus on which HKGs are most stably expressed in endometrial tissue so this study aimed to identify the most stable HKG in the endometrium of women with recurrent implantation failure (RIF) and recurrent miscarriages (RM). Inclusion criteria were women between 25–45 years (n = 45) suffering recurrent miscarriage (RM), recurrent implantation failure (RIF) or fertile controls. Endometrial biopsies were taken and total RNA extraction, cDNA synthesis and PCR was performed using 10 candidate HKG. The genes were arranged in terms of stability and normalisation was determined. Several HKGs not previously tested in endometrial samples were found to be more stable than those previously identified as the most stable. Of these, the 5 most stable HKG (in order of stability) were *Prdm4* (PR domain 4) > *Ube4a* (Ubiquitin-Conjugating Enzyme 4a) > *Enox2* (Ecto-NOX Disulfide-Thiol Exchanger 2) > *Ube2d2* (Ubiquitin-conjugating enzyme E2D 2) > *Actb* (Actin beta). We therefore recommend using at least four of the aforementioned HKG for normalisation of endometrial tissues taken from patients with RM and RIF.

The measurement of mRNA expression is a widely accepted and useful method of describing and quantifying gene expression in any tissue. It offers a high-turnover and the accurate expression profiling of selected genes. This offers a means of investigating tissue such as the endometrium to allow the identification of markers that could potentially play a role in endometrial function, implantation of an embryo and continuation of successful pregnancy.

The use of mRNA expression by quantitative reverse transcription polymerase chain reaction (qRT-PCR) requires normalisation to constitutively expressed genes, known as housekeeping genes (HKG). HKG are genes that are required for the maintenance of basal cellular functions that are essential for the existence of a cell, regardless of its specific role in the tissue or organism[1]. Comparison of the gene of interest to these conserved HKG is vital to adjust for potential experimental variables such as the quantity of starting material, enzymatic activity and any differences in overall transcription between tissues[2]. This is especially prudent in cells undergoing growth and differentiation, often driven by subtle changes in gene expression. Thus, it is ideal to use HKG that are sufficiently expressed in the tissue of interest, have minimal variability and high stability irrespective of physiological or pathological conditions.

The most commonly used HKG have been shown to vary considerably across samples and tissues[2,3]. This includes glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), but has now been demonstrated to be less stably expressed in some tissues than is generally assumed[4,5]. Despite this, validation of the presumed stability of HKG in

[1]University Hospital Southampton NHS Foundation Trust, Princess Anne Hospital, Division of Women and Newborn, Coxford Road, Southampton, SO16 5YA, UK. [2]Institute of Developmental Sciences, University of Southampton, Faculty of Medicine, Tremona Road, Southampton, SO16 6YD, UK. [3]Complete Fertility Centre, Princess Anne Hospital, Division of Women and Newborn, Coxford Road, Southampton, SO16 5YA, UK. Correspondence and requests for materials should be addressed to L.S. (email: l.stocker@soton.ac.uk)

Clinical Review
State of the Art Review

Health consequences of shift work and insufficient sleep

BMJ 2016 ; 355 doi: https://doi.org/10.1136/bmj.i5210 (Published 01 November 2016)
Cite this as: *BMJ* 2016;355:i5210

Reproductive consequences of shift work and insufficient sleep deserve consideration.

09 November 2016
Linden J Stocker
O&G Registrar
Ying Cheong, Professor of Obstetrics and Gynaecology, University of Southampton. Susan Bewley, Professor of Women's Health, King's College London
University of Southampton NHS Foundation Trust
6
@linden_stocker

As obstetricians and gynaecologists, we read about the adverse health consequences of shift work and insufficient sleep (doi: http://dx.doi.org/10.1136/bmj.i5210) with both personal and professional interest. Although the authors state, "some people are more resilient to shift work than others", they do not mention women: who make up a large proportion of shift and night workers, and undoubtedly the majority in the NHS.

Women account for almost half the working population, contribute significantly to the world economy and the majority of workers are of reproductive age. Circadian control is found throughout the hypothalamic-pituitary axis in women, playing a role in the timing of ovulation and progesterone secretion. Inappropriate exposure to light in order to deliberately deregulate circadian rhythm has been shown to negatively impact upon mammalian implantation and pregnancy success (1, 2). Human reproductive outcomes are reliant on circadian timings and previous systematic reviews have revealed associations between shift work and increased risks of preterm birth, low birth weight, fetal loss as well as menstruation, fecundity and early pregnancy complications (3-6). The workforce, and the NHS in particular, depends on women. Whilst the associations may be affected, or even explained, by confounders (i.e. class, income, smoking etc.), women's specific reproductive concerns and amelioration should have been considered.

1. Sellix MT, Menaker M. Circadian clocks in mammalian reproductive physiology: effects of the "other" biological clock on fertility. Discov Med. 2011;11(59):273-81.
2. Summa KC, Vitaterna MH, Turek FW. Environmental perturbation of the circadian clock disrupts pregnancy in the mouse. PLoS One. 2012;7(5):e37668.
3. Physical and shift work in pregnancy : occupational aspects of management: a national guideline. London: Royal College of Physicians of London; 2009.
4. Knutsson A. Health disorders of shift workers. Occup Med (Lond). 2003;53(2):103-8.
5. Palmer KT, Bonzini M, Bonde JP, Multidisciplinary Guideline Development G, Health, Work Development U, et al. Pregnancy: occupational aspects of management: concise guidance. Clin Med (Lond). 2013;13 (1):75-9.
6. Stocker LJ, Macklon NS, Cheong YC, Bewley SJ. Influence of shift work on early reproductive outcomes: a systematic review and meta-analysis. Obstet Gynecol. 2014;124(1):99-110.

Competing interests: No competing interests

Appendix B Patient consent, information and questionnaires

University of Southampton

University Hospital Southampton NHS
NHS Foundation Trust

Consent form

Patient identification number:

Project title:
Biological cycles and the reproductive tract

Please initial box

1. I confirm that I have read and understand the information sheet dated 16/11/2012 (version 2.0) of the above study. I have had time to consider the information, ask questions and have had these satisfactorily answered. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my notes and data collected during the study may be looked at by individuals from University Hospitals Southampton NHS Foundation Trust where it is relevant to me taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I agree to wear the Actiwatch as described to me by the research team and keep a sleep diary. ☐
5. I agree to have a blood and urine sample taken as part of this research project and that this will only be disposed of once the study is complete. ☐
6. I agree to have a sample of endometrial fluid (fluid from inside the womb) and a biopsy of my endometrium (lining of the womb) taken as part of this research project and that these will only be disposed of once the study is complete. ☐
7. I agree to have a sample of peritoneum and peritoneal fluid taken as part of this research project and that this will only be disposed of once the study is complete. ☐
8. I agree to take part in the above study. ☐

Name of participant	Signature	Date

Patient consent form version 2.0 16/11/2012
REC 12/SC/0568

University of Southampton | University Hospital Southampton NHS NHS Foundation Trust

Participant information sheet: control patients

Project title:
Biological cycles and the reproductive tract

We would like to invite you to take part in our research study looking at factors which influence the way your body regulates the times when you are asleep or awake, and whether these are related to your body's reproductive system. We would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. We suggest this should take about 5 minutes.

Purpose of the study
This study aims to provide us with a better understanding of the factors that control the timings of different events within the female reproductive tract such as periods, ovulation and pregnancy, and what happens if these events don't happen in the correct way. It is thought that there are ways in which your body controls these events which are related to the building blocks which are unique to the way in which your body is made (your genes).

The reproductive tract in a woman includes her ovaries, uterus (womb), fallopian tubes (oviducts), cervix (neck of the womb) and vagina. For the purposes of this study we are mainly interested in looking at the endometrium, which is the lining of the womb and/or your sleep.

What does participating in this study involve?
You will be asked to sign a consent form. Following this, the study has different parts:

a) You will be asked to fill in short questionnaires about your sleep.
b) You will be asked to wear an Actiwatch for one week and fill in a sleep diary. This device is similar in appearance to a wrist-watch. It monitors whether or not you are exposed to light or darkness and whether you are moving or not.
c) We will ask you for approximately a 5ml blood sample (the equivalent of a teaspoonful) and a urine sample for the purposes of the research.
d) The study will involve the collection of some endometrial fluid (a very small amount of fluid that is contained within the womb and is produced by the lining cells), and a tiny sample of endometrium (womb lining) via the use of a suction device. This involves a thin tube of plastic being placed inside the uterus, through the vagina to obtain the samples. This may be being done as part of your treatment, or done additionally for the research if this is not the case.
e) If you are having an operation on your tummy (laparoscopy) as part of your routine care, we will take a small amount of peritoneal tissue (the tissue that lines the inside of where the surgeons will operate) and the peritoneal fluid (surplus fluid that is contained in your tummy). This may be being done anyway as part of your treatment but if not, it will be done for the purposes of the study.
f) The samples of blood, urine, endometrium, endometrial fluid, peritoneum and peritoneal fluid will be analysed in a laboratory.
g) We will perform various tests on these samples which will include looking at your DNA (the building-blocks of your genes, which are the way your body is made)

You will not have to make additional visits to the hospital except those needed for your routine care.

Why have I been invited to take part?
You have been invited to take part because you are attending the hospital as part of your routine care and fulfil the criteria for recruitment in to our study. You are a woman who has no known reproductive problems and we wish to compare your results to women who do. We are not investigating your gynaecology complaint.

Will anyone know that I'm taking part?
The data collected will be completely confidential within the hospital. All of the findings will be kept confidential in accordance with standards followed by medical researchers in compliance with national laws. Your sample will be anonymous which means that people looking at the study results will not know that you were involved. The data from the Actiwatch will be treated in the same way.

Do I have to take part?
It is your choice whether or not you take part. If you would like to take part you will be asked to sign a consent form. You may withdraw from the study at any time and this will not affect your future care in any

University of Southampton | University Hospital Southampton NHS NHS Foundation Trust

way. If you withdraw from the study, we will destroy all your identifiable samples, but we would use the data collected up to your withdrawal.

What will happen if I don't want to continue taking part in the study?
You may withdraw from the study at any time and this will not affect your future care in any way. If you withdraw from the study we would use the data collected up to the point of your withdrawal.

What are the benefits to taking part?
We hope that your help will help us to gain better understanding of the mechanism involved in the way the reproductive tract functions. In the long term we hope that this work will contribute to the development of strategies to predict, prevent or treat reproductive disorders.

What are the disadvantages to taking part?
The study will not affect the treatment you receive in anyway. You may have some slight vaginal spotting of blood or some mild period type pains after the procedure. These normally go away within a few hours. If you are having an operation, this is no different from the routine part of your operation.

Will any genetic test be done?
We are not looking at specific genetic disorders or diseases. We are looking at the samples you provide to examine your, 'gene expression', which is a process by which your body dictates its functions. This is not likely to provide any information directly relevant to you or your family. If we think that it will be relevant to you, we will tell you.

What will happen to the information gathered by this study?
It is hoped that this information will be printed in a medical journal as a source of information for other researchers and doctors. The results may be presented at academic meetings and a summary of the results will be put on the departmental website. At all times the information will be confidential and any information used in analysing the results would not be linked to you in any way. If you would like to be informed as to the outcomes of the study, please provide the researcher with your contact address, and relevant information may be sent to you on completion of the study.

What if there is a problem?
Any concerns or complaints you have about the way you have been approached or treated during the study will be listened to and dealt with. Contact information is available from departmental staff in the first instance. If you have concerns about the care provided to you or the Trust services, the Patient Support Service (PSS). PSS may be contacted by telephone 023 8079 8498, e-mail PSS@uhs.nhs.uk. If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

What will happen to my sample at the end of the study?
At the end of the study your sample will be destroyed.

Who is organising and funding the research?
It is organised and funded jointly by the University of Southampton and University Hospitals Southampton NHS Foundation Trust.

Who has approved this study?
All research in the NHS is looked at by an independent group of people call a research ethics committee to protect you safety, rights, wellbeing and dignity. This study has been given a favourable opinion by South Central – Southampton B ethics committee.

Who can I speak to for more information or if I have questions?
Main investigator:
Dr L Stocker, Gynaecology and obstetrics specialist registrar and academic clinical fellow
E-mail: l.stocker@soton.ac.uk
Telephone 02380 796033
Consultant and joint investigator:
Dr Y Cheong, Gynaecology consultant and senior lecturer

Thank you for reading this

Patient Information leaflet version 1.0 10/12/12
Applies to protocol version 1.0 21/09/2012, consent form version 2.0 16/11/12
REC 12/SC/0568

Appendix B

University of Southampton — University Hospital Southampton NHS Foundation Trust

Participant information sheet: gynaecology patients

Project title:
Biological cycles and the reproductive tract

We would like to invite you to take part in our research study looking at factors which influence the way your body regulates the times when you are asleep or awake, and whether these are related to your body's reproductive system. We would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. We suggest this should take about 5 minutes.

Purpose of the study
This study aims to provide us with a better understanding of the factors that control the timings of different events within the female reproductive tract such as periods, ovulation and pregnancy, and what happens if these events don't happen in the correct way. It is thought that there are ways in which your body controls these events which are related to the building blocks which are unique to the way in which your body is made (your genes).

The reproductive tract in a woman includes her ovaries, uterus (womb), fallopian tubes (oviducts), cervix (neck of the womb) and vagina. For the purposes of this study we are mainly interested in looking at the endometrium, which is the lining of the womb and/or your sleep.

What does participating in this study involve?
You will be asked to sign a consent form. Following this, the study has different parts:

a) You will be asked to fill in short questionnaires about your sleep.
b) You will be asked to wear an Actiwatch for one week and fill in a sleep diary. This device is similar in appearance to a wrist-watch. It monitors whether or not you are exposed to light or darkness and whether you are moving or not.
c) We will ask you for approximately a 5ml blood sample (the equivalent of a teaspoonful) and a urine sample for the purposes of the research.
d) The study will involve the collection of some endometrial fluid (a very small amount of fluid that is contained within the womb and is produced by the lining cells), and a tiny sample of endometrium (womb lining) via the use of a suction device. This involves a thin tube of plastic being placed inside the uterus, through the vagina to obtain the samples. This may be being done as part of your treatment, or done additionally for the research if this is not the case.
e) If you are having an operation on your tummy (laparoscopy) as part of your routine care, we will take a small amount of peritoneal tissue (the tissue that lines the inside of where the surgeons will operate) and the peritoneal fluid (surplus fluid that is contained in your tummy). This may be being done anyway as part of your treatment but if not, it will be done for the purposes of the study.
f) The samples of blood, urine, endometrium, endometrial fluid, peritoneum and peritoneal fluid will be analysed in a laboratory.
g) We will perform various tests on these samples which will include looking at your DNA (the building-blocks of your genes, which are the way your body is made)

You will not have to make additional visits to the hospital except those needed for your routine care.

Why have I been invited to take part?
You have been invited to take part because you are attending the hospital as part of your routine care and fulfil the criteria for recruitment in to our study.

Will anyone know that I'm taking part?
The data collected will be completely confidential within the hospital. All of the findings will be kept confidential in accordance with standards followed by medical researchers in compliance with national laws. Your sample will be anonymous which means that people looking at the study results will not know that you were involved. The data from the Actiwatch will be treated in the same way.

Do I have to take part?
It is your choice whether or not you take part. If you would like to take part you will be asked to sign a consent form. You may withdraw from the study at any time and this will not affect your future care in any way. If you withdraw from the study, we will destroy all your identifiable samples, but we would use the data collected up to your withdrawal.

University of Southampton — University Hospital Southampton NHS Foundation Trust

What will happen if I don't want to continue taking part in the study?
You may withdraw from the study at any time and this will not affect your future care in any way. If you withdraw from the study we would use the data collected up to the point of your withdrawal.

What are the benefits to taking part?
We hope that your help will help us to gain better understanding of the mechanism involved in the way the reproductive tract functions. In the long term we hope that this work will contribute to the development of strategies to predict, prevent or treat reproductive disorders.

What are the disadvantages to taking part?
The study will not affect the treatment you receive in anyway. You may have some slight vaginal spotting of blood or some mild period type pains after the procedure. These normally go away within a few hours. If you are having an operation, this is no different from the routine part of your operation.

Will any genetic test be done?
We are not looking at specific genetic disorders or diseases. We are looking at the samples you provide to examine your, 'gene expression', which is a process by which your body dictates its functions. This is not likely to provide any information directly relevant to you or your family. If we think that it will be relevant to you, we will tell you.

What will happen to the information gathered by this study?
It is hoped that this information will be printed in a medical journal as a source of information for other researchers and doctors. The results may be presented at academic meetings and a summary of the results will be put on the departmental website. At all times the information will be confidential and any information used in analysing the results would not be linked to you in anyway. If you would like to be informed as to the outcomes of the study, please provide the researcher with your contact address, and relevant information may be sent to you on completion of the study.

What if there is a problem?
Any concerns or complaints you have about the way you have been approached or treated during the study will be listened to and dealt with. Contact information is available from departmental staff in the first instance. If you have concerns about the care provided to you or the Trust services, the Patient Support Service (PSS). PSS may be contacted by telephone 023 8079 8498, e-mail PSS@uhs.nhs.uk. If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

What will happen to my sample at the end of the study?
At the end of the study your sample will be destroyed.

Who is organising and funding the research?
It is organised and funded jointly by the University of Southampton and University Hospitals Southampton NHS Foundation Trust.

Who has approved this study?
All research in the NHS is looked at by an independent group of people call a research ethics committee to protect you safety, rights, wellbeing and dignity. This study has been given a favourable opinion by South Central – Southampton B ethics committee.

Who can I speak to for more information or if I have questions?
Main investigator:
Dr L Stocker, Gynaecology and obstetrics specialist registrar and academic clinical fellow
E-mail: l.stocker@soton.ac.uk
Telephone 02380 796033

Consultant and joint investigator:
Dr Y Cheong, Gynaecology consultant and senior lecturer

Thank you for reading this

Patient Information leaflet version 3.0 10/12/12
Applies to protocol version 1.0 21/08/2012, consent form version 2.0 16/11/12
REC 12/SC/0568

University of Southampton
University Hospital Southampton NHS
NHS Foundation Trust

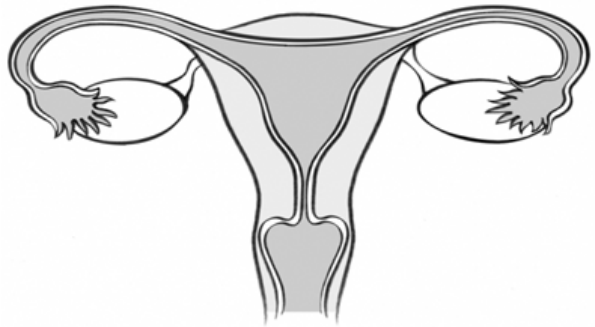
Gynaecological Research Patient Proforma

This information is to be gathered with the help of the researcher and will be used to help form valid conclusions from the samples obtained.

Recruitment Number	
Age	
Height (cm)	
Weight (kg)	
BMI kg/m2	
Ethnicity	
Current gynaecological pathology	
Normal length of menstrual cycle: (K = bleed days/total cycle length)	K= /
LMP date	
Day of cycle on day sample taken (Days since LMP)	
Current contraceptive use: *(if hormone method check inclusion criteria)*	
Pregnancies G = total no of pregnancies P = number of deliveries after 24 weeks	G P
Number of live births	
Details (gestation, outcome) Eg normal delivery, miscarriages, terminations etc	
Previous fertility treatment (details and dates)	
Does the patient smoke?	Cigs/day
Does the patient drink alcohol?	Units/day
Which samples were collected?	Endometrial biopsy ☐ Endometrial fluid ☐ Peritoneal biopsy ☐ Peritoneal fluid ☐ Serum ☐ Urine ☐
Day Actiwatch given	
Number of Actiwatch given	
Day Actiwatch returned	

University of Southampton
University Hospital Southampton NHS
NHS Foundation Trust

Medical Information:

Medical Conditions (including psychiatric conditions) *(include when diagnosed)*	
Previous operations (incl ERPCs/STOPs)	
Regular Medication: *(include dosage and what it is for)*	
Previous gynaecological problems? (History of PCOS? Endometriosis?)	
History of S.T.I.'s (please describe if yes)	
Occupation (shift work?) What sort of shifts How long for	
Contraceptive hx? What and how long for? How long ago? When discontinued?	
Fertility problems? (describe if yes)	
Pain? (duration and details)	
What did the operation show? Time of sample: Date of operation	
Annotate if required: 	

Patient proforma gynaecology patients 1.2 21/05/13
Applies to protocol version 1.0 21/08/2012, consent form version 2.0 16/11/12
REC 12/SC/0568

Appendix B

University of Southampton — University Hospital Southampton NHS Foundation Trust

Subject's ID_____ Date_____

PITTSBURGH SLEEP QUALITY INDEX

INSTRUCTIONS:
The following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month.

Please answer all questions

1.During the past month, what time have you usually gone to bed at night?	BED TIME_____
2. During the past month, how long (in minutes) has it usually taken you to fall asleep each night?	_____MINUTES
3. During the past month, what time have you usually been getting up in the morning?	GETTING UP TIME_____
4. During the past month, how many hours of actual sleep did you get at night? (This may be different than the number of hours you spent in bed).	_____HOURS

For each of the remaining questions, tick the one best response. Please answer all questions.
5. During the past month, how often have you had trouble sleeping because you:

	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
a) Cannot get to sleep within 30 minutes				
b) Wake up in the middle of the night or early morning				
c) Have to get up to use the toilet				
d) Cannot breathe comfortably				
e) Cough or snore loudly				
f) Feel too cold				
g) Feel too hot				
h) Had bad dreams				
i) Have pain				
j) Other reason(s), please describe_____				

6. During the past month, how would you rate your sleep quality overall?

Very good ☐

Fairly good ☐

Fairly bad ☐

Very bad ☐

University of Southampton — University Hospital Southampton NHS Foundation Trust

	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
7. During the past month, how often have you taken medicine to help you sleep (prescribed or "over the counter")?				
8. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?				
9. During the past month, how much of a problem has it been for you to keep up enough enthusiasm to get things done?				

10. Do you have a bed partner or room mate?

No bed partner or room mate ☐

Partner/room mate in other room ☐

Partner in same room, but not same bed ☐

Partner in same bed ☐

If you have a room mate or bed partner, ask him/her how often in the past month you have had:

	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
a) Loud snoring				
b) Long pauses between breaths while asleep				
c) Legs twitching or jerking while you sleep				
d) Episodes of disorientation or confusion during sleep				
e) Other restlessness while you sleep; please describe_____				

Buysse DJ, Reynolds CF, Monk TH, Berman SR, Kupfer DJ: Psychiatry Research, 28:193-213, 1989.

Rec 12/SC/0568
PSQI questionnaire

Epworth Sleepiness scale questionnaire

University of Southampton

University Hospital Southampton NHS
NHS Foundation Trust

Epworth Sleepiness Scale

Name: _____ Today's date: _____

Your age (Yrs): _____ Your sex (Male = M, Female = F): _____

How likely are you to doze off or fall asleep in the following situations, in comparison to feeling just tired?

This refers to your usual way of life in recent times.

Even if you haven't done some of these things recently, try to work out how they would have affected you.

Use the following scale to choose the **most appropriate number** for each situation:

0 = would **never** doze
1 = **slight chance** of dozing
2 = **moderate chance** of dozing
3 = **high chance** of dozing

It is important that you answer each question as best you can.

Situation	**Chance of Dozing (0-3)**
Sitting and reading	___
Watching TV	___
Sitting / inactive in a public place (e.g. a theatre, a cinema or a meeting)	___
As a passenger in a car for an hour without a break	___
Lying down to rest in the afternoon when circumstances permit	___
Sitting and talking to someone	___
Sitting quietly after a lunch without alcohol	___
In a car, while stopped for a few minutes in traffic	___

THANK YOU FOR YOUR COOPERATION

Rec 12/SC/0568
ESS questionnaire

Appendix C Supplementary data

Protocol for Systematic review and meta analysis

Systematic review and meta-analysis to determine the relation between shift work and early reproductive outcomes.
Objective To determine whether there is an association between shift work and early reproductive outcomes.
Inclusion criteria *Study type* All study types will be eligible. Must describe shift-work and either • menstrual disorders • subfertility • pregnancy loss
Participants Women of all age groups and occupations will be included.
Definition of exposure All studies which report women working shifts in comparison to a group of women who do not. Within shift work we will accept anything that is classified as a shift by the author. If possible, we will perform sub-group analyses for different types of shift i.e. nights only.
Outcome variable All studies which report the number of women in each reproductive pathology group will be included. 1. Menstrual dysfunction/deregulation Either a short cycle (< 25 days) or a long cycle (>31 days) (207). 2. Subfertility A time to pregnancy (TTP) interval of greater than 12 months (208, 312). 3. Miscarriage Spontaneous loss of pregnancy before 24 completed weeks of pregnancy (209). If miscarriages are defined as fewer than 24 weeks, we will include them.
Outcome measures The OR will either be calculated or if the necessary data is not reported or cannot be readily extracted from the published data, the reviewers will contact the corresponding authors for additional information (i.e. data

provided in 2x2 contingency tables).

Publication type

These could be published or unpublished, in full text, abstract or poster form with no restriction on publication date, study design or status. Limited to English language.

Search Methods

We will search the following electronic databases:

Medline

Embase

BIOSIS

Web of Science

Google scholar

The Cochrane library

Online publications and guidelines of:

The colleges or associations of obstetricians and gynaecologists (UK/USA/Australia)

The colleges or associations of physicians (UK/USA/Australia)

The colleges or associations of surgeons (UK/USA/Australia)

In these databases, we will search according to the thesaurus of the NCBI MeSH browser the following terms and combinations of keywords in full text:

The following keywords will be employed:

Work Schedule Tolerance

"shift* *work" (adjacent)

Fertility (truncated)

Reproduction (explode)

Menstruation (truncated)

Abortion (truncated/explode)

Miscarriage (truncated)

Pregnancy Complications (explode)

Pregnancy (truncated)

Subfertility (truncated/explode)

Fertility (truncated/explode)

Fecundity

Subfecundity

Additionally, bibliographies of identified publications and published reviews will be hand searched for relevant articles. Authors will be contacted if data, methods and/or parameter definitions provided from the studies are unclear.

Reviews

All references cited in the identified reviews will be manually searched for potentially relevant studies.

Data collection

LS will search the list of titles and the abstracts, to determine potential usefulness of the article. Final selection will be based on the full text of potentially relevant articles by two reviewers independently (LS, YC). In cases of disagreement, a third reviewer (NM) will examine such articles.

The study characteristics will be extracted as per the standardised data collection form.

An independent reviewer will confirm all data entries and will check for completeness and accuracy.

Quality assessment

Will be performed by 2 independent reviewers. The Newcastle Ottawa Scoring system (Wells) will be used (compared studies will not be randomised).

Meta-analysis

Dichotomous comparisons

Data on numbers of subjects exposed or not to shift work will be collated. The numbers of poor reproductive outcomes in each group will be compared (using RevMan V5.1). Fixed-effects as well as random-effects models to estimate the pooled odds ratios for risk of shift work will be constructed across all studies.

Assessment of heterogeneity

Impact of heterogeneity will be assessed by calculating the I^2 according to Higgins et al (Higgins 2003). If >50%, use random effects model.

- Low (<25%)
- Medium (25-50%)
- High (>50%)
- Considerable (>75%)

Sensitivity analyses

Robustness of the pooled estimates will be checked by influence analyses and to identify potential sources of heterogeneity and sources of bias. The studies of interest will be retained and irrelevant studies individually omitted from the data set, followed in each case by recalculation of the pooled estimate of the remaining studies. Studies will be stratified by:

- Study design to assess potential recall bias.
- Further stratifications will be made if any studies are deemed to be outliers on basis of study size, country of origin, population or method.

Meta-regression

Will be considered if there are greater than ten studies in the individual meta-analyses (Cochrane 2011).

Evaluation of bias and confounding

Publication bias

Funnel plot asymmetry to detect publication bias and Egger's regression test to measure any asymmetry will be used if warranted.

Discussion and Evaluating

The results will be critically discussed.

References

Higgins, J.P., et al., *Measuring inconsistency in meta-analyses.* BMJ, 2003. **327**(7414): p. 557-60.

Sterne, J.A., M. Egger, and G.D. Smith, *Systematic reviews in health care: Investigating and dealing with publication and other biases in meta-analysis.* BMJ (Clinical research ed), 2001. **323**(7304): p. 101-5.

The Cochrane Collaboration, *Cochrane Handbook for Systematic Reviews of Interventions*. Version 5.1.0 ed, ed. J. Higgins, Green, S.2011, http://handbook.cochrane.org.

Wells, G., Shea,B O'Connell, D Peterson, J Welch,V Losos, M Tugwell, P *The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses*: http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp.

Appendix C

Databases used for systematic review and meta analysis

Electronic database/online resource	Years covered	Platform used
Medline	1946-present	OvidSP 2012
Embase	1947-present	OvidSP 2012
Web of Science	1970-present	Thompson Reuters 2012
Google Scholar	Present	Google 2013
The Cochrane library	Present	Wiley online library 2012
The colleges/associations of obstetricians and gynaecologists	Present	Websites (UK/USA/Australia)
The colleges/associations of physicians (UK/USA/Australia)	Present	Websites (UK/USA/Australia)
The colleges/associations of surgeons (UK/USA/Australia)	Present	Websites (UK/USA/Australia)
ClinicalTrials.gov	2000-Present	Website ClinicalTrials.gov

(Database: Ovid MEDLINE) 1946 to January Week 4 2013 (other searches done by same methodology)

Example search string for for systematic review and meta analysis

1 Work Schedule Tolerance/ (4761)
2 (shift$1 adj2 work$).mp. (mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept, rare disease supplementary concept, unique identifier) (3821)
3 1 or 2 (7325)
4 Fertility/ (30915)
5 fertility.mp. (69680)
6 exp Reproduction/ (838087)
7 Menstruation/ (14217)
8 reproduction.mp. (93215)
9 (menstruation or menstrual).mp. (48830)
10 exp Abortion, Spontaneous/ (27838)
11 miscarriage.mp. (5425)
12 exp Pregnancy Complications/ (323158)
13 pregnan$.mp. (734861)
14 (subfertil$ or infertil$).mp. (64261)
15 fecundity.mp. (6476)
16 subfecundity.mp. (108)
17 abort$.mp. (85087)
18 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 (1055883)
19 3 and 18 (249)
20 *Work Schedule Tolerance/ (2881)
21 (shift$1 adj2 work$).ti. (1169)
22 20 or 21 (3381)

23	19 and 22 (78)
24	limit 23 to english language (61)

Information from: A shift in the right direction: RCN guidance on the occupational health and safety of shift work in the nursing workforce, 2012, The Royal College of Nursing: London, UK available at: http://www.rcn.org.uk/__data/assets/pdf_file/0004/479434/004285.pdf

Shift definitions for systematic review and meta analysis

		Common Nomenclature	Type of shift pattern	Terms used by authors
Comparison group		Day shift	Typical working day 0800-1800.	'days', 'day shift', 'daytime', 'days only', 'no shift work', 'fixed days', 'always a day'
All included as shift work		Night shifts	Typically starts anywhere between 2000-2200 and lasts for 10-12 hours.	'nights', 'night shift', 'nights only', 'permanent nights', 'fixed night shift', 'always a night'
	Subgroup analysis	Rotating shifts	Working a pattern of days and nights (or occasionally evenings). Shifts can be forward (from morning to afternoon to night shifts) or backwards rotating. Rotation can be fast; every day or so, or doing a week of 'earlies' (typically 0700-1500) then a week of 'lates' (typically 1400-2200).	'rotating shift', 'changing shift', 'shift work', 'rotating shifts including nights', 'day/evening rotating', 'irregular shifts'
		Split shifts	The shift is split into two parts with a gap in between; often 0600-2200 and 1600-2000.	'irregular shifts'
		Continental shift	Continuous three-shift system that rotates rapidly; for example three mornings, then two afternoons, then two nights.	'irregular shifts'
		Three-shift system	The day is divided into three working periods – morning, afternoon and night. This kind of shift work can involve a week of mornings followed by a week on evenings and a week of nights.	'three-shift schedule', 'rotating 3-shift','irregular shifts'
		Two-shift system	Normally two shifts of eight hours each.	'two-shift schedule', 'rotating 2-shift', 'irregular shifts'
		Evening shifts	Typically starts anywhere between 1800-2200 and lasts for 6-8 hours.	'evening shift', 'evening work'

Appendix C

Standard data extracted for systematic review and meta analysis

Source

- Citation and year

Eligibility

- Confirm eligibility for review
- Reason for exclusion

Methods

- Study design
- Total study duration
- Concerns about bias

Participants

- Total number
- Diagnostic criteria
- Age
- Setting/country
- Date of study

Exposure

- Total number of exposure groups
- Specific exposure (type of shift)
- Intervention details (authors' description)

Outcomes

- Outcomes and time points (i) collected; (ii) reported

For each outcome of interest:

- Outcome definition

Results

- Number of participants allocated to each exposure group

For each outcome of interest:

- Sample size
- Missing participants/response rates
- Summary data for each exposure group
- Estimate of effect with confidence interval; P value

Miscellaneous

- Key conclusions of the study authors
- Correspondence required

Newcastle-Ottawa scale

	Menstrual disruption studies				Subfertility studies					Miscarriage studies						
	Chung 2005	Hatch 1999	Lawson 2011	Su 2008	Bisanti 1996 (1)	Bisanti 1996 (2)	Spinelli 1997	Tuntiseranee 1998	Zhu 2003	Axelsson 1989	Axelsson 1996	Eskenazi 1994	Fenster 1997	Hemminiki 1985	Lawson 2012	Whealan 2007
Selection (maximum 4 stars)	★★	★★★	★★★	★★	★★★★	★★	★★	★★★	★★★	★★★★	★★	★★★★	★★★★	★★★★	★★★	★★★
Comparability (maximum 2 stars)	★	★	★★	★★	★	★	★	★	★	★	★	★	★	★	★★	★★
Outcome (maximum 3 stars)	★★★	★★	★★	★★★	★★	★	★★★	★★★	★★	★★★	★★	★★	★★★	★★	★★	★★
TOTAL % of maximum score	67	67	78	78	78	45	67	78	67	89	56	□8	89	□8	78	78
QUALITY	High	High	High	High	High	Medium	High	High	High	High	Medium	High	High	High	High	High

Appendix C

Included studies for systematic review and meta analysis

Menstrual disturbance

Author (year of publication)	Method (years of data collection)	Location	Participants (age in years)	Controls	Number of women	Method of working times assessment (response/participation rate)	Method of outcome assessment	Working time categories (exposure period)	Qualification of 'nights'	Primary exposure	Definition of outcome
Chung (2005)	Cross-sectional (not stated)	Taiwan	Nurses in one hospital (21-44)	Working nurses	151	Questionnaire and 'life/work diary' (75%)	Contemporaneous diary over three and a half months.	'Day shift', evening shift', 'night shift', 'rotating shift' (during shift work)	0000-0800	Various work related risk factors	Either short cycle <25 days or long cycle >35 days
Hatch (1999)	Prospective cohort (not stated)	USA, Italy	Nurses from two hospitals (<50)	Working nurses	124	Postal questionnaire (61%)	Contemporaneous diary over three months.	'Day shift only', 'night shift only', 'rotating shift' (during shift work)	Night shift only	Various work related risk factors	Either short cycle <25 days or long cycle >33 days
Lawson (2011)	Cross-sectional (1993)	USA	A nested cohort from the Nurse's Health Study II (USA) (<45)	Working nurses	71 077	Questionnaire (92%)	Same questionnaire.	Rotating night shifts and at least 3 nights/month in addition to other days and evenings. (during shift work)	N/A	Shift work	Cycle length >31 days or < 21 days. NB excluded >51 days from analysis

Menstrual disturbance

Author (year of publication)	Method (years of data collection)	Location	Participants (age in years)	Controls	Number of women	Method of working times assessment (response/participation rate)	Method of outcome assessment	Working time categories (exposure period)	Qualification of 'nights'	Primary exposure	Definition of outcome
Su (2008)	Cross-sectional (2004-2005)	Taiwan	Factory workers (pre-menopausal)	1 control per five cases; office workers	329	Questionnaire (85%)	Monthly 'updates' provided by participants for eight months.	Shift workers: 12 hour shifts; day (0730-1930), night (1930-0730 -two days off) (during shift work)	N/A	Shift work	Mean cycle length >35 days or <25 days

Appendix C

Subfertility											
Author (year of publication)	**Method (years of data collection)**	**Country**	**Participants (age in years)**	**Comparison group**	**Number of women**	**Method of working times assessment (response/participation rate)**	**Method of outcome assessment**	**Working time categories (exposure period)**	**Qualification of 'nights'**	**Primary exposure**	**Definition of outcome**
Bisanti (1996)	Cross sectional (1991-1993)	Denmark, Germany, Italy, Poland, Spain	1. Population based ('random' sample of women) (25-44)	Working and non-working women (authors' analysis limited to working women)	3092	Face-to-face interview (54-88%)	Same interview	'Shift work'; changing or rotating shifts including daytime, evening, night time or rotating (at time of initial unprotected sexual intercourse UPSI)	N/A	Shift work	Time to pregnancy ≥15.5 months (data for 9.5-15.4 months available but excluded)
	Cross sectional (1992)	Denmark, France Germany, Italy, Sweden	2. Pregnancy based (consecutive antenatal attendees > 20/40) (no restriction)	Working and non-working women (authors' analysis limited to working women)	2420	Postal questionnaire (70-98%)	Same questionnaire		N/A	Shift work	
Spinelli (1997)	Cross Sectional (1993)	Italy	All women delivering a live born child in four hospitals in different towns (no restriction)	Working or not working (150 not working)	622	Face-to-face interview (98%)	Same interview	'No shift work', 'shift work'; no further qualification offered ('prior to conception)	N/A	Type of occupation	Time to pregnancy interval >12 months
Tuntiseranee	Cross Sectional (1995)	Thailand	All pregnant women attending for antenatal care	Working and non-working women (authors'	907	Face-to-face interview (82%)	Same interview	'No shift work', 'shift work'; which includes rotating shift,	N/A	Shift work and hours spent working	Time to pregnancy interval >12

Subfertility

Author (year of publication)	Method (years of data collection)	Country	Participants (age in years)	Comparison group	Number of women	Method of working times assessment (response/participation rate)	Method of outcome assessment	Working time categories (exposure period)	Qualification of 'nights'	Primary exposure	Definition of outcome
(1998)			at two hospitals in different towns (no restriction)	analysis limited to women in paid employment prior to conception)				fixed afternoon shift or fixed night shift (unclear, likely at time of UPSI)			months
Zhu (2003)	Retrospective Cohort (1998-2000)	Denmark	Danish National Birth Cohort (nationwide study of pregnant women attending family doctor who wanted to continue with their pregnancy) (no restriction)	All working women	21438	Telephone interview (60%)	Same interview (	'Daytime work', 'fixed evening work', fixed night work', rotating shift work without nights', 'rotating shift work with nights' (unclear, likely at 12/40)	Fixed night (mutually exclusive category)	Shift work	Time to pregnancy >12 months

Appendix C

ESPL											
Author (year of publication)	**Method (years of data collection)**	**Country**	**Participants (age in years)**	**Comparison group**	**Number of women (pregnancies)**	**Method of working times assessment (response/participation rate)**	**Method of outcome assessment**	**Working time categories (exposure period)**	**Qualification of 'nights'**	**Primary exposure**	**Definition of outcome**
Axelsson (1989)	Cross sectional (1980-1984)	Sweden	All women employed by a hospital (mixed occupations) (≤50)	Non-shift workers of matched occupation	463 (707)	Employment register and postal questionnaire (81%)	Verified by medical records and questionnaire	'Always a day', always an evening', 'always a night', 'irregular time', 'rotating shift' (1st trimester)	Always night	Shift work	'miscarriage'; <28/40 with 99% before 16/40
Axelsson (1996)	Cross sectional (1989)	Sweden	Female members of the Swedish midwives association (<49)	Non-shift workers of matched occupation	1587 (1711)	Postal questionnaire (83%)	Same questionnaire	'Always a day', always a night, 'two-shift schedule, 'three-shift schedule' (1st trimester)	Always night	Shift work and nitrous oxide	<29/40 (early <13/40, late 13-29/40). Only early data used
Eskenazi (1994)	Case control (1986-1987)	USA	Women attending one hospital with a miscarriage (>18)	2 per case; live births matched for Last Menstrual Period (LMP). Only working women	included 1341 (1341)	Telephone interview (81% cases, 88% controls)	Pathology records and medical records (71% included)	'Day', 'evening/night', 'variable' (<20 weeks pregnancy)	Evening/night combined so excluded	Various work related risk factors	<20/40

ESPL

Author (year of publication)	Method (years of data collection)	Country	Participants (age in years)	Comparison group	Number of women (pregnancies)	Method of working times assessment (response/participation rate)	Method of outcome assessment	Working time categories (exposure period)	Qualification of 'nights'	Primary exposure	Definition of outcome
Fenster (1997)	Prospective cohort (1990-1991)	USA	Members of a private health insurer (>18)	All working women	4058 (4058)	Telephone interview (84%)	Medical records (89%) or patient recall (11%)	'Day', 'evening/night', 'variable' (1st trimester)	Evening/night combined so excluded	Various work related risk factors	<20/40
Hemminiki (1985)	Case control (1972-1979)	Finland	Pregnant nurses from a central register of health workers suffering a miscarriage (no restriction)	3 per case; nurses who had a normal birth, matched for employment and age	624 (624)	Questionnaire completed by a site co-ordinator (no information on employment for 87.1% cases, 87.8% controls)	Hospital and central discharge registers	'No shift work', 'rotating 2-shift', 'rotating 3-shift', 'permanent night shift', 'telephone duty' (1st trimester)	Permanent nights	Various work related risk factors	Diagnosis of miscarriage International Classification of Disease eighth revision, diagnoses 643 and 645
Lawson (2012)	Retrospective cohort (1993-2001)	USA	A nested cohort from the Nurse's Health Study II (USA) (25-42)	Nurses working at least 1 hour per week	7482 (7482)	Postal questionnaire (76%)	Same questionnaire	'Nights only', 'rotating shifts including nights', 'day/evening rotating, no nights' (1st trimester)	Nights only	Various work related risk factors	<20/40 (early <12/40, late 12-20/40)
Whealan (2007)	Prospective cohort (1993-2001)	USA	A nested cohort from the Nurse's Health Study II (US) (25-42)	Working nurses	7688 (7688)	Postal questionnaire (89%)	Same questionnaire	'Days only', 'days/evenings with no nights', 'nights only', 'rotating shifts with nights' (1st trimester)	Nights only	Shift work	<20/40 definition of miscarriage

Appendix C

Excluded studies for systematic review and meta analysis

Author	Method (years of data collection)	Country	Outcome measure	Participants (age in years)	Comparison group	Number of women (pregnancies)	Major potentials for selection bias	Effect measure (as quoted by author)	Reason for exclusion of study
Attarchi 2012 (389)	Cross sectional (2010)	Tehran	Miscarriage	Female factory workers (20-40)	Employed factory workers	406	Unclear if induced abortions included	Adjusted OR of miscarriage 4.13 (95% CI 1.70-10.02) shift work.	Unable to extract numbers
			Subfertility			406	Only included women who conceived	Not reported	Unable to extract numbers
Ahlborg 1996 (390)	Cross sectional (1989)	Sweden	Subfertility	All members of the Swedish Midwives Association (<49)	Analysis limited to midwives working more than 20 hours a week	746	Only women who conceived. Planned pregnancies only.	FR 0.82 (95%CI 0.56-1.04) nights, 0.78 (95%CI 0.65-0.94) two-shift, 0.77 (0.61-0.98) three-shift.	Incompatible definition of outcome
Axelsson 1984 (229)	Cross sectional (1968-1979)	Sweden	Miscarriage	Female laboratory workers employed by a university (≤44)	Women in same or different employment, studying or housewives	556 (1160)	Included induced abortions. Non responders excluded	Age-adjusted RR shift work 3.19 (95% CI 1.36-7.47)	Incompatible definition of outcome
Barzilai-Pesach 2006 (391)	Prospective cohort (1999-2000)	Israel	Subfertility	Female attendants to a fertility clinic (no restriction)	All working women	75	Included women who conceived and those who did not. Infertility patients only.	Shift work RR, 1.52 (95% CI 0.89 –2.61)	Incompatible definition of outcome
Bryant 1991 (392)	Case control (1984-1985)	Canada	Miscarriage	Gynaecology admissions to hospital with miscarriages (no restriction)	Used 2 equal sized control groups: pregnant (<25/40) and delivered	981 (981)	'Delivered' controls included only normal term d deliveries	Not reported	Unable to extract numbers
Chen 2002 (232)	Retrospective cohort (1997)	Taiwan	Miscarriage	Female factory workers (no restriction)	Matched employment	173 (292)	Did include induced abortions	Not reported for miscarriage.	Unable to extract numbers
			Subfertility			173	Only women who conceived.	Crude fecundity ratio (FR) fixed night shift 1.45 (95% CI 0.93-2.25), long day shift FR 1.13 (95% CI 0.85–1.50)	Unable to extract numbers

Author	Method (years of data collection)	Country	Outcome measure	Participants (age in years)	Comparison group	Number of women (pregnancies)	Major potentials for selection bias	Effect measure (as quoted by author)	Reason for exclusion of study
El Metwalli 2000 (393)	Case control(1998-1999)	Egypt	Miscarriage	All women admitted to a hospital with a miscarriage (no restriction)	2 per case; routine hospital attenders with completed pregnancies during same time (working or non-working figures provided)	1200	No mention of induced abortions	OR 1.57 (95% CI 1.25-1.97)	Incompatible definition of outcome
Hjollund 1998 (394)	Prospective cohort (1992-1994)	Denmark	Subfertility	Members of a trade union (20-35)	All working women	297	Only nulliparous women	Evening/daytime OR 1.5 (95% CI 0.9-2.4) night work 1.2 (95% CI 0.7-1.9)	Incompatible definition of outcome
Infante-Rivarde 1993 (395)	Case control (1987-1989)	Canada	Miscarriage	All women admitted to a hospital with a miscarriage (no restriction)	3 per case; pregnant routine hospital attenders matched for gestation (working or non-working figures provided)	1324 (1324)	Neither cases nor controls had suffered a previous miscarriages	Adjusted OR fixed evenings 4.17 (95% CI 2.19-7.92). Rotating schedule with nights OR 0.87 (95% CI 0.46-1.62), fixed nights OR 2.68 (95% CI 0.53-13.43)	Incompatible definition of outcome
Labyak 2002 (396)	Prospective cohort (not stated)	USA	Menstrual dysfunction	A nested cohort from a larger study of nurses (<40)	Working nurses	68	78% using hormonal contraceptives	53% of shift workers noted change in cycle length.	Incompatible definition of outcome. Unable to extract numbers
Lohstroh 2003 (397)	Cohort (1996-1998)	China	Menstrual dysfunction	A cohort of textile mill workers (23-31)	No comparison group	12	No mention of contraception use	Not reported	Incompatible definition of exposure (no comparison group)
McDonald 1986 (398)	Cross sectional (1982-1984)	Canada	Miscarriage	All women admitted to a hospital with a miscarriage/ delivery (current and previous pregnancies) (no restriction)	All working women	56012 (104620)	Unclear if induced abortions included	Observed:expected (O/E ratio) 1.01-1.11($p<0.10$)	Incompatible definition of outcome. Unable to extract numbers
McDonald 1988 (399)	Cross sectional (1982-1984)	Canada	Miscarriage	All women admitted to 11 hospitals with a miscarriage/	All working women	56067 (78680)	Unclear if induced abortions included	Shift work RR 1.45 (90% CI 1.0-1.9).	Incompatible definition of outcome. Unable to extract

Author	Method (years of data collection)	Country	Outcome measure	Participants (age in years)	Comparison group	Number of women (pregnancies)	Major potentials for selection bias	Effect measure (as quoted by author)	Reason for exclusion of study
				delivery (current and previous pregnancies) (no restriction)					numbers. Same data as McDonald 1986
Messing 1992 (400)	Cross sectional (1987-1988)	France	Menstrual dysfunction	Slaughter house and cannery workers (pre-menopausal)	Employed factory workers	726	35% using hormonal contraceptives	Variable beginning to shift OR 2.2 (95% CI 1.0-4.7) for long cycle. OR 3.9 (95%CI 1.3-11.4) if variable ending to shift, OR 2.6 (95% CI 1.2-5.6) if variable hours a week.	Incompatible definition of outcome. Unable to extract numbers
Samarawee ra 2010 (194)	Case control (2004)	Sri Lanka	Miscarriage	All women admitted to one hospital with a miscarriage/ antenatal care (current and previous pregnancies) (no restriction)	Women in employment or housewives	731	Unclear if induced abortions included	Sleeping<8hours a day risk factor for first trimester miscarriage OR 3.8 (95% CI 1.01-14.3)	Incompatible definition of exposure
Swan 1995 (401)	(1986-1989)	USA	Miscarriage	Workers of 14 different companies (18-44)	All working women	891	Excluded induced abortions, ectopics and molar pregnancies	Night shifts RR 1.7 (95% CI 0.73-3.7) – rotating shifts 0.81 (95% CI 0.33-2.0) in non chemically exposed women	Unable to extract numbers.
Uehata 1982 (402)	Cross sectional (not stated)	Japan	Miscarriage	Female members of 33 working unions (15-44)	All working women	229 (229)	Unclear if induced abortions included	18 ‘abortions’ in shift-workers compared with 36 in non-shift workers, p<0.05	Incompatible definition of outcome
			Menstrual dysfunction			1822	No mention of contraception use	Not reported	Incompatible definition of outcome

Adjusted data for systematic review and meta analysis

Author	Comparison	Adjusted for	Effect measure	Shifts	Unadjusted OR (95%CI)	Shift adjusted OR (95%CI)	Nights	Unadjusted OR (95%CI)	Night adjusted OR (95%CI)
Menstrual disruption									
Chung 2005	19/72	Work related factors, working hours, age, marital status, education,	N/A	35/79	2.22 (1.12-4.41)	Not available	7/12	3.91 (1.11-13.79)	Not available
Hatch 1999	20/83	Age	N/A	12/41	1.30 (0.56-3.02)	Not available	3/16	0.73 (0.19-2.81)	Not available
Lawson 2011	7505/57 728	Age, age at menarche, race, parity, smoking, alcohol, physical activity, BMI	Risk ratio	2047/13349	1.21 (1.15-1.28)	<10 months 1.27 (1.09-1.48) 10-19 months 1.01 (0.96-1.06) >19 months 1.07 (0.98-1.17)	-	N/A	N/A
Su 2008	17/49	Shift work history, employment duration, caffeine, pre-employment menstrual irregularity	Odds ratio	113/280	1.27 (0.68-2.40)	1.71 (1.03-2.88)	-	N/A	N/A

Appendix C

Author	Comparison	Adjusted for	Effect measure	Shifts	Unadjusted OR (95%CI)	Shift adjusted OR (95%CI)	Nights	Unadjusted OR (95%CI)	Night adjusted OR (95%CI)
Subfertility									
Bisanti 1996 'population-based'	259/2911	Age, smoking, partner's occupation, partner's shift work, gravidity	Odds ratio	36/181	2.54 (1.73-3.74)	1.2 (0.2-8.3)	-	N/A	N/A
Bisanti 1996 'pregnancy-based'	156/2209	Age, smoking, partner's occupation, partner's shift work, gravidity	Odds ratio	44/211	3.47 (2.4-5.02)	2.0 (1.3-3.2)	-	N/A	N/A
Spinelli 1997	58/555	Working hours, work related factors, partner's occupation, smoking (maternal and paternal), caffeine, alcohol, age, parity, coital frequency	Rate ratio	12/67	1.87 (0.95-3.69)	0.90 (0.67-1.2)	-	N/A	N/A

Author	Comparison	Adjusted for	Effect measure	Shifts	Unadjusted OR (95%CI)	Shift adjusted OR (95%CI)	Nights	Unadjusted OR (95%CI)	Night adjusted OR (95%CI)
Tuntiseranee 1998	85/605	Age, education, BMI, menstrual regularity, obstetric disease, medical disease, work related factors (maternal and paternal), coital frequency, hormonal injection use in past,	Odds ratio	51/302	1.26 (0.85-1.84)	1.2 (0.7-1.9)	-	N/A	N/A
Zhu 2003	1797/17531	Age, gravidity, BMI, smoking, alcohol, occupation (maternal and paternal), working hours	Odds ratio	225/2065	0.96 (0.85-1.08)	0.96 (0.88-1.05)	29/177	1.72 (1.15-2.56)	0.80 (0.63-1.00)
Miscarriage									
Axelsson 1989	17/202	Parity, smoking (not adjusted for age as no difference seen)	Risk ratio	55/505	1.33 (0.75-2.35)	1.39 (0.86-2.25) Evenings 0.78 (0.11-5.42) Irregular 1.42 (0.80-2.52)	19/182	1.27 (0.64-2.52)	0.93 (0.49-1.78)

Appendix C

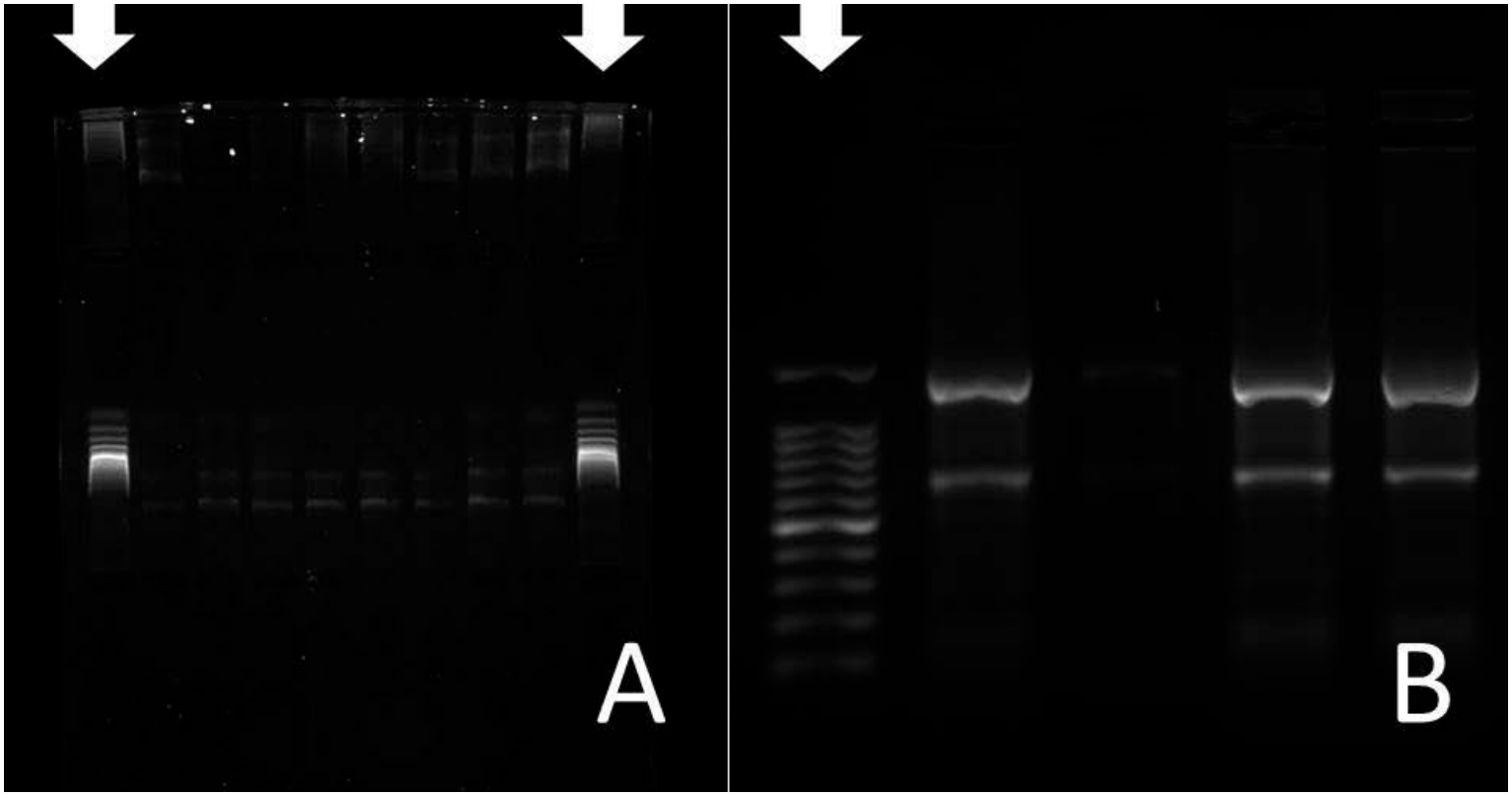
Author	Comparison	Adjusted for	Effect measure	Shifts	Unadjusted OR (95%CI)	Shift adjusted OR (95%CI)	Nights	Unadjusted OR (95%CI)	Night adjusted OR (95%CI)
						Rotating 1.50 (0.55-4.09)			
Axelsson 1996	50/421	Calendar year, age, previous miscarriage, smoking, infection	Odds ratio	150/1290	0.98 (0.69-1.37)	1.20 (0.92-1.58) Three shift 1.23 (0.78-1.94) Two shift 1.19 (0.85-1.67)	34/285	1.01 (0.63-1.60)	1.30 (0.83-2.01)
Eskenazi 1994	353/1057	Race, age, previous miscarriage, smoking, alcohol and caffeine, tap water consumption, marital status, medical insurance status, parity, education, nausea	N/A	79/284	0.77 (0.58-1.03)	Variable 0.60 (0.36-1.00) Evening/night 0.80 (0.53-1.20)	-	N/A	N/A
Fenster 1997	344/3427	Age, gestation at interview, pregnancy history, smoking, alcohol, caffeine, marital status	Odds ratio	66/631	1.05 (0.79-1.38)	Variable 1.34 (0.77-2.34) Evening/night 1.03 (0.75-1.41)	-	N/A	N/A

Author	Comparison	Adjusted for	Effect measure	Shifts	Unadjusted OR (95%CI)	Shift adjusted OR (95%CI)	Nights	Unadjusted OR (95%CI)	Night adjusted OR (95%CI)
Hemminki 1985	57/221	Work related factors (controls/cases matched for age)	N/A	105/403	1.01 (0.70-1.47)	1.5 (0.9-2.5)	4/12	1.44 (0.42-4.96)	Not available
Lawson 2012	536/5109	Age	N/A	239/2373	0.96 (0.81- 1.12)	Not available	89/664	1.32 (1.04-1.68)	Not available
Whealan 2007	541/5242	Age, parity, hours worked, BMI, medication, previous miscarriage, caffeine (work- related factors did not alter results so not included)	Risk ratio	245/2446	0.97 (0.82-1.13)	0.96 (0.65-1.42) Rotating 0.80 (0.70-0.91) Rotating with night 1.20 (0.90-1.60)	91/680	1.34 (1.06-1.70)	1.6 (1.2-2.0)

Appendix C

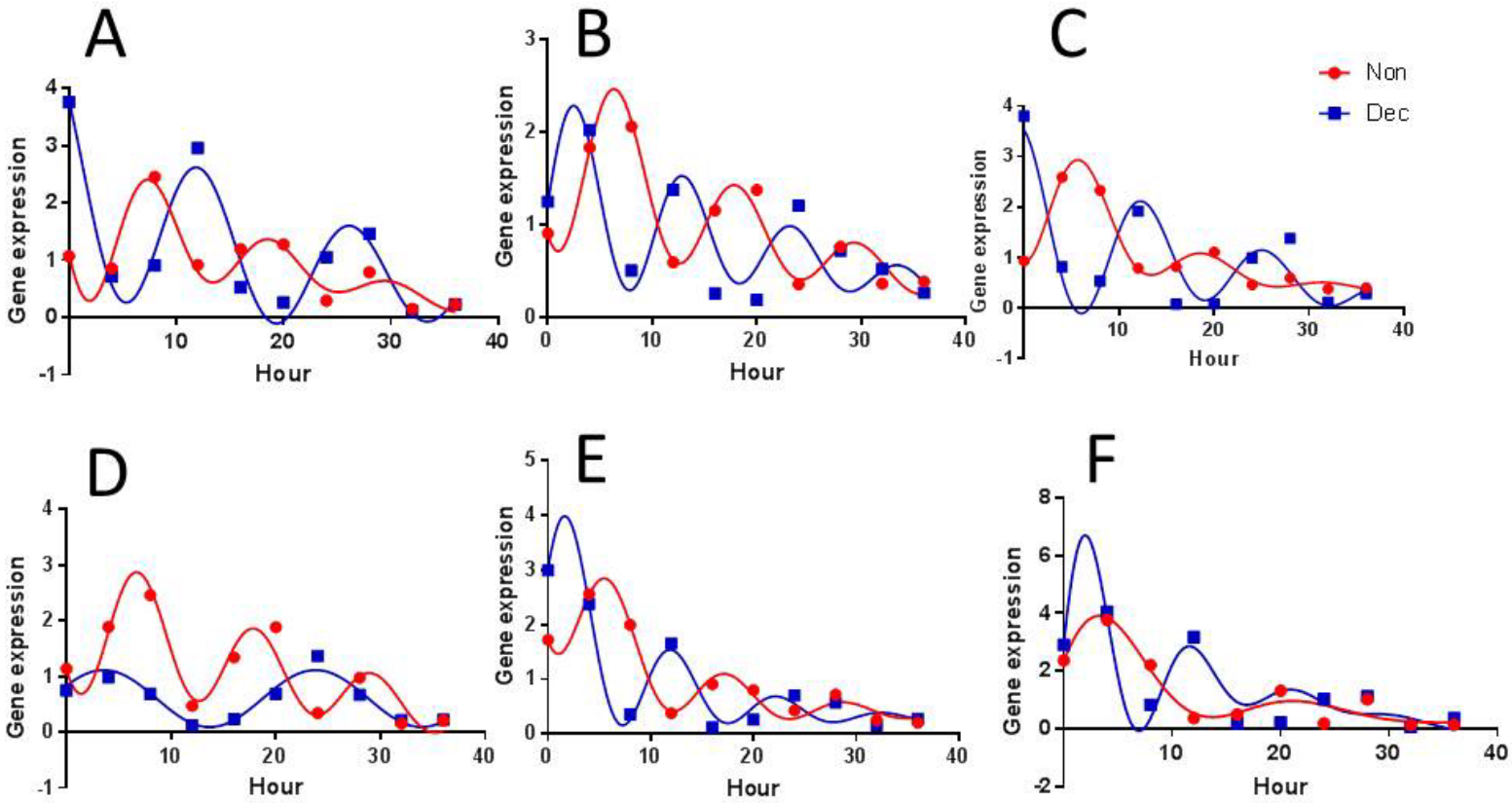
Integrity of RNA (chapter four)

Shown on agarose gel stained with ethidium bromide (A) No magnification (B) Magnified x10 to demonstrate 18S/26S bands (faint bands in diluted sample). Arrows show ladder positions.



Curve fitting for the control patients (chapter four)

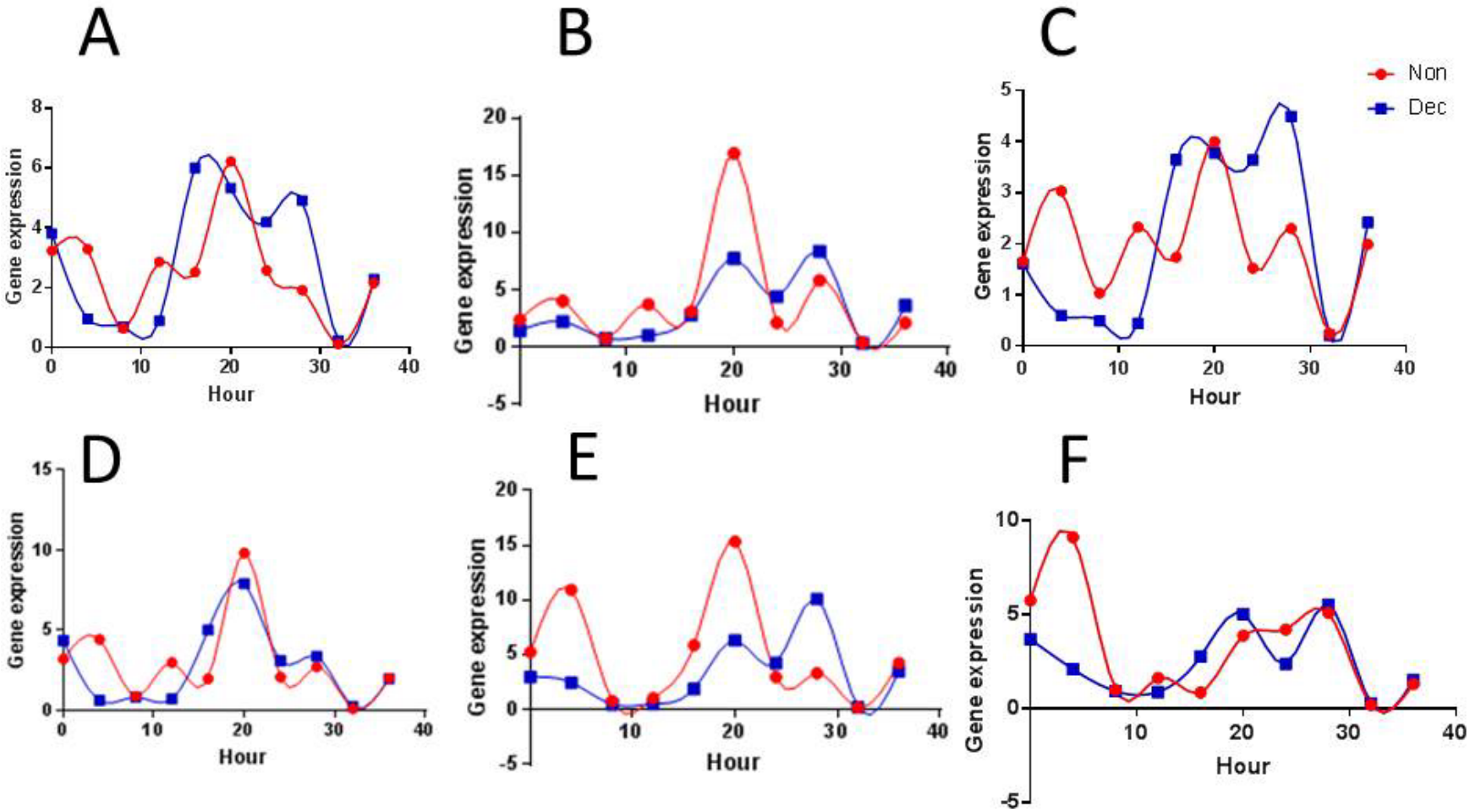
Clock gene expression over 24 hours in control women Sine wave curve fitting was applied (mean r2=0.92, range 0.78-0.99) with non decidualised cells (r2=0.95) showing stronger adherence to the fitted curve than decidualised (r2=0.89). (A) *bmal1* (B) *clock* (C) *cry1* (D) *cry2* (E) *per1* (F) *per2*



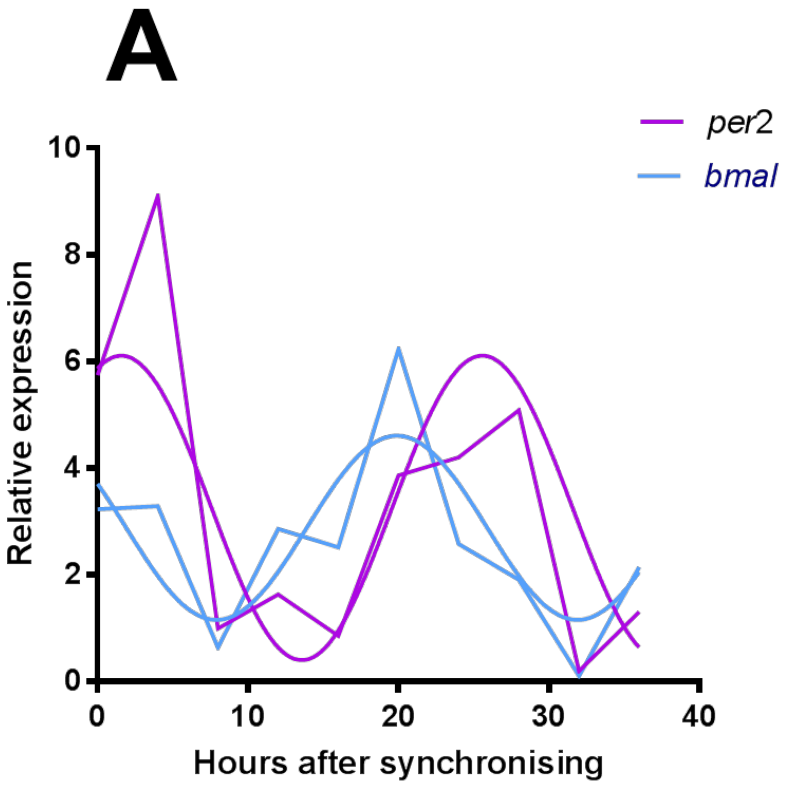
Appendix C

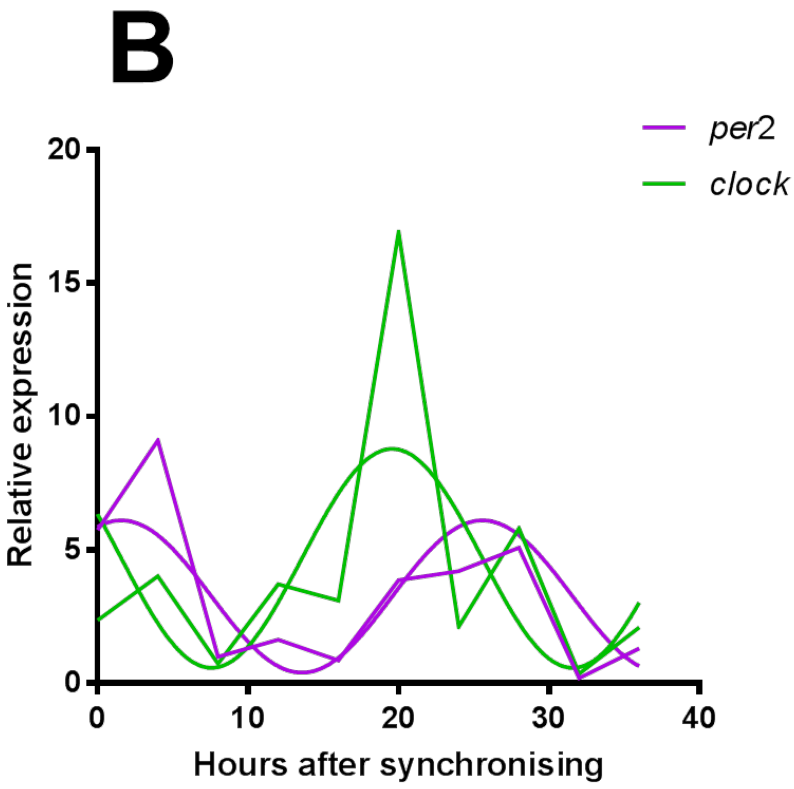
Curve fitting for the RIF patients (chapter four)

Clock gene expression over 24 hours in RIF women after curve fitting was applied (A) *bmal1* (B) *clock* (C) *cry1* (D) *cry2* (E) *per1* (F) *per2*



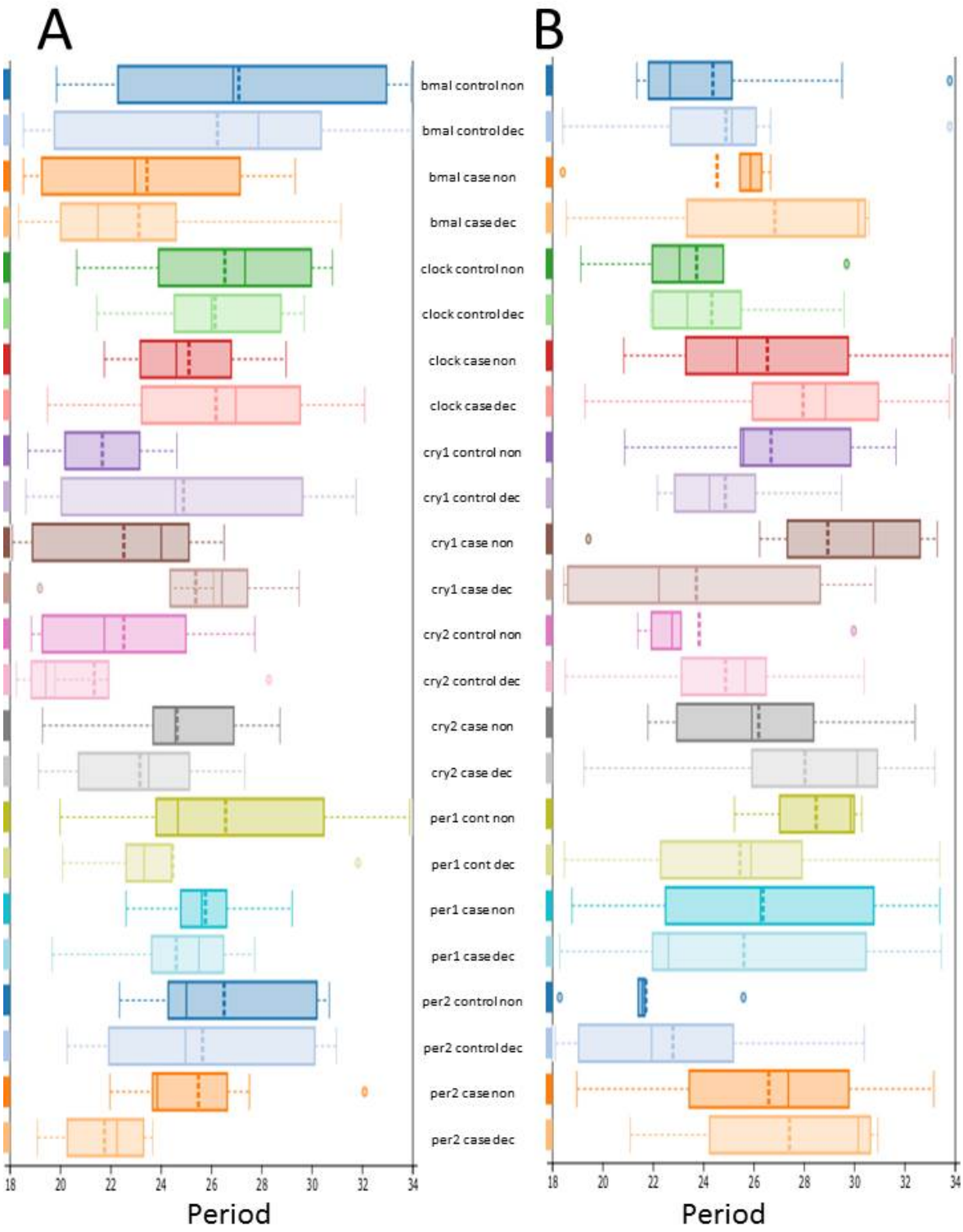
The expression of *bmal1/clock* and *per2* is out of phase





Appendix C

Mean ESC clock gene Period (A) MESA (B) MFF (chapter four)

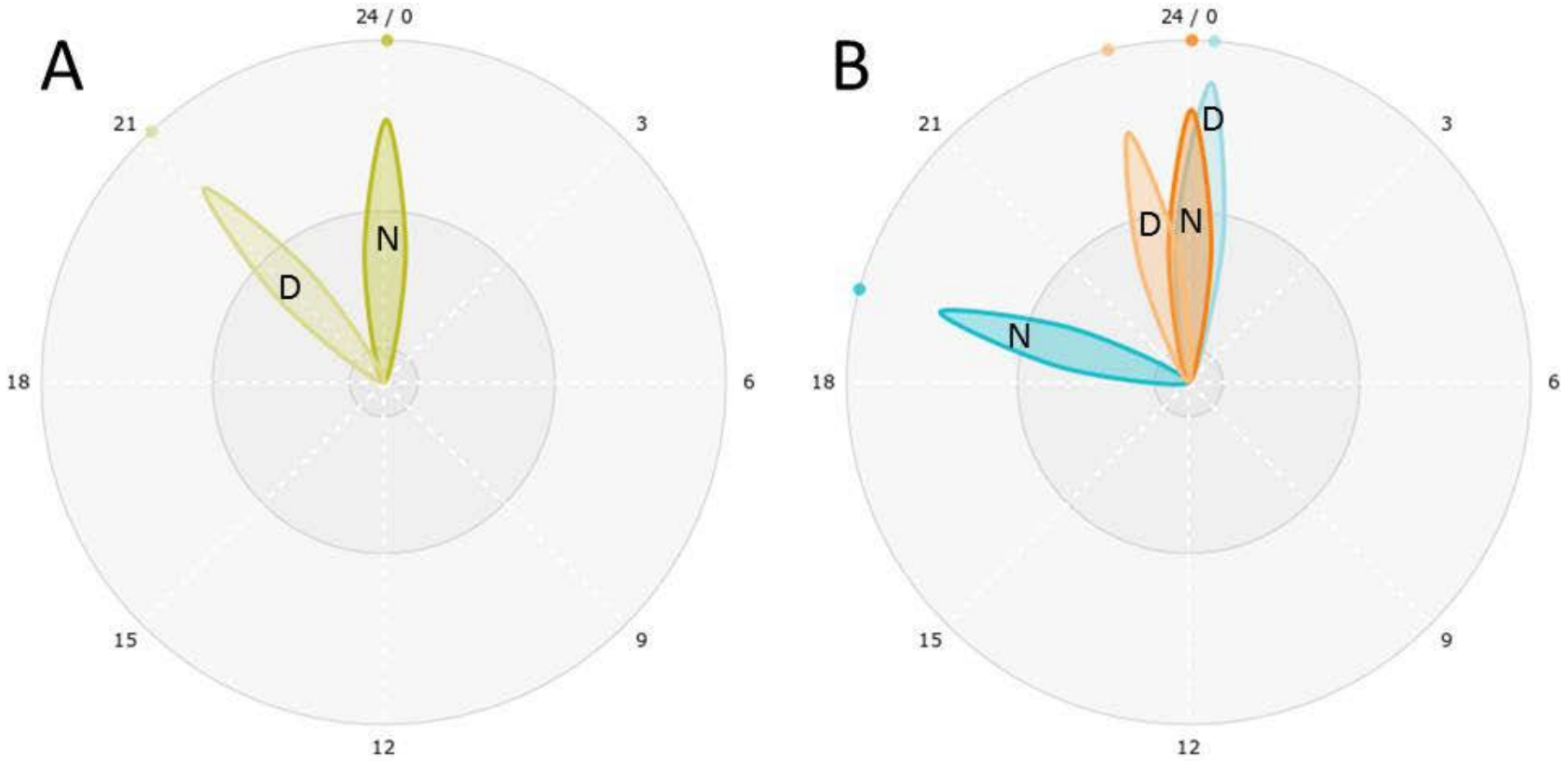


Core clock genes phase shifted by decidualisation

Polar plots demonstrating the circadian phase over a 24 hour periods. The size of the 'petal' represents variance (smaller petal, larger variance) (A) Controls (B) RIF

N: Non decidualised (darker colour), D: (D) decidualised (lighter colour)

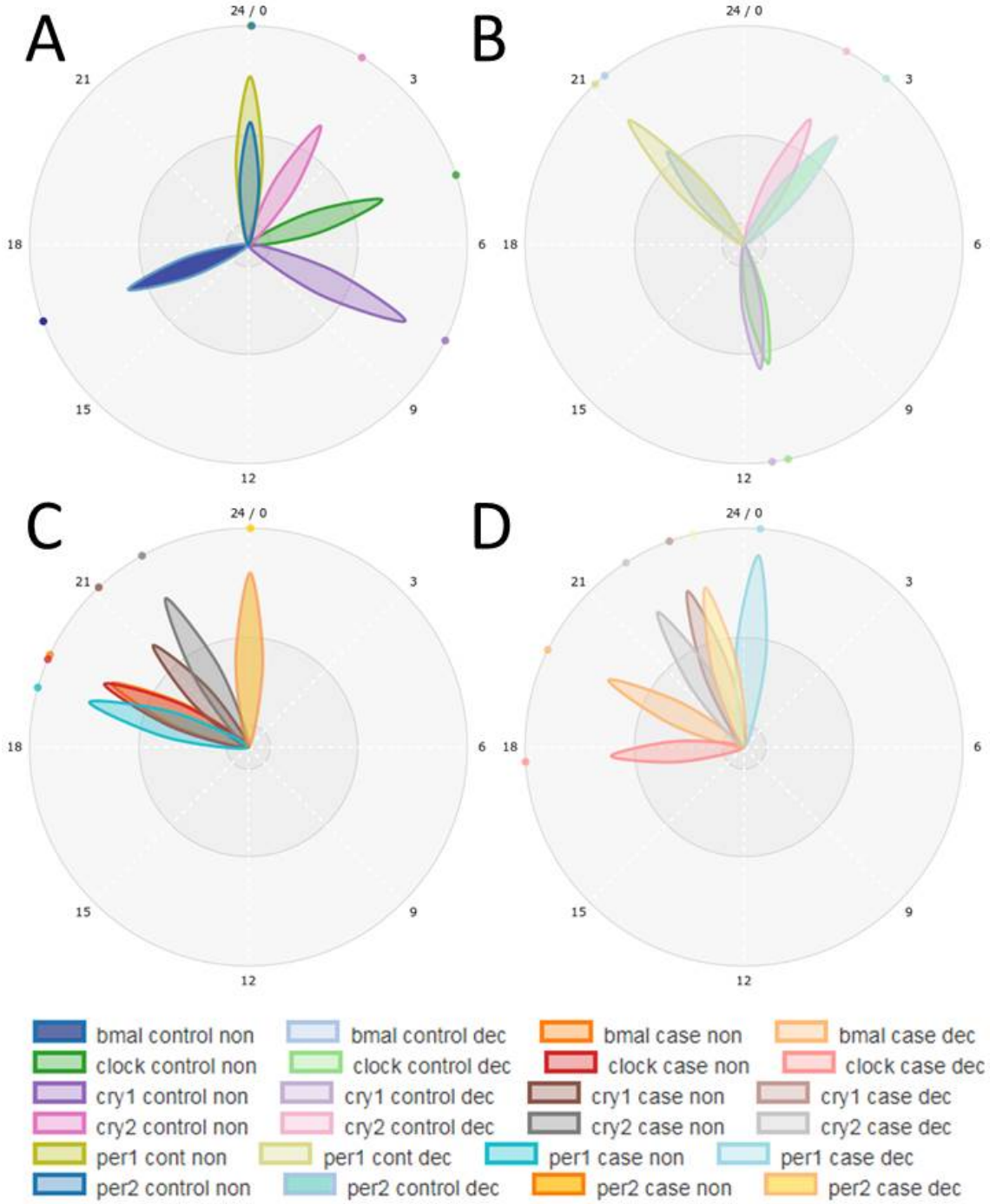
Green: *per1* controls; Blue *per1* RIF ;Orange *per2* RIF



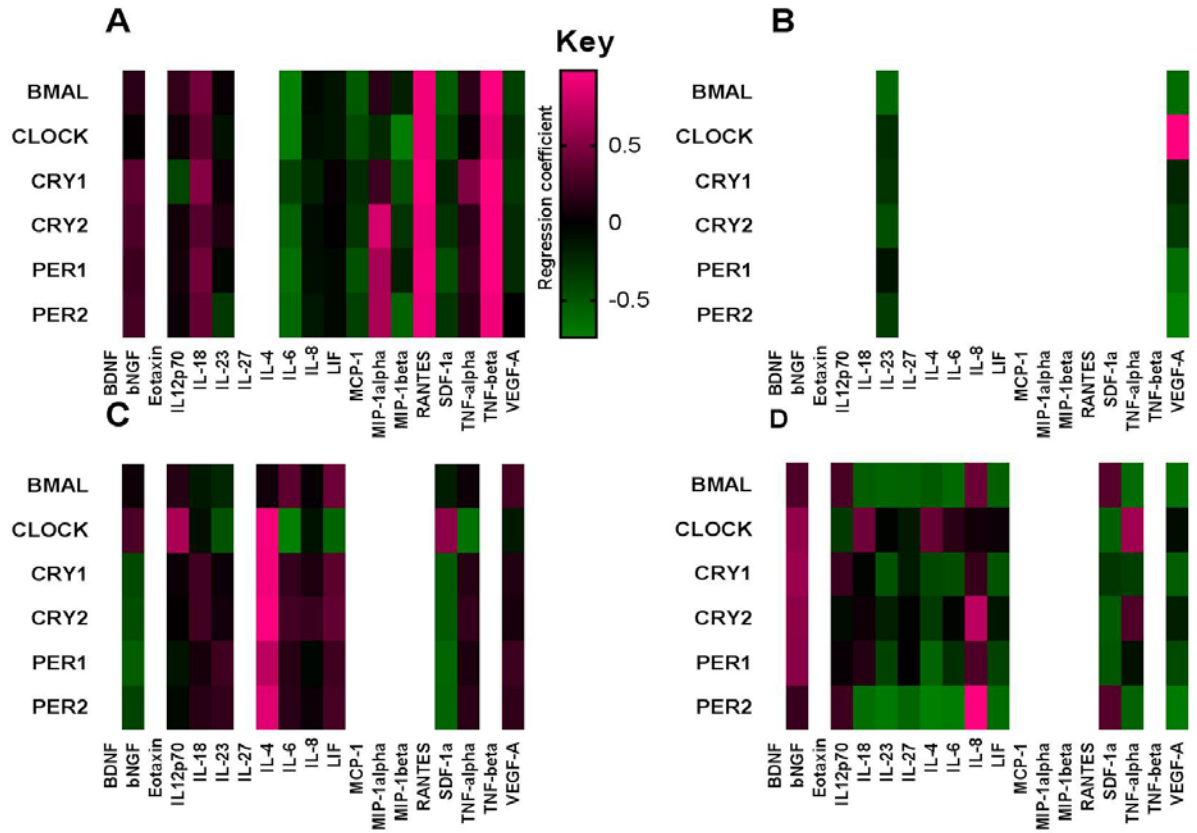
Appendix C

The phases of all of the core clock genes

Polar plots demonstrating phase. The size of the 'petal' represents variance (smaller petal, larger variance) (A) non decidualised controls (B) decidualised controls (C) non decidualised cases (D) decidualised cases

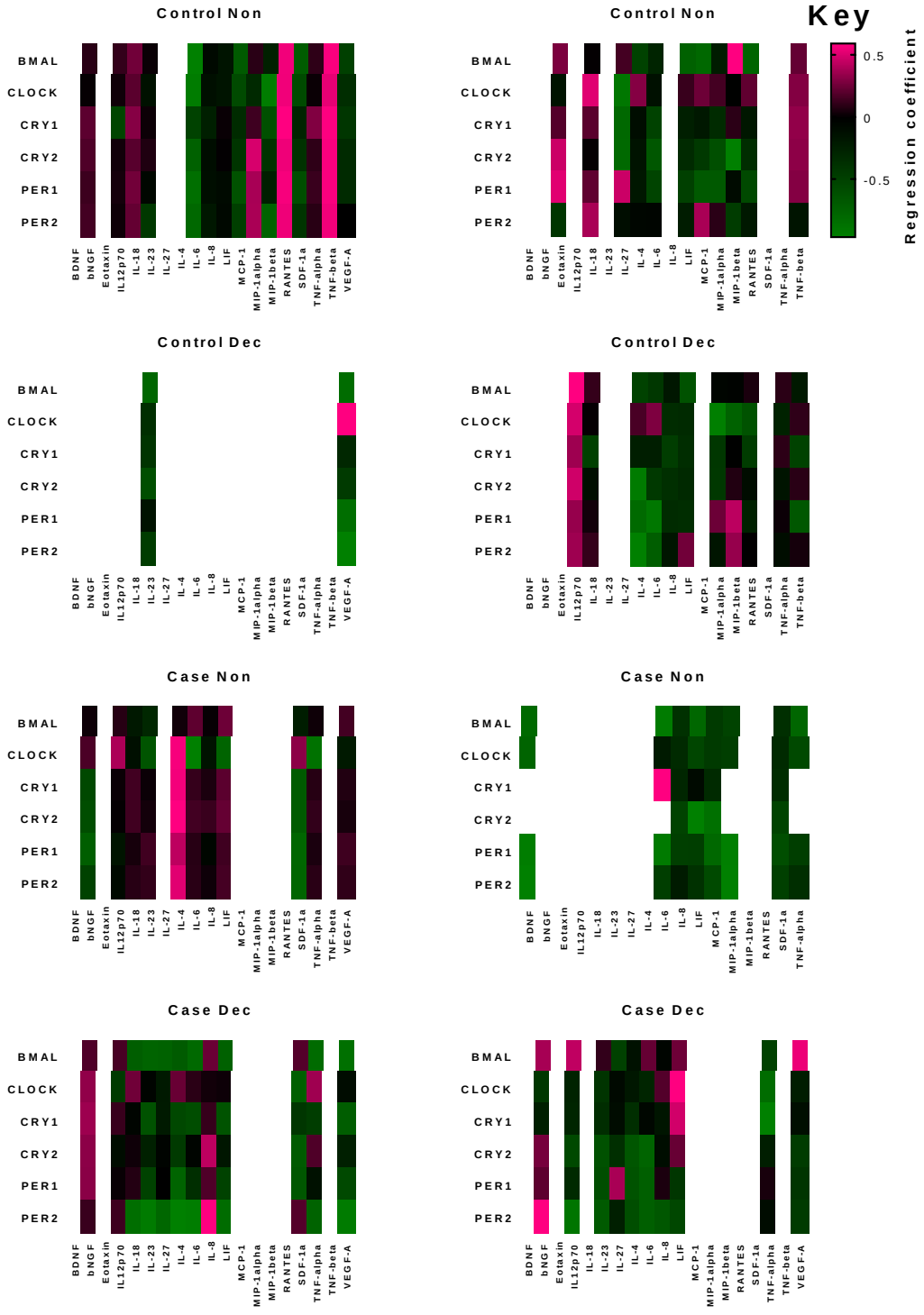


Correlations of clock genes and immunomodulators of the other case (not shown in chapter four) and control



Appendix C

Correlations of clock genes and immunomodulators all the individual patients



Appendix D Reagents

Tissue culture reagents and media

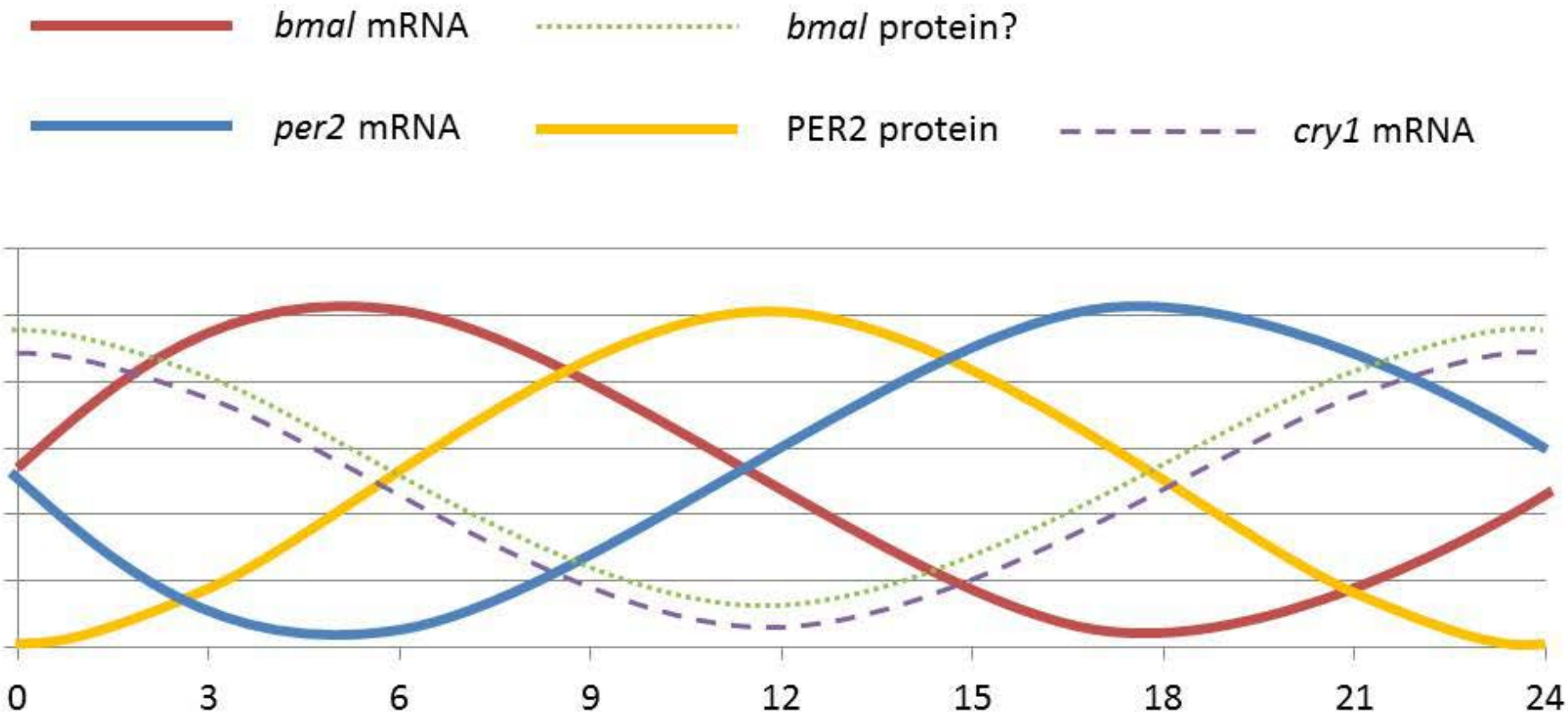
Medium	**Manufacturer**	**Storage temperature (˚C)**
Tissue culture		
Foetal Calf Serum	P.A.A.	-20
Dimethyl sulfoxide	AnalaR, BHD	Room temperature
Phosphate buffered saline	Prepared in house	Room temperature
Trypsin-EDTA (1x)	Sigma	-20
Tryptan Blue (0.4%)	Sigma	Room temperature
Tuerk Solution	Life Technologies	Room temperature
DMEM/F1 (no phenol red)	Gibco	4-6
DMEM/F (no phenol red)	Gibco	4-6
8-Br-cAMP	Sigma	-20
Medroxyprogesterone acetate	Sigma	-20
Penicillin-streptokinase	Gibco	-20
Amphotericin-B	Sigma	-20
L-Glutamin 100x	Gibco	-20
FBS	Gibco	-20
Charcoal- stripped FCS	Gibco	-20
Insulin bovine pancreas	Sigma	-20
Collagenase, crude: type 1A	Sigma	-20
Immunocytochemisty		
Diff-Quik (fix, I (red) and II (bleu))	Medion Diagnostics	+4
Falcon 8-well chambers slides (12/pack)	BD	Room temperature
Vectastain-elite abc peroxidase kit	Brunschwig	+4
Peroxidase substrate kit DAB	Brunschwig	+4
Peroxidase substrate kit SG	Brunschwig	+4
Cytokeratin-7 (CK-7); mouse mAb	DAKO	+4
Vimentin	DAKO	+4

Appendix E Additional relevant information

Clock genes in mammals and *Drosophila*; a brief overview of mutational effects

Mammalian gene	Effects	Mutation (human) and circadian function	Drosophila homologue
Period *(per)*	The transcription factor heterodimerises with *cry* and binds to the E-box Binds with CRY and among PER proteins Activator of *bmal1*	Mutation of *per 2* associated with Familial Advanced Sleep Phase Syndrome (FASPS) and morning preference((403)	*period (per)*
Timeless *(tim)*	Not known	Homozygous null mutant is lethal?(404)	*timeless (*closest = *timeout)*
Casein kinase 1 (*ck1*)	Phosphorylates PER and affects PER stability	Mutation of *per 2* associated with Familial Advanced Sleep Phase Syndrome (FASPS) (405)	*doubletime (dbt)*
Circadian locomotor output cycle kaput *(clock)*	The transcription factor heterodimerises with BMAL1 and binds to the E-box to promote *per* and *cry* transcription	Delayed Sleep Phase Syndrome (DSPS) and diurnal preference, although evidence conflicting (406-408) (409)	*dClock (dClk)*
Brain and muscle ARNT (Arylhydrocarbon Receptor Nuclear Translocator)-like protein *(bmal1)/*mediator of paramutation *(mop3)/*aryl hydrocarbon receptor nuclear translocator-like protein *(arntl)*	The transcription factor heterodimerises with CLOCK and binds to the E-box to promotes *per* and *cry* transcription	Some redundancy in the circadian function of Arntl with its paralog? (bmal2) (410)	*cycle (cyc)*
Cryptochrome *(cry)*	Physically associates with and stabilises PER Inhibits *per* and *cry*	Mutations alter rhythmicity in mice (404)	*cryptochrome (cry)*

The ZT timingsof relative core clock gene expression. Time 'inverted' for nocturnal mammals (333, 411-413).



Bibliography

1. Bretzl H, Alexander tGBCBCAIIM. Botanische Forschungen des Alexanderzuges ... Mit elf Abbildungen und vier Kartenskizzen, etc: Leipzig; 1903.
2. Riccio AP, Goldman BD. Circadian rhythms of locomotor activity in naked mole-rats (Heterocephalus glaber). Physiology & behavior. 2000;71(1-2):1-13.
3. Heath-Heckman EA, Peyer SM, Whistler CA, Apicella MA, Goldman WE, McFall-Ngai MJ. Bacterial bioluminescence regulates expression of a host cryptochrome gene in the squid-Vibrio symbiosis. mBio. 2013;4(2).
4. Underwood H, Goldman BD. Vertebrate circadian and photoperiodic systems: role of the pineal gland and melatonin. Journal of biological rhythms. 1987;2(4):279-315.
5. Hogg C, Neveu M, Stokkan KA, Folkow L, Cottrill P, Douglas R, et al. Arctic reindeer extend their visual range into the ultraviolet. The Journal of experimental biology. 2011;214(Pt 12):2014-9.
6. Stokkan KA, Folkow L, Dukes J, Neveu M, Hogg C, Siefken S, et al. Shifting mirrors: adaptive changes in retinal reflections to winter darkness in Arctic reindeer. Proceedings Biological sciences / The Royal Society. 2013;280(1773):20132451.
7. Pittendrigh CS. Temporal organization: reflections of a Darwinian clock-watcher. Annual review of physiology. 1993;55:16-54.
8. van Oort BE, Tyler NJ, Gerkema MP, Folkow L, Stokkan KA. Where clocks are redundant: weak circadian mechanisms in reindeer living under polar photic conditions. Die Naturwissenschaften. 2007;94(3):183-94.
9. Aschoff J. Exogenous and endogenous components in circadian rhythms. Cold Spring Harbor symposia on quantitative biology. 1960;25:11-28.
10. Smals AG, Kloppenborg PW, Benraad TJ. Circannual cycle in plasma testosterone levels in man. The Journal of clinical endocrinology and metabolism. 1976;42(5):979-82.
11. Bellastella A, Criscuolo T, Mango A, Perrone L, Sinisi AA, Faggiano M. Circannual rhythms of plasma luteinizing hormone, follicle-stimulating hormone, testosterone, prolactin and cortisol in prepuberty. Clinical endocrinology. 1983;19(4):453-9.
12. Roenneberg T, Aschoff J. Annual rhythm of human reproduction: I. Biology, sociology, or both? Journal of biological rhythms. 1990;5(3):195-216.
13. Harlow SD, Ephross SA. Epidemiology of menstruation and its relevance to women's health. Epidemiologic reviews. 1995;17(2):265-86.
14. Swaab DF, Van Someren EJ, Zhou JN, Hofman MA. Biological rhythms in the human life cycle and their relationship to functional changes in the suprachiasmatic nucleus. Progress in brain research. 1996;111:349-68.
15. McFarland D. A dictionary of animal behaviour. Oxford: Oxford University Press; 2006.
16. Lincoln GA. Melatonin entrainment of circannual rhythms. Chronobiology international. 2006;23(1-2):301-6.
17. Gwinner E. Circannual rythms : endogenous annual clocks in the organization of seasonal processes. Berlin ; London: Springer; 1986.
18. Sothern RB, Smaaland R, Moore JG. Circannual rhythm in DNA synthesis (S-phase) in healthy human bone marrow and rectal mucosa. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1995;9(5):397-403.

19. Gangwisch JE. Seasonal variation in metabolism: evidence for the role of circannual rhythms in metabolism? Hypertension research : official journal of the Japanese Society of Hypertension. 2013;36(5):392-3.
20. Zahorska-Markiewicz B, Markiewicz A. Circannual rhythm of exercise metabolic rate in humans. European journal of applied physiology and occupational physiology. 1984;52(3):328-30.
21. Tjoa WS, Smolensky MH, Hsi BP, Steinberger E, Smith KD. Circannual rhythm in human sperm count revealed by serially independent sampling. Fertility and sterility. 1982;38(4):454-9.
22. Rojansky N, Brzezinski A, Schenker JG. Seasonality in human reproduction: an update. Hum Reprod. 1992;7(6):735-45.
23. Wirz-Justice A, Krauchi K, Graw P. An underlying circannual rhythm in seasonal affective disorder? Chronobiology international. 2001;18(2):309-13.
24. Disanto G, Handel AE, Para AE, Ramagopalan SV, Handunnetthi L. Season of birth and anorexia nervosa. The British journal of psychiatry : the journal of mental science. 2011;198(5):404-5.
25. Hine R. A dictionary of biology. 6th ed. ed. Oxford: Oxford University Press; 2008.
26. Kaiser TS, Neumann D, Heckel DG. Timing the tides: genetic control of diurnal and lunar emergence times is correlated in the marine midge Clunio marinus. BMC genetics. 2011;12:49.
27. Tessmar-Raible K, Raible F, Arboleda E. Another place, another timer: Marine species and the rhythms of life. BioEssays : news and reviews in molecular, cellular and developmental biology. 2011;33(3):165-72.
28. Foster RG, Roenneberg T. Human responses to the geophysical daily, annual and lunar cycles. Current biology : CB. 2008;18(17):R784-R94.
29. Cajochen C, Altanay-Ekici S, Munch M, Frey S, Knoblauch V, Wirz-Justice A. Evidence that the lunar cycle influences human sleep. Current biology : CB. 2013;23(15):1485-8.
30. Cutler WB, Schleidt WM, Friedmann E, Preti G, Stine R. Lunar influences on the reproductive cycle in women. Human biology. 1987;59(6):959-72.
31. Law SP. The regulation of menstrual cycle and its relationship to the moon. Acta obstetricia et gynecologica Scandinavica. 1986;65(1):45-8.
32. Pochobradsky J. Independence of human menstruation on lunar phases and days of the week. American journal of obstetrics and gynecology. 1974;118(8):1136-8.
33. Haus E, Lakatua DJ, Swoyer J, Sackett-Lundeen L. Chronobiology in hematology and immunology. The American journal of anatomy. 1983;168(4):467-517.
34. Levi F, Halberg F. Circaseptan (about-7-day) bioperiodicity--spontaneous and reactive--and the search for pacemakers. La Ricerca in clinica e in laboratorio. 1982;12(2):323-70.
35. Lee MS, Lee JS, Lee JY, Cornelissen G, Otsuka K, Halberg F. About 7-day (circaseptan) and circadian changes in cold pressor test (CPT). Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2003;57 Suppl 1:39s-44s.
36. Pittendrigh CS. On Temperature Independence in the Clock System Controlling Emergence Time in Drosophila. Proceedings of the National Academy of Sciences of the United States of America. 1954;40(10):1018-29.
37. McClung CR. Plant circadian rhythms. The Plant cell. 2006;18(4):792-803.
38. Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, et al. Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nature reviews Genetics. 2005;6(7):544-56.

39. Lowrey PL, Takahashi JS. Mammalian circadian biology: elucidating genome-wide levels of temporal organization. Annual review of genomics and human genetics. 2004;5:407-41.
40. Takahashi JS, Hong HK, Ko CH, McDearmon EL. The genetics of mammalian circadian order and disorder: implications for physiology and disease. Nature reviews Genetics. 2008;9(10):764-75.
41. Aschoff J, Fatranska M, Giedke H, Doerr P, Stamm D, Wisser H. Human circadian rhythms in continuous darkness: entrainment by social cues. Science. 1971;171(3967):213-5.
42. Pittendrigh CS. Circadian rhythms and the circadian organization of living systems. Cold Spring Harbor symposia on quantitative biology. 1960;25:159-84.
43. Hastings M, O'Neill JS, Maywood ES. Circadian clocks: regulators of endocrine and metabolic rhythms. The Journal of endocrinology. 2007;195(2):187-98.
44. Krauchi K. How is the circadian rhythm of core body temperature regulated? Clinical autonomic research : official journal of the Clinical Autonomic Research Society. 2002;12(3):147-9.
45. Wunderlich KRA. The course of temperature in disease: a guide to clinical thermometry. American Journal Medical Sciences 1869;57:425-47.
46. Kelly G. Body temperature variability (Part 1): a review of the history of body temperature and its variability due to site selection, biological rhythms, fitness, and aging. Alternative medicine review : a journal of clinical therapeutic. 2006;11(4):278-93.
47. Kelly GS. Body temperature variability (Part 2): masking influences of body temperature variability and a review of body temperature variability in disease. Alternative medicine review : a journal of clinical therapeutic. 2007;12(1):49-62.
48. Mastorakos G, Pavlatou M, Diamanti-Kandarakis E, Chrousos GP. Exercise and the stress system. Hormones (Athens). 2005;4(2):73-89.
49. Narasimamurthy R, Hatori M, Nayak SK, Liu F, Panda S, Verma IM. Circadian clock protein cryptochrome regulates the expression of proinflammatory cytokines. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(31):12662-7.
50. Suzuki S, Toyabe S, Moroda T, Tada T, Tsukahara A, Iiai T, et al. Circadian rhythm of leucocytes and lymphocytes subsets and its possible correlation with the function of the autonomic nervous system. Clinical and experimental immunology. 1997;110(3):500-8.
51. Aschoff J, Daan S, Groos GA. Vertebrate circadiar systems : structure and physiology. Berlin: Springer; 1982.
52. Baker FC, Waner JI, Vieira EF, Taylor SR, Driver HS, Mitchell D. Sleep and 24 hour body temperatures: a comparison in young men, naturally cycling women and women taking hormonal contraceptives. The Journal of physiology. 2001;530(Pt 3):565-74.
53. Cagnacci A, Arangino S, Tuveri F, Paoletti AM, Volpe A. Regulation of the 24h body temperature rhythm of women in luteal phase: role of gonadal steroids and prostaglandins. Chronobiology international. 2002;19(4):721-30.
54. Coyne MD, Kesick CM, Doherty TJ, Kolka MA, Stephenson LA. Circadian rhythm changes in core temperature over the menstrual cycle: method for noninvasive monitoring. American journal of physiology Regulatory, integrative and comparative physiology. 2000;279(4):R1316-20.
55. Simic N, Ravlic A. Changes in basal body temperature and simple reaction times during the menstrual cycle. Arhiv za higijenu rada i toksikologiju. 2013;64(1):99-106.

56. Hunter RHF. Physiology of the Graafian follicle and ovulation. Cambridge: Cambridge University Press; 2003.
57. Luck MR, Griffiths S, Gregson K, Watson E, Nutley M, Cooper A. Follicular fluid responds endothermically to aqueous dilution. Hum Reprod. 2001;16(12):2508-14.
58. Hunter RH, Einer-Jensen N, Greve T. Presence and significance of temperature gradients among different ovarian tissues. Microscopy research and technique. 2006;69(6):501-7.
59. Bahat A, Eisenbach M, Tur-Kaspa I. Periovulatory increase in temperature difference within the rabbit oviduct. Hum Reprod. 2005;20(8):2118-21.
60. Hunter WC, Miyaoka RS, Macdonald LR, Lewellen TK. Measured Temperature Dependence of Scintillation Camera Signals Read Out by Geiger-Muller Mode Avalanche Photodiodes. IEEE Nuclear Science Symposium conference record Nuclear Science Symposium. 2009;2009:2662-5.
61. Hunter RH. Temperature gradients in female reproductive tissues. Reproductive biomedicine online. 2012;24(4):377-80.
62. Grinsted J, Blendstrup K, Andreasen MP, Byskov AG. Temperature measurements of rabbit antral follicles. Journal of reproduction and fertility. 1980;60(1):149-55.
63. Grinsted J, Kjer JJ, Blendstrup K, Pedersen JF. Is low temperature of the follicular fluid prior to ovulation necessary for normal oocyte development? Fertility and sterility. 1985;43(1):34-9.
64. Hunter RH, Nichol R. A preovulatory temperature gradient between the isthmus and ampulla of pig oviducts during the phase of sperm storage. Journal of reproduction and fertility. 1986;77(2):599-606.
65. El-Sheikh Ali H, Kitahara G, Tamura Y, Kobayashi I, Hemmi K, Torisu S, et al. Presence of a temperature gradient among genital tract portions and the thermal changes within these portions over the estrous cycle in beef cows. The Journal of reproduction and development. 2013;59(1):59-65.
66. Hardeland R, Pandi-Perumal SR, Cardinali DP. Melatonin. The international journal of biochemistry & cell biology. 2006;38(3):313-6.
67. Stankov B, Reiter RJ. Melatonin receptors: current status, facts, and hypotheses. Life sciences. 1990;46(14):971-82.
68. Slominski RM, Reiter RJ, Schlabritz-Loutsevitch N, Ostrom RS, Slominski AT. Melatonin membrane receptors in peripheral tissues: distribution and functions. Molecular and cellular endocrinology. 2012;351(2):152-66.
69. Hardeland R, Madrid JA, Tan DX, Reiter RJ. Melatonin, the circadian multioscillator system and health: the need for detailed analyses of peripheral melatonin signaling. Journal of pineal research. 2012;52(2):139-66.
70. Reiter RJ, Tamura H, Tan DX, Xu XY. Melatonin and the circadian system: contributions to successful female reproduction. Fertility and sterility. 2014;102(2):321-8.
71. Acuna-Castroviejo D, Reiter RJ, Menendez-Pelaez A, Pablos MI, Burgos A. Characterization of high-affinity melatonin binding sites in purified cell nuclei of rat liver. Journal of pineal research. 1994;16(2):100-12.
72. Becker-Andre M, Wiesenberg I, Schaeren-Wiemers N, Andre E, Missbach M, Saurat JH, et al. Pineal gland hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. The Journal of biological chemistry. 1994;269(46):28531-4.
73. Wirz-Justice A. How to measure circadian rhythms in humans. Medicographia. 2007;29(1):84-90.
74. Nakamura Y, Tamura H, Kashida S, Takayama H, Yamagata Y, Karube A, et al. Changes of serum melatonin level and its relationship to feto-placental unit during pregnancy. Journal of pineal research. 2001;30(1):29-33.

75. Seko LM, Moroni RM, Leitao VM, Teixeira DM, Nastri CO, Martins WP. Melatonin supplementation during controlled ovarian stimulation for women undergoing assisted reproductive technology: systematic review and meta-analysis of randomized controlled trials. Fertility and sterility. 2014;101(1):154-61 e4.
76. Showell MG, Brown J, Clarke J, Hart RJ. Antioxidants for female subfertility. Cochrane Database Syst Rev. 2013(8):CD007807.
77. Wurtman RJ, Axelrod J, Chu EW. Melatonin, a pineal substance: effect on the rat ovary. Science. 1963;141(3577):277-8.
78. Reiter RJ. The melatonin rhythm: both a clock and a calendar. Experientia. 1993;49(8):654-64.
79. Itoh MT, Ishizuka B, Kuribayashi Y, Amemiya A, Sumi Y. Melatonin, its precursors, and synthesizing enzyme activities in the human ovary. Molecular human reproduction. 1999;5(5):402-8.
80. Itoh MT, Ishizuka B, Kudo Y, Fusama S, Amemiya A, Sumi Y. Detection of melatonin and serotonin N-acetyltransferase and hydroxyindole-O-methyltransferase activities in rat ovary. Molecular and cellular endocrinology. 1997;136(1):7-13.
81. Lanoix D, Beghdadi H, Lafond J, Vaillancourt C. Human placental trophoblasts synthesize melatonin and express its receptors. Journal of pineal research. 2008;45(1):50-60.
82. Kivela A, Kauppila A, Ylostalo P, Vakkuri O, Leppaluoto J. Seasonal, menstrual and circadian secretions of melatonin, gonadotropins and prolactin in women. Acta physiologica Scandinavica. 1988;132(3):321-7.
83. Eryilmaz OG, Devran A, Sarikaya E, Aksakal FN, Mollamahmutoglu L, Cicek N. Melatonin improves the oocyte and the embryo in IVF patients with sleep disturbances, but does not improve the sleeping problems. Journal of assisted reproduction and genetics. 2011;28(9):815-20.
84. Batioglu AS, Sahin U, Gurlek B, Ozturk N, Unsal E. The efficacy of melatonin administration on oocyte quality. Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology. 2012;28(2):91-3.
85. Rizzo P, Raffone E, Benedetto V. Effect of the treatment with myo-inositol plus folic acid plus melatonin in comparison with a treatment with myo-inositol plus folic acid on oocyte quality and pregnancy outcome in IVF cycles. A prospective, clinical trial. Eur Rev Med Pharmacol Sci. 2010;14(6):555-61.
86. Tamura H, Takasaki A, Taketani T, Tanabe M, Kizuka F, Lee L, et al. The role of melatonin as an antioxidant in the follicle. J Ovarian Res. 2012;5:5.
87. du Plessis SS, Hagenaar K, Lampiao F. The in vitro effects of melatonin on human sperm function and its scavenging activities on NO and ROS. Andrologia. 2010;42(2):112-6.
88. Ortiz A, Espino J, Bejarano I, Lozano GM, Monllor F, Garcia JF, et al. High endogenous melatonin concentrations enhance sperm quality and short-term in vitro exposure to melatonin improves aspects of sperm motility. Journal of pineal research. 2011;50(2):132-9.
89. Taketani T, Tamura H, Takasaki A, Lee L, Kizuka F, Tamura I, et al. Protective role of melatonin in progesterone production by human luteal cells. Journal of pineal research. 2011;51(2):207-13.
90. Takasaki A, Tamura H, Taniguchi K, Asada H, Taketani T, Matsuoka A, et al. Luteal blood flow and luteal function. J Ovarian Res. 2009;2:1.
91. Wood S, Quinn A, Troupe S, Kingsland C, Lewis-Jones I. Seasonal variation in assisted conception cycles and the influence of photoperiodism on outcome in in vitro fertilization cycles. Hum Fertil (Camb). 2006;9(4):223-9.

92. Srinivasan V, Spence WD, Pandi-Perumal SR, Zakharia R, Bhatnagar KP, Brzezinski A. Melatonin and human reproduction: shedding light on the darkness hormone. Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology. 2009;25(12):779-85.
93. Barron ML. Light exposure, melatonin secretion, and menstrual cycle parameters: an integrative review. Biol Res Nurs. 2007;9(1):49-69.
94. Littner M, Kushida CA, Anderson WM, Bailey D, Berry RB, Davila DG, et al. Practice parameters for the role of actigraphy in the study of sleep and circadian rhythms: an update for 2002. Sleep. 2003;26(3):337-41.
95. Pollak CP, Tryon WW, Nagaraja H, Dzwonczyk R. How accurately does wrist actigraphy identify the states of sleep and wakefulness? Sleep. 2001;24(8):957-65.
96. Gironda RJ, Lloyd J, Clark ME, Walker RL. Preliminary evaluation of reliability and criterion validity of Actiwatch-Score. Journal of rehabilitation research and development. 2007;44(2):223-30.
97. Zulley J, Wever R, Aschoff J. The dependence of onset and duration of sleep on th circadian rhythm of rectal temperature. Pflugers Archiv : European journal of physiology. 1981;391(4):314-8.
98. Adan A, Archer SN, Hidalgo MP, Di Milia L, Natale V, Randler C. Circadian typology: a comprehensive review. Chronobiology international. 2012;29(9):1153-75.
99. Rusak B, Zucker I. Neural regulation of circadian rhythms. Physiological reviews. 1979;59(3):449-526.
100. Richter CP. Biological clocks in medicine and psychiatry: Springfield Il. : C C Thomas, 1965 (1979).
101. Moore RY, Eichler VB. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain research. 1972;42(1):201-6.
102. Stephan FK, Zucker I. Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. Proceedings of the National Academy of Sciences of the United States of America. 1972;69(6):1583-6.
103. Moore RY, Lenn NJ. A retinohypothalamic projection in the rat. The Journal of comparative neurology. 1972;146(1):1-14.
104. Inouye ST, Kawamura H. Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus. Proceedings of the National Academy of Sciences of the United States of America. 1979;76(11):5962-6.
105. Feldman JF. Lengthening the period of a biological clock in Euglena by cycloheximide, an inhibitor of protein synthesis. Proceedings of the National Academy of Sciences of the United States of America. 1967;57(4):1080-7.
106. Karakashian MW, Hastings JW. The inhibition of a biological clock by actinomycin D. Proceedings of the National Academy of Sciences of the United States of America. 1962;48:2130-7.
107. Konopka RJ, Benzer S. Clock mutants of Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America. 1971;68(9):2112-6.
108. Hardin PE, Hall JC, Rosbash M. Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. Nature. 1990;343(6258):536-40.
109. Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, et al. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science. 1994;264(5159):719-25.

110. Toh KL, Jones CR, He Y, Eide EJ, Hinz WA, Virshup DM, et al. An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. Science. 2001;291(5506):1040-3.
111. Dunlap JC. Molecular bases for circadian clocks. Cell. 1999;96(2):271-90.
112. Gu YZ, Hogenesch JB, Bradfield CA. The PAS superfamily: sensors of environmental and developmental signals. Annual review of pharmacology and toxicology. 2000;40:519-61.
113. Abildskov JA, Rush S, Burgess MJ, Millar K. Graphic studies concerning the body surface distribution of T potentials. Am Heart J. 1972;83(2):283-5.
114. Lehman MN, Silver R, Gladstone WR, Kahn RM, Gibson M, Bittman EL. Circadian rhythmicity restored by neural transplant. Immunocytochemical characterization of the graft and its integration with the host brain. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1987;7(6):1626-38.
115. Weaver DR. The suprachiasmatic nucleus: a 25-year retrospective. Journal of biological rhythms. 1998;13(2):100-12.
116. Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, et al. Resetting central and peripheral circadian oscillators in transgenic rats. Science. 2000;288(5466):682-5.
117. Balsalobre A, Damiola F, Schibler U. A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell. 1998;93(6):929-37.
118. Welsh DK, Yoo SH, Liu AC, Takahashi JS, Kay SA. Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. Current biology : CB. 2004;14(24):2289-95.
119. Yagita K, Okamura H. Forskolin induces circadian gene expression of rPer1, rPer2 and dbp in mammalian rat-1 fibroblasts. FEBS letters. 2000;465(1):79-82.
120. Debruyne JP, Noton E, Lambert CM, Maywood ES, Weaver DR, Reppert SM. A clock shock: mouse CLOCK is not required for circadian oscillator function. Neuron. 2006;50(3):465-77.
121. Messersmith D LD, Vargas C, Keller E, Takahashi J. The Mammalian Molecular Clock Model https://www.hhmi.org/biointeractive/mammalian-molecular-clock-model: Howard Hughes Medical Institute; 2014 [
122. Franken P, Thomason R, Heller HC, O'Hara BF. A non-circadian role for clock-genes in sleep homeostasis: a strain comparison. BMC neuroscience. 2007;8:87.
123. McNamara P, Barton RA, Nunn CL. Evolution of sleep : phylogenetic and functional perspectives. Cambridge: Cambridge University Press; 2010.
124. Krueger JM, Obal F, Jr. Sleep function. Frontiers in bioscience : a journal and virtual library. 2003;8:d511-9.
125. Krueger JM, Fang J, Hansen MK, Zhang J, Obal F, Jr. Humoral Regulation of Sleep. News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society. 1998;13:189-94.
126. Kovanen L, Saarikoski ST, Aromaa A, Lonnqvist J, Partonen T. ARNTL (BMAL1) and NPAS2 gene variants contribute to fertility and seasonality. PloS one. 2010;5(4):e10007.
127. Tasaki H, Zhao L, Isayama K, Chen H, Nobuhiko Y, Yasufumi S, et al. Profiling of circadian genes expressed in the uterus endometrial stromal cells of pregnant rats as revealed by DNA microarray coupled with RNA interference. Frontiers in endocrinology. 2013;4:82.
128. Sellix MT, Menaker M. Circadian clocks in the ovary. Trends in endocrinology and metabolism: TEM. 2010;21(10):628-36.

129. Everett JW, Sawyer CH. A 24-hour periodicity in the "LH-release apparatus" of female rats, disclosed by barbiturate sedation. Endocrinology. 1950;47(3):198-218.
130. Yamamoto T, Nakahata Y, Soma H, Akashi M, Mamine T, Takumi T. Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. BMC molecular biology. 2004;5:18.
131. Lemos DR, Downs JL, Urbanski HF. Twenty-four-hour rhythmic gene expression in the rhesus macaque adrenal gland. Mol Endocrinol. 2006;20(5):1164-76.
132. Dolatshad H, Campbell EA, O'Hara L, Maywood ES, Hastings MH, Johnson MH. Developmental and reproductive performance in circadian mutant mice. Hum Reprod. 2006;21(1):68-79.
133. He PJ, Hirata M, Yamauchi N, Hattori MA. Up-regulation of Per1 expression by estradiol and progesterone in the rat uterus. The Journal of endocrinology. 2007;194(3):511-9.
134. Richards JS, Russell DL, Ochsner S, Espey LL. Ovulation: new dimensions and new regulators of the inflammatory-like response. Annual review of physiology. 2002;64:69-92.
135. Morris JK, Richards JS. An E-box region within the prostaglandin endoperoxide synthase-2 (PGS-2) promoter is required for transcription in rat ovarian granulosa cells. The Journal of biological chemistry. 1996;271(28):16633-43.
136. Simon C, Mercader A, Gimeno MJ, Pellicer A. The interleukin-1 system and human implantation. Am J Reprod Immunol. 1997;37(1):64-72.
137. Hambartsoumian E. Leukemia inhibitory factor (LIF) production by human decidua and its relationship with pregnancy hormones. Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology. 1998;12(1):17-22.
138. Fahrenkrug J, Georg B, Hannibal J, Hindersson P, Gras S. Diurnal rhythmicity of the clock genes Per1 and Per2 in the rat ovary. Endocrinology. 2006;147(8):3769-76.
139. Karman BN, Tischkau SA. Circadian clock gene expression in the ovary: Effects of luteinizing hormone. Biology of reproduction. 2006;75(4):624-32.
140. He PJ, Hirata M, Yamauchi N, Hashimoto S, Hattori MA. The disruption of circadian clockwork in differentiating cells from rat reproductive tissues as identified by in vitro real-time monitoring system. The Journal of endocrinology. 2007;193(3):413-20.
141. He PJ, Hirata M, Yamauchi N, Hashimoto S, Hattori MA. Gonadotropic regulation of circadian clockwork in rat granulosa cells. Molecular and cellular biochemistry. 2007;302(1-2):111-8.
142. Yoshikawa T, Sellix M, Pezuk P, Menaker M. Timing of the ovarian circadian clock is regulated by gonadotropins. Endocrinology. 2009;150(9):4338-47.
143. Johnson MH, Lim A, Fernando D, Day ML. Circadian clockwork genes are expressed in the reproductive tract and conceptus of the early pregnant mouse. Reproductive biomedicine online. 2002;4(2):140-5.
144. Gras S, Georg B, Jorgensen HL, Fahrenkrug J. Expression of the clock genes Per1 and Bmal1 during follicle development in the rat ovary. Effects of gonadotropin stimulation and hypophysectomy. Cell and tissue research. 2012;350(3):539-48.
145. Chu G, Misawa I, Chen H, Yamauchi N, Shigeyoshi Y, Hashimoto S, et al. Contribution of FSH and triiodothyronine to the development of circadian clocks during granulosa cell maturation. American journal of physiology Endocrinology and metabolism. 2012;302(6):E645-53.

146. Touzet S, Rabilloud M, Boehringer H, Barranco E, Ecochard R. Relationship between sleep and secretion of gonadotropin and ovarian hormones in women with normal cycles. Fertility and sterility. 2002;77(4):738-44.
147. Sellix MT, Menaker M. Circadian clocks in mammalian reproductive physiology: effects of the "other" biological clock on fertility. Discovery medicine. 2011;11(59):273-81.
148. Ratajczak CK, Herzog ED, Muglia LJ. Clock gene expression in gravid uterus and extra-embryonic tissues during late gestation in the mouse. Reproduction, fertility, and development. 2010;22(5):743-50.
149. Ratajczak CK, Asada M, Allen GC, McMahon DG, Muglia LM, Smith D, et al. Generation of myometrium-specific Bmal1 knockout mice for parturition analysis. Reproduction, fertility, and development. 2012;24(5):759-67.
150. Ratajczak CK, Boehle KL, Muglia LJ. Impaired steroidogenesis and implantation failure in Bmal1-/- mice. Endocrinology. 2009;150(4):1879-85.
151. Horard B, Rayet B, Triqueneaux G, Laudet V, Delaunay F, Vanacker JM. Expression of the orphan nuclear receptor ERRalpha is under circadian regulation in estrogen-responsive tissues. Journal of molecular endocrinology. 2004;33(1):87-97.
152. Nakamura TJ, Moriya T, Inoue S, Shimazoe T, Watanabe S, Ebihara S, et al. Estrogen differentially regulates expression of Per1 and Per2 genes between central and peripheral clocks and between reproductive and nonreproductive tissues in female rats. Journal of neuroscience research. 2005;82(5):622-30.
153. Yeh KT, Yang MY, Liu TC, Chen JC, Chan WL, Lin SF, et al. Abnormal expression of period 1 (PER1) in endometrial carcinoma. J Pathol. 2005;206(1):111-20.
154. Shih HC, Choo KB, Chang TJ, Yang MY, Shih MC, Yeh KT, et al. Disturbance of circadian gene expression in endometrial cancer: detection by real-time quantitative RT-PCR. Oncol Rep. 2005;14(6):1533-8.
155. Hirata M, He PJ, Shibuya N, Uchikawa M, Yamauchi N, Hashimoto S, et al. Progesterone, but not estradiol, synchronizes circadian oscillator in the uterus endometrial stromal cells. Molecular and cellular biochemistry. 2009;324(1-2):31-8.
156. Akiyama S, Ohta H, Watanabe S, Moriya T, Hariu A, Nakahata N, et al. The uterus sustains stable biological clock during pregnancy. The Tohoku journal of experimental medicine. 2010;221(4):287-98.
157. Uchikawa M, Kawamura M, Yamauchi N, Hattori MA. Down-regulation of circadian clock gene period 2 in uterine endometrial stromal cells of pregnant rats during decidualization. Chronobiology international. 2011;28(1):1-9.
158. Isayama K, Chen H, Yamauchi N, Hattori MA. REV-ERBalpha inhibits the PTGS2 expression in bovine uterus endometrium stromal and epithelial cells exposed to ovarian steroids. J Reprod Dev. 2014;60(5):362-70.
159. Muter J, Lucas ES, Chan YW, Brighton PJ, Moore JD, Lacey L, et al. The clock protein period 2 synchronizes mitotic expansion and decidual transformation of human endometrial stromal cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2015;29(4):1603-14.
160. Tasaki H, Zhao L, Isayama K, Chen H, Yamauchi N, Shigeyoshi Y, et al. Inhibitory role of REV-ERBalpha in the expression of bone morphogenetic protein gene family in rat uterus endometrium stromal cells. Am J Physiol Cell Physiol. 2015;308(7):C528-38.
161. Zhao L, Isayama K, Chen H, Yamauchi N, Shigeyoshi Y, Hashimoto S, et al. The nuclear receptor REV-ERBalpha represses the transcription of growth/differentiation factor 10 and 15 genes in rat endometrium stromal cells. Physiol Rep. 2016;4(2).

162. Macklon NS, Brosens JJ. The human endometrium as a sensor of embryo quality. Biology of reproduction. 2014;91(4):98.
163. Gnainsky Y, Granot I, Aldo P, Barash A, Or Y, Mor G, et al. Biopsy-induced inflammatory conditions improve endometrial receptivity: the mechanism of action. Reproduction. 2015;149(1):75-85.
164. Lass A, Weiser W, Munafo A, Loumaye E. Leukemia inhibitory factor in human reproduction. Fertility and sterility. 2001;76(6):1091-6.
165. Capp E, Milner CM, Williams J, Hauck L, Jauckus J, Strowitzki T, et al. Modulation of tumor necrosis factor-stimulated gene-6 (TSG-6) expression in human endometrium. Archives of gynecology and obstetrics. 2014;289(4):893-901.
166. Sundar IK, Rashid K, Sellix MT, Rahman I. The nuclear receptor and clock gene REV-ERBalpha regulates cigarette smoke-induced lung inflammation. Biochemical and biophysical research communications. 2017;493(4):1390-5.
167. Yoshida K, Hashimoto T, Sakai Y, Hashiramoto A. Involvement of the circadian rhythm and inflammatory cytokines in the pathogenesis of rheumatoid arthritis. J Immunol Res. 2014;2014:282495.
168. Ando N, Nakamura Y, Aoki R, Ishimaru K, Ogawa H, Okumura K, et al. Circadian Gene Clock Regulates Psoriasis-Like Skin Inflammation in Mice. The Journal of investigative dermatology. 2015;135(12):3001-8.
169. Sato S, Sakurai T, Ogasawara J, Takahashi M, Izawa T, Imaizumi K, et al. A circadian clock gene, Rev-erbalpha, modulates the inflammatory function of macrophages through the negative regulation of Ccl2 expression. J Immunol. 2014;192(1):407-17.
170. Siklosi GS, Banhidy FG, Acs N. Fundamental role of folliculo-luteal function in recurrent miscarriage. Archives of gynecology and obstetrics. 2012;286(5):1299-305.
171. Sonntag B, Ludwig M. An integrated view on the luteal phase: diagnosis and treatment in subfertility. Clinical endocrinology. 2012;77(4):500-7.
172. Burney RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. Fertility and sterility. 2012;98(3):511-9.
173. Banu SK, Lee J, Speights VO, Jr., Starzinski-Powitz A, Arosh JA. Cyclooxygenase-2 regulates survival, migration, and invasion of human endometriotic cells through multiple mechanisms. Endocrinology. 2008;149(3):1180-9.
174. Weimar CH, Post Uiterweer ED, Teklenburg G, Heijnen CJ, Macklon NS. In-vitro model systems for the study of human embryo-endometrium interactions. Reproductive biomedicine online. 2013;27(5):461-76.
175. Sugimoto Y, Narumiya S. Prostaglandin E receptors. The Journal of biological chemistry. 2007;282(16):11613-7.
176. Das M, Holzer HE. Recurrent implantation failure: gamete and embryo factors. Fertility and sterility. 2012;97(5):1021-7.
177. Teklenburg G, Salker M, Molokhia M, Lavery S, Trew G, Aojanepong T, et al. Natural selection of human embryos: decidualizing endometrial stromal cells serve as sensors of embryo quality upon implantation. PloS one. 2010;5(4):e10258.
178. Reppert SM, Schwartz WJ. Maternal endocrine extirpations do not abolish maternal coordination of the fetal circadian clock. Endocrinology. 1986;119(4):1763-7.
179. Jud C, Albrecht U. Circadian rhythms in murine pups develop in absence of a functional maternal circadian clock. Journal of biological rhythms. 2006;21(2):149-54.
180. Weimar CH, Kavelaars A, Brosens JJ, Gellersen B, de Vreeden-Elbertse JM, Heijnen CJ, et al. Endometrial stromal cells of women with recurrent

miscarriage fail to discriminate between high- and low-quality human embryos. PloS one. 2012;7(7):e41424.
181. Larsen EC, Christiansen OB, Kolte AM, Macklon N. New insights into mechanisms behind miscarriage. BMC Med. 2013;11:154.
182. Everson CA, Toth LA. Systemic bacterial invasion induced by sleep deprivation. American journal of physiology Regulatory, integrative and comparative physiology. 2000;278(4):R905-16.
183. Horne J. Working throughout the night: beyond 'sleepiness'--impairments to critical decision making. Neuroscience and biobehavioral reviews. 2012;36(10):2226-31.
184. Tempesta D, Couyoumdjian A, Curcio G, Moroni F, Marzano C, De Gennaro L, et al. Lack of sleep affects the evaluation of emotional stimuli. Brain research bulletin. 2010;82(1-2):104-8.
185. Garaulet M, Lee YC, Shen J, Parnell LD, Arnett DK, Tsai MY, et al. Genetic variants in human CLOCK associate with total energy intake and cytokine sleep factors in overweight subjects (GOLDN population). European journal of human genetics : EJHG. 2010;18(3):364-9.
186. Tan DX, Manchester LC, Fuentes-Broto L, Paredes SD, Reiter RJ. Significance and application of melatonin in the regulation of brown adipose tissue metabolism: relation to human obesity. Obesity reviews : an official journal of the International Association for the Study of Obesity. 2011;12(3):167-88.
187. Leproult R, Van Cauter E. Role of sleep and sleep loss in hormonal release and metabolism. Endocrine development. 2010;17:11-21.
188. Davis S, Mirick DK. Circadian disruption, shift work and the risk of cancer: a summary of the evidence and studies in Seattle. Cancer causes & control : CCC. 2006;17(4):539-45.
189. Wulff K, Gatti S, Wettstein JG, Foster RG. Sleep and circadian rhythm disruption in psychiatric and neurodegenerative disease. Nature reviews Neuroscience. 2010;11(8):589-99.
190. Kennaway DJ, Boden MJ, Varcoe TJ. Circadian rhythms and fertility. Molecular and cellular endocrinology. 2012;349(1):56-61.
191. Palma JA, Urrestarazu E, Iriarte J. Sleep loss as risk factor for neurologic disorders: a review. Sleep medicine. 2013;14(3):229-36.
192. Puttonen S, Harma M, Hublin C. Shift work and cardiovascular disease - pathways from circadian stress to morbidity. Scandinavian journal of work, environment & health. 2010;36(2):96-108.
193. Menegaux F, Truong T, Anger A, Cordina-Duverger E, Lamkarkach F, Arveux P, et al. Night work and breast cancer: a population-based case-control study in France (the CECILE study). Int J Cancer. 2013;132(4):924-31.
194. Samaraweera Y, Abeysena C. Maternal sleep deprivation, sedentary lifestyle and cooking smoke: Risk factors for miscarriage: A case control study. The Australian & New Zealand journal of obstetrics & gynaecology. 2010;50(4):352-7.
195. Reinberg A, Andlauer P, De Prins J, Malbecq W, Vieux N, Bourdeleau P. Desynchronization of the oral temperature circadian rhythm and intolerance to shift work. Nature. 1984;308(5956):272-4.
196. Richards J, Gumz ML. Advances in understanding the peripheral circadian clocks. FASEB J. 2012;26(9):3602-13.
197. Mitchell PJ, Hoese EK, Liu L, Fogg LF, Eastman CI. Conflicting bright light exposure during night shifts impedes circadian adaptation. J Biol Rhythms. 1997;12(1):5-15.
198. Bracci M, Copertaro A, Manzella N, Staffolani S, Strafella E, Nocchi L, et al. Influence of night-shift and napping at work on urinary melatonin, 17-beta-

estradiol and clock gene expression in pre-menopausal nurses. J Biol Regul Homeost Agents. 2013;27(1):267-74.
199. The Office of National Statistics. Changes in shift work patterns over the last ten years (1999 to 2009). London: Health and Safety Executive; 2011.
200. Bureau of Labor Statistics. Labor Force Statistics from the Current Population Survey, http://www.bls.gov/cps/cpsaat11.pdf. 2014.
201. Summa KC, Vitaterna MH, Turek FW. Environmental perturbation of the circadian clock disrupts pregnancy in the mouse. PLoS One. 2012;7(5):e37668.
202. Boden MJ, Varcoe TJ, Kennaway DJ. Circadian regulation of reproduction: From gamete to offspring. Prog Biophys Mol Biol. 2013;113(3):387-97.
203. Knutsson A. Health disorders of shift workers. Occup Med (Lond). 2003;53(2):103-8.
204. NHS Plus. Physical and shift work in pregnancy: Occupational aspects of management : a national guideline. Faculty of Occupational Medicine, editor. London: Royal College of Physicians of London; 2009.
205. Stroup DF, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, et al. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. JAMA. 2000;283(15):2008-12.
206. The Royal College of Nursing. A shift in the right direction: RCN guidance on the occupational health and safety of shift work in the nursing workforce. London, UK: The Royal College of Nursing; 2012.
207. Belsey EM, Pinol AP. Menstrual bleeding patterns in untreated women. Task Force on Long-Acting Systemic Agents for Fertility Regulation. Contraception. 1997;55(2):57-65.
208. National Institute for Health and Clinical Excellence. Fertility: Assessment and treatment for people with fertility problems (update). Clinical guideline 156. guidance.nice.org.uk/cg1562013.
209. Farquharson RG, Jauniaux E, Exalto N. Updated and revised nomenclature for description of early pregnancy events. Hum Reprod. 2005;20(11):3008-11.
210. The Cochrane Collaboration. Cochrane Handbook for Systematic Reviews of Interventions. Version 5.1.0 ed. Higgins J, Green, S., editor. http://handbook.cochrane.org2011.
211. Wells G, Shea,B O'Connell, D Peterson, J Welch,V Losos, M Tugwell, P The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses.
212. Mantel N, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. Journal of the National Cancer Institute. 1959;22(4):719-48.
213. DerSimonian R, Laird N. Meta-analysis in clinical trials. Control Clin Trials. 1986;7(3):177-88.
214. Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. Bmj. 2003;327(7414):557-60.
215. Chung FF, Yao CC, Wan GH. The associations between menstrual function and life style/working conditions among nurses in Taiwan. J Occup Health. 2005;47(2):149-56.
216. Hatch MC, Figa-Talamanca I, Salerno S. Work stress and menstrual patterns among American and Italian nurses. Scandinavian journal of work, environment & health. 1999;25(2):144-50.
217. Su SB, Lu CW, Kao YY, Guo HR. Effects of 12-hour rotating shifts on menstrual cycles of photoelectronic workers in Taiwan. Chronobiology international. 2008;25(2):237-48.

218. Bisanti L, Olsen J, Basso O, Thonneau P, Karmaus W. Shift work and subfecundity: a European multicenter study. European Study Group on Infertility and Subfecundity. J Occup Environ Med. 1996;38(4):352-8.
219. Spinelli A, Figa-Talamanca I, Osborn J. Time to pregnancy and occupation in a group of Italian women. Int J Epidemiol. 1997;26(3):601-9.
220. Tuntiseranee P, Olsen J, Geater A, Kor-anantakul O. Are long working hours and shiftwork risk factors for subfecundity? A study among couples from southern Thailand. Occup Environ Med. 1998;55(2):99-105.
221. Zhu JL, Hjollund NH, Boggild H, Olsen J. Shift work and subfecundity: a causal link or an artefact? Occup Environ Med. 2003;60(9):E12.
222. Axelsson G, Rylander R, Molin I. Outcome of pregnancy in relation to irregular and inconvenient work schedules. Br J Ind Med. 1989;46(6):393-8.
223. Axelsson G, Ahlborg G, Jr., Bodin L. Shift work, nitrous oxide exposure, and spontaneous abortion among Swedish midwives. Occup Environ Med. 1996;53(6):374-8.
224. Lawson CC, Rocheleau CM, Whelan EA, Lividoti Hibert EN, Grajewski B, Spiegelman D, et al. Occupational exposures among nurses and risk of spontaneous abortion. American journal of obstetrics and gynecology. 2012;206(4):327 e1-8.
225. Whelan EA, Lawson CC, Grajewski B, Hibert EN, Spiegelman D, Rich-Edwards JW. Work schedule during pregnancy and spontaneous abortion. Epidemiology. 2007;18(3):350-5.
226. Eskenazi B, Fenster L, Wight S, English P, Windham GC, Swan SH. Physical exertion as a risk factor for spontaneous abortion. Epidemiology. 1994;5(1):6-13.
227. Hemminki K, Kyyronen P, Lindbohm ML. Spontaneous abortions and malformations in the offspring of nurses exposed to anaesthetic gases, cytostatic drugs, and other potential hazards in hospitals, based on registered information of outcome. J Epidemiol Community Health. 1985;39(2):141-7.
228. Lawson CC, Whelan EA, Lividoti Hibert EN, Spiegelman D, Schernhammer ES, Rich-Edwards JW. Rotating shift work and menstrual cycle characteristics. Epidemiology. 2011;22(3):305-12.
229. Axelsson G, Lutz C, Rylander R. Exposure to solvents and outcome of pregnancy in university laboratory employees. British journal of industrial medicine. 1984;41(3):305-12.
230. Fenster L, Hubbard AE, Windham GC, Waller KO, Swan SH. A prospective study of work-related physical exertion and spontaneous abortion. Epidemiology. 1997;8(1):66-74.
231. Attarchi MS, Ashouri M, Labbafinejad Y, Mohammadi S. Assessment of time to pregnancy and spontaneous abortion status following occupational exposure to organic solvents mixture. Int Arch Occup Environ Health. 2012;85(3):295-303.
232. Chen PC, Hsieh GY, Wang JD, Cheng TJ. Prolonged time to pregnancy in female workers exposed to ethylene glycol ethers in semiconductor manufacturing. Epidemiology. 2002;13(2):191-6.
233. Bonde JP, Jorgensen KT, Bonzini M, Palmer KT. Miscarriage and occupational activity: a systematic review and meta-analysis regarding shift work, working hours, lifting, standing, and physical workload. Scandinavian journal of work, environment & health. 2013;39(4):325-34.
234. Grundy A, Richardson H, Burstyn I, Lohrisch C, Sengupta SK, Lai AS, et al. Increased risk of breast cancer associated with long-term shift work in Canada. Occup Environ Med. 2013.

235. McMichael AJ. Standardized mortality ratios and the "healthy worker effect": Scratching beneath the surface. Journal of occupational medicine : official publication of the Industrial Medical Association. 1976;18(3):165-8.
236. Shapley M, Jordan K, Croft PR. An epidemiological survey of symptoms of menstrual loss in the community. The British journal of general practice : the journal of the Royal College of General Practitioners. 2004;54(502):359-63.
237. Small CM, Manatunga AK, Klein M, Feigelson HS, Dominguez CE, McChesney R, et al. Menstrual cycle characteristics: associations with fertility and spontaneous abortion. Epidemiology (Cambridge, Mass). 2006;17(1):52-60.
238. Wang JX, Davies MJ, Norman RJ. Obesity increases the risk of spontaneous abortion during infertility treatment. Obes Res. 2002;10(6):551-4.
239. Cnattingius S, Signorello LB, Anneren G, Clausson B, Ekbom A, Ljunger E, et al. Caffeine intake and the risk of first-trimester spontaneous abortion. The New England journal of medicine. 2000;343(25):1839-45.
240. Ballantyne JW. The Problem of the Premature Infant. Br Med J. 1902;1(2159):1196-200.
241. Baba S, Noda H, Nakayama M, Waguri M, Mitsuda N, Iso H. Risk factors of early spontaneous abortions among Japanese: a matched case-control study. Hum Reprod. 2011;26(2):466-72.
242. Madsen M, Jorgensen T, Jensen ML, Juhl M, Olsen J, Andersen PK, et al. Leisure time physical exercise during pregnancy and the risk of miscarriage: a study within the Danish National Birth Cohort. BJOG. 2007;114(11):1419-26.
243. Revonta M, Raitanen J, Sihvo S, Koponen P, Klemetti R, Mannisto S, et al. Health and life style among infertile men and women. Sex Reprod Healthc. 2010;1(3):91-8.
244. National Institute for Health and Clinical Excellence. Ectopic pregnancy and miscarriage: Diagnosis and initial management in early pregnancy of ectopic pregnancy and miscarriage. http://www.nice.org.uk/nicemedia/live/14000/61854/61854.pdf2012.
245. Kloss JD, Perlis ML, Zamzow JA, Culnan EJ, Gracia CR. Sleep, sleep disturbance, and fertility in women. Sleep medicine reviews. 2014.
246. Stocker LJ, Macklon NS, Cheong YC, Bewley SJ. Influence of shift work on early reproductive outcomes: a systematic review and meta-analysis. Obstet Gynecol. 2014;124(1):99-110.
247. Knutsson A, Boggild H. Shiftwork and cardiovascular disease: review of disease mechanisms. Rev Environ Health. 2000;15(4):359-72.
248. Frost P, Kolstad HA, Bonde JP. Shift work and the risk of ischemic heart disease - a systematic review of the epidemiologic evidence. Scandinavian journal of work, environment & health. 2009;35(3):163-79.
249. Esquirol Y, Bongard V, Mabile L, Jonnier B, Soulat JM, Perret B. Shift work and metabolic syndrome: respective impacts of job strain, physical activity, and dietary rhythms. Chronobiology international. 2009;26(3):544-59.
250. Pietroiusti A, Neri A, Somma G, Coppeta L, Iavicoli I, Bergamaschi A, et al. Incidence of metabolic syndrome among night-shift healthcare workers. Occup Environ Med. 2010;67(1):54-7.
251. Maury E, Ramsey KM, Bass J. Circadian rhythms and metabolic syndrome: from experimental genetics to human disease. Circ Res. 2010;106(3):447-62.
252. Kloss JD, Perlis ML, Zamzow JA, Culnan EJ, Gracia CR. Sleep, sleep disturbance, and fertility in women. Sleep Med Rev. 2015;22:78-87.
253. Palubska S, Adamiak-Godlewska A, Winkler I, Romanek-Piva K, Rechberger T, Gogacz M. Hyperprolactinaemia - a problem in patients from the reproductive period to the menopause. Prz Menopauzalny. 2017;16(1):1-7.

254. Shreeve N, Cagampang F, Sadek K, Tolhurst M, Houldey A, Hill CM, et al. Poor sleep in PCOS; is melatonin the culprit? Hum Reprod. 2013;28(5):1348-53.
255. Nakamura K, Sheps S, Arck PC. Stress and reproductive failure: past notions, present insights and future directions. Journal of assisted reproduction and genetics. 2008;25(2-3):47-62.
256. Louis GM, Lum KJ, Sundaram R, Chen Z, Kim S, Lynch CD, et al. Stress reduces conception probabilities across the fertile window: evidence in support of relaxation. Fertility and sterility. 2011;95(7):2184-9.
257. Vgontzas AN, Legro RS, Bixler EO, Grayev A, Kales A, Chrousos GP. Polycystic ovary syndrome is associated with obstructive sleep apnea and daytime sleepiness: role of insulin resistance. The Journal of clinical endocrinology and metabolism. 2001;86(2):517-20.
258. Aldabal L, Bahammam AS. Metabolic, endocrine, and immune consequences of sleep deprivation. Open Respir Med J. 2011;5:31-43.
259. Kotronoulas G, Stamatakis A, Stylianopoulou F. Hormones, hormonal agents, and neuropeptides involved in the neuroendocrine regulation of sleep in humans. Hormones (Athens). 2009;8(4):232-48.
260. Tasali E, Van Cauter E, Ehrmann DA. Polycystic Ovary Syndrome and Obstructive Sleep Apnea. Sleep medicine clinics. 2008;3(1):37-46.
261. Shilaih M, Clerck V, Falco L, Kubler F, Leeners B. Pulse Rate Measurement During Sleep Using Wearable Sensors, and its Correlation with the Menstrual Cycle Phases, A Prospective Observational Study. Sci Rep. 2017;7(1):1294.
262. King AE, Critchley HO. Oestrogen and progesterone regulation of inflammatory processes in the human endometrium. J Steroid Biochem Mol Biol. 2010;120(2-3):116-26.
263. Spiegel K, Sheridan JF, Van Cauter E. Effect of sleep deprivation on response to immunization. JAMA. 2002;288(12):1471-2.
264. Turner K, Reynolds-May MF, Zitek EM, Tisdale RL, Carlisle AB, Westphal LM. Stress and anxiety scores in first and repeat IVF cycles: a pilot study. PloS one. 2013;8(5):e63743.
265. Smeenk JM, Verhaak CM, Vingerhoets AJ, Sweep CG, Merkus JM, Willemsen SJ, et al. Stress and outcome success in IVF: the role of self-reports and endocrine variables. Hum Reprod. 2005;20(4):991-6.
266. Hammerli K, Znoj H, Barth J. The efficacy of psychological interventions for infertile patients: a meta-analysis examining mental health and pregnancy rate. Human reproduction update. 2009;15(3):279-95.
267. de Liz TM, Strauss B. Differential efficacy of group and individual/couple psychotherapy with infertile patients. Hum Reprod. 2005;20(5):1324-32.
268. Buysse DJ, Reynolds CF, 3rd, Monk TH, Berman SR, Kupfer DJ. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. Psychiatry research. 1989;28(2):193-213.
269. Johns MW. A new method for measuring daytime sleepiness: the Epworth sleepiness scale. Sleep. 1991;14(6):540-5.
270. Johns M. Epworth Sleepiness Scale 2017 [Available from: http://epworthsleepinessscale.com/about-the-ess/.
271. Littner M, Hirshkowitz M, Kramer M, Kapen S, Anderson WM, Bailey D, et al. Practice parameters for using polysomnography to evaluate insomnia: an update. Sleep. 2003;26(6):754-60.
272. Kayumov L, Casper RF, Hawa RJ, Perelman B, Chung SA, Sokalsky S, et al. Blocking low-wavelength light prevents nocturnal melatonin suppression with no adverse effect on performance during simulated shift work. The Journal of clinical endocrinology and metabolism. 2005;90(5):2755-61.
273. Rosner B. Fundamentals of biostatistics. 7th ed., International ed. ed. Pacific Grove, Calif.: Brooks/Cole, Cengage Learning; 2011.

274. Dewan K, Benloucif S, Reid K, Wolfe LF, Zee PC. Light-induced changes of the circadian clock of humans: increasing duration is more effective than increasing light intensity. Sleep. 2011;34(5):593-9.
275. Chang AM, Scheer FA, Czeisler CA, Aeschbach D. Direct effects of light on alertness, vigilance, and the waking electroencephalogram in humans depend on prior light history. Sleep. 2013;36(8):1239-46.
276. Czeisler CA, Allan JS, Strogatz SH, Ronda JM, Sanchez R, Rios CD, et al. Bright light resets the human circadian pacemaker independent of the timing of the sleep-wake cycle. Science. 1986;233(4764):667-71.
277. Grandner MA, Kripke DF, Yoon IY, Youngstedt SD. Criterion validity of the Pittsburgh Sleep Quality Index: Investigation in a non-clinical sample. Sleep Biol Rhythms. 2006;4(2):129-39.
278. Colten HR, Altevogt BM. Sleep disorders and sleep deprivation : an unmet public health problem. Washington, DC: Institute of Medicine : National Academies Press; 2006.
279. Shrivastava D, Jung S, Saadat M, Sirohi R, Crewson K. How to interpret the results of a sleep study. J Community Hosp Intern Med Perspect. 2014;4(5):24983.
280. Aarts JW, van Empel IW, Boivin J, Nelen WL, Kremer JA, Verhaak CM. Relationship between quality of life and distress in infertility: a validation study of the Dutch FertiQoL. Hum Reprod. 2011;26(5):1112-8.
281. Stocker LJ HK, Cheong YC A Randomized Controlled Trial Assessing Whether Listening to Music at Time of Embryo Transfer Effects Anxiety Levels. Gynecology and Obstetrics (Sunnyvale). 2016;6(401).
282. Coughlan C, Walters S, Ledger W, Li TC. A comparison of psychological stress among women with and without reproductive failure. Int J Gynaecol Obstet. 2014;124(2):143-7.
283. Gooley JJ, Chamberlain K, Smith KA, Khalsa SB, Rajaratnam SM, Van Reen E, et al. Exposure to room light before bedtime suppresses melatonin onset and shortens melatonin duration in humans. J Clin Endocrinol Metab. 2011;96(3):E463-72.
284. Brown-Grant K, Raisman G. Abnormalities in reproductive function associated with the destruction of the suprachiasmatic nuclei in female rats. Proc R Soc Lond B Biol Sci. 1977;198(1132):279-96.
285. Takasu NN, Nakamura TJ, Tokuda IT, Todo T, Block GD, Nakamura W. Recovery from Age-Related Infertility under Environmental Light-Dark Cycles Adjusted to the Intrinsic Circadian Period. Cell Rep. 2015;12(9):1407-13.
286. Duffy JF, Czeisler CA. Effect of Light on Human Circadian Physiology. Sleep Med Clin. 2009;4(2):165-77.
287. Amaral FG, Castrucci AM, Cipolla-Neto J, Poletini MO, Mendez N, Richter HG, et al. Environmental control of biological rhythms: effects on development, fertility and metabolism. J Neuroendocrinol. 2014;26(9):603-12.
288. Anujuo K, Stronks K, Snijder MB, Jean-Louis G, Rutters F, van den Born BJ, et al. Relationship between short sleep duration and cardiovascular risk factors in a multi-ethnic cohort - the helius study. Sleep Med. 2015;16(12):1482-8.
289. Jike M, Itani O, Watanabe N, Buysse DJ, Kaneita Y. Long sleep duration and health outcomes: A systematic review, meta-analysis and meta-regression. Sleep Med Rev. 2017.
290. Belenky G, Wesensten NJ, Thorne DR, Thomas ML, Sing HC, Redmond DP, et al. Patterns of performance degradation and restoration during sleep restriction and subsequent recovery: a sleep dose-response study. J Sleep Res. 2003;12(1):1-12.

291. Vgontzas AN, Papanicolaou DA, Bixler EO, Lotsikas A, Zachman K, Kales A, et al. Circadian interleukin-6 secretion and quantity and depth of sleep. J Clin Endocrinol Metab. 1999;84(8):2603-7.
292. Nilsson JP, Soderstrom M, Karlsson AU, Lekander M, Akerstedt T, Lindroth NE, et al. Less effective executive functioning after one night's sleep deprivation. J Sleep Res. 2005;14(1):1-6.
293. Copinschi G. Metabolic and endocrine effects of sleep deprivation. Essent Psychopharmacol. 2005;6(6):341-7.
294. Smith C. Sleep states and memory processes in humans: procedural versus declarative memory systems. Sleep Med Rev. 2001;5(6):491-506.
295. Strine TW, Chapman DP. Associations of frequent sleep insufficiency with health-related quality of life and health behaviors. Sleep Med. 2005;6(1):23-7.
296. Kapur VK, Redline S, Nieto FJ, Young TB, Newman AB, Henderson JA, et al. The relationship between chronically disrupted sleep and healthcare use. Sleep. 2002;25(3):289-96.
297. Kryger MH, Roth T, Dement WC. Principles and practice of sleep medicine. 5th ed. ed. Philadelphia, PA: Saunders/Elsevier; 2011.
298. Kryger M RT, Dement W. Principles and Practice of Sleep Medicine: Elsevier; 2016.
299. Davis S, Mirick DK, Chen C, Stanczyk FZ. Night shift work and hormone levels in women. Cancer Epidemiol Biomarkers Prev. 2012;21(4):609-18.
300. Daynes RA, Araneo BA, Ershler WB, Maloney C, Li GZ, Ryu SY. Altered regulation of IL-6 production with normal aging. Possible linkage to the age-associated decline in dehydroepiandrosterone and its sulfated derivative. J Immunol. 1993;150(12):5219-30.
301. Dodson ER, Zee PC. Therapeutics for Circadian Rhythm Sleep Disorders. Sleep Med Clin. 2010;5(4):701-15.
302. Weitzman ED, Czeisler CA, Coleman RM, Spielman AJ, Zimmerman JC, Dement W, et al. Delayed sleep phase syndrome. A chronobiological disorder with sleep-onset insomnia. Arch Gen Psychiatry. 1981;38(7):737-46.
303. Regestein QR, Monk TH. Delayed sleep phase syndrome: a review of its clinical aspects. Am J Psychiatry. 1995;152(4):602-8.
304. Wakatsuki Y, Kudo T, Shibata S. Constant light housing during nursing causes human DSPS (delayed sleep phase syndrome) behaviour in Clock-mutant mice. Eur J Neurosci. 2007;25(8):2413-24.
305. Archer SN, Robilliard DL, Skene DJ, Smits M, Williams A, Arendt J, et al. A length polymorphism in the circadian clock gene Per3 is linked to delayed sleep phase syndrome and extreme diurnal preference. Sleep. 2003;26(4):413-5.
306. Patke A, Murphy PJ, Onat OE, Krieger AC, Ozcelik T, Campbell SS, et al. Mutation of the Human Circadian Clock Gene CRY1 in Familial Delayed Sleep Phase Disorder. Cell. 2017;169(2):203-15 e13.
307. Ebisawa T. Circadian rhythms in the CNS and peripheral clock disorders: human sleep disorders and clock genes. Journal of pharmacological sciences. 2007;103(2):150-4.
308. Sack RL, Auckley D, Auger RR, Carskadon MA, Wright KP, Jr., Vitiello MV, et al. Circadian rhythm sleep disorders: part I, basic principles, shift work and jet lag disorders. An American Academy of Sleep Medicine review. Sleep. 2007;30(11):1460-83.
309. Fleming TP, Lucas ES, Watkins AJ, Eckert JJ. Adaptive responses of the embryo to maternal diet and consequences for post-implantation development. Reprod Fertil Dev. 2011;24(1):35-44.
310. Fowden AL, Forhead AJ, Coan PM, Burton GJ. The placenta and intrauterine programming. J Neuroendocrinol. 2008;20(4):439-50.

311. Weimar CH, Macklon NS, Post Uiterweer ED, Brosens JJ, Gellersen B. The motile and invasive capacity of human endometrial stromal cells: implications for normal and impaired reproductive function. Human reproduction update. 2013;19(5):542-57.
312. Evers JL. Female subfertility. Lancet. 2002;360(9327):151-9.
313. Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF, et al. Endometrial glands are required for preimplantation conceptus elongation and survival. Biol Reprod. 2001;64(6):1608-13.
314. Spencer TE, Gray CA. Sheep uterine gland knockout (UGKO) model. Methods Mol Med. 2006;121:85-94.
315. Russell P, Hey-Cunningham A, Berbic M, Tremellen K, Sacks G, Gee A, et al. Asynchronous glands in the endometrium of women with recurrent reproductive failure. Pathology. 2014;46(4):325-32.
316. Cheong Y, Boomsma C, Heijnen C, Macklon N. Uterine secretomics: a window on the maternal-embryo interface. Fertil Steril. 2013;99(4):1093-9.
317. Boomsma CM, Kavelaars A, Eijkemans MJ, Lentjes EG, Fauser BC, Heijnen CJ, et al. Endometrial secretion analysis identifies a cytokine profile predictive of pregnancy in IVF. Hum Reprod. 2009;24(6):1427-35.
318. Ledee-Bataille N, Bonnet-Chea K, Hosny G, Dubanchet S, Frydman R, Chaouat G. Role of the endometrial tripod interleukin-18, -15, and -12 in inadequate uterine receptivity in patients with a history of repeated in vitro fertilization-embryo transfer failure. Fertil Steril. 2005;83(3):598-605.
319. Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, et al. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature. 1992;359(6390):76-9.
320. Olivennes F, Ledee-Bataille N, Samama M, Kadoch J, Taupin JL, Dubanchet S, et al. Assessment of leukemia inhibitory factor levels by uterine flushing at the time of egg retrieval does not adversely affect pregnancy rates with in vitro fertilization. Fertil Steril. 2003;79(4):900-4.
321. Heng S, Hannan NJ, Rombauts LJ, Salamonsen LA, Nie G. PC6 levels in uterine lavage are closely associated with uterine receptivity and significantly lower in a subgroup of women with unexplained infertility. Hum Reprod. 2011;26(4):840-6.
322. Hannan NJ, Paiva P, Meehan KL, Rombauts LJ, Gardner DK, Salamonsen LA. Analysis of fertility-related soluble mediators in human uterine fluid identifies VEGF as a key regulator of embryo implantation. Endocrinology. 2011;152(12):4948-56.
323. Fitzgerald HC, Salamonsen LA, Rombauts LJ, Vollenhoven BJ, Edgell TA. The proliferative phase underpins endometrial development: Altered cytokine profiles in uterine lavage fluid of women with idiopathic infertility. Cytokine. 2016;88:12-9.
324. Ledee-Bataille N, Lapree-Delage G, Taupin JL, Dubanchet S, Frydman R, Chaouat G. Concentration of leukaemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation. Hum Reprod. 2002;17(1):213-8.
325. Ulbrich SE, Schulke K, Groebner AE, Reichenbach HD, Angioni C, Geisslinger G, et al. Quantitative characterization of prostaglandins in the uterus of early pregnant cattle. Reproduction. 2009;138(2):371-82.
326. Filant J, Spencer TE. Endometrial glands are essential for blastocyst implantation and decidualization in the mouse uterus. Biol Reprod. 2013;88(4):93.
327. van Mourik MS, Macklon NS, Heijnen CJ. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. J Leukoc Biol. 2009;85(1):4-19.

328. Maybin JA, Critchley HO, Jabbour HN. Inflammatory pathways in endometrial disorders. Mol Cell Endocrinol. 2011;335(1):42-51.
329. Greening DW, Nguyen HP, Evans J, Simpson RJ, Salamonsen LA. Modulating the endometrial epithelial proteome and secretome in preparation for pregnancy: The role of ovarian steroid and pregnancy hormones. J Proteomics. 2016;144:99-112.
330. Reppert SM, Weaver DR. Molecular analysis of mammalian circadian rhythms. Annu Rev Physiol. 2001;63:647-76.
331. McNamara P, Seo SB, Rudic RD, Sehgal A, Chakravarti D, FitzGerald GA. Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock. Cell. 2001;105(7):877-89.
332. Reppert SM, Weaver DR. Coordination of circadian timing in mammals. Nature. 2002;418(6901):935-41.
333. Lee C, Etchegaray JP, Cagampang FR, Loudon AS, Reppert SM. Posttranslational mechanisms regulate the mammalian circadian clock. Cell. 2001;107(7):855-67.
334. Miller BH, Olson SL, Turek FW, Levine JE, Horton TH, Takahashi JS. Circadian clock mutation disrupts estrous cyclicity and maintenance of pregnancy. Curr Biol. 2004;14(15):1367-73.
335. Carter SJ, Durrington HJ, Gibbs JE, Blaikley J, Loudon AS, Ray DW, et al. A matter of time: study of circadian clocks and their role in inflammation. J Leukoc Biol. 2016;99(4):549-60.
336. Cutolo M, Seriolo B, Craviotto C, Pizzorni C, Sulli A. Circadian rhythms in RA. Ann Rheum Dis. 2003;62(7):593-6.
337. Stapleton CM, Jaradat M, Dixon D, Kang HS, Kim SC, Liao G, et al. Enhanced susceptibility of staggerer (RORalphasg/sg) mice to lipopolysaccharide-induced lung inflammation. Am J Physiol Lung Cell Mol Physiol. 2005;289(1):L144-52.
338. Dzhagalov I, Giguere V, He YW. Lymphocyte development and function in the absence of retinoic acid-related orphan receptor alpha. J Immunol. 2004;173(5):2952-9.
339. Delerive P, Monte D, Dubois G, Trottein F, Fruchart-Najib J, Mariani J, et al. The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response. EMBO Rep. 2001;2(1):42-8.
340. He C, Wang J, Li Y, Zhu K, Xu Z, Song Y, et al. Melatonin-related genes expressed in the mouse uterus during early gestation promote embryo implantation. Journal of pineal research. 2015;58(3):300-9.
341. Jung-Hynes B, Huang W, Reiter RJ, Ahmad N. Melatonin resynchronizes dysregulated circadian rhythm circuitry in human prostate cancer cells. J Pineal Res. 2010;49(1):60-8.
342. Gallo V, Egger M, McCormack V, Farmer PB, Ioannidis JP, Kirsch-Volders M, et al. STrengthening the Reporting of OBservational studies in Epidemiology--Molecular Epidemiology (STROBE-ME): an extension of the STROBE Statement. PLoS Med. 2011;8(10):e1001117.
343. Clinical endocrinology [electronic resource]: [Oxford] : [Blackwell Science]; [
344. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell. 1982;31(1):11-24.
345. Kempers MJ, Kuiper RP, Ockeloen CW, Chappuis PO, Hutter P, Rahner N, et al. Risk of colorectal and endometrial cancers in EPCAM deletion-positive Lynch syndrome: a cohort study. The lancet oncology. 2011;12(1):49-55.

346. Kimura H, Kato H, Faried A, Sohda M, Nakajima M, Fukai Y, et al. Prognostic significance of EpCAM expression in human esophageal cancer. International journal of oncology. 2007;30(1):171-9.
347. Schwab KE, Hutchinson P, Gargett CE. Identification of surface markers for prospective isolation of human endometrial stromal colony-forming cells. Hum Reprod. 2008;23(4):934-43.
348. Pilling D, Fan T, Huang D, Kaul B, Gomer RH. Identification of markers that distinguish monocyte-derived fibrocytes from monocytes, macrophages, and fibroblasts. PloS one. 2009;4(10):e7475.
349. Stocker L, Cagampang F, Cheong Y. Identifying stably expressed housekeeping genes in the endometrium of fertile women, women with recurrent implantation failure and recurrent miscarriages. Sci Rep. 2017;7(1):14857.
350. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-8.
351. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology. 2002;3(7):RESEARCH0034.
352. Edwards KD, Akman OE, Knox K, Lumsden PJ, Thomson AW, Brown PE, et al. Quantitative analysis of regulatory flexibility under changing environmental conditions. Mol Syst Biol. 2010;6:424.
353. Moore A, Zielinski T, Millar AJ. Online period estimation and determination of rhythmicity in circadian data, using the BioDare data infrastructure. Methods Mol Biol. 2014;1158:13-44.
354. Burg J. The relationship between maximum entropy spectra and maximum likelihood spectra. Geophysics. 1972;37(2):375-6.
355. Zielinski T, Moore AM, Troup E, Halliday KJ, Millar AJ. Strengths and limitations of period estimation methods for circadian data. PLoS One. 2014;9(5):e96462.
356. Pandian Z, Marjoribanks J, Ozturk O, Serour G, Bhattacharya S. Number of embryos for transfer following in vitro fertilisation or intra-cytoplasmic sperm injection. Cochrane Database Syst Rev. 2013(7):CD003416.
357. Harrison EM, Gorman MR. Changing the waveform of circadian rhythms: considerations for shift-work. Front Neurol. 2012;3:72.
358. Farrar JD, Carson DD. Differential temporal and spatial expression of mRNA encoding extracellular matrix components in decidua during the peri-implantation period. Biol Reprod. 1992;46(6):1095-108.
359. Paria BC, Ma W, Tan J, Raja S, Das SK, Dey SK, et al. Cellular and molecular responses of the uterus to embryo implantation can be elicited by locally applied growth factors. Proc Natl Acad Sci U S A. 2001;98(3):1047-52.
360. Potdar N, Gelbaya T, Nardo LG. Endometrial injury to overcome recurrent embryo implantation failure: a systematic review and meta-analysis. Reprod Biomed Online. 2012;25(6):561-71.
361. Kalma Y, Granot I, Gnainsky Y, Or Y, Czernobilsky B, Dekel N, et al. Endometrial biopsy-induced gene modulation: first evidence for the expression of bladder-transmembranal uroplakin Ib in human endometrium. Fertility and sterility. 2009;91(4):1042-9, 9 e1-9.
362. Salleh N, Giribabu N. Leukemia inhibitory factor: roles in embryo implantation and in nonhormonal contraception. ScientificWorldJournal. 2014;2014:201514.
363. Arici A, Engin O, Attar E, Olive DL. Modulation of leukemia inhibitory factor gene expression and protein biosynthesis in human endometrium. The Journal of clinical endocrinology and metabolism. 1995;80(6):1908-15.

364. Kimber SJ. Leukaemia inhibitory factor in implantation and uterine biology. Reproduction. 2005;130(2):131-45.
365. Pan H, Zhu L, Deng Y, Pollard JW. Microarray analysis of uterine epithelial gene expression during the implantation window in the mouse. Endocrinology. 2006;147(10):4904-16.
366. Paria BC, Wang H, Dey SK. Endocannabinoid signaling in synchronizing embryo development and uterine receptivity for implantation. Chem Phys Lipids. 2002;121(1-2):201-10.
367. Leese HJ. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. BioEssays : news and reviews in molecular, cellular and developmental biology. 2002;24(9):845-9.
368. Strowitzki T, Germeyer A, Popovici R, von Wolff M. The human endometrium as a fertility-determining factor. Hum Reprod Update. 2006;12(5):617-30.
369. van der Gaast MH, Beier-Hellwig K, Fauser BC, Beier HM, Macklon NS. Endometrial secretion aspiration prior to embryo transfer does not reduce implantation rates. Reprod Biomed Online. 2003;7(1):105-9.
370. Gene database [Internet]. 2014. Available from: http://www.ncbi.nlm.nih.gov/gene.
371. Robb L, Li R, Hartley L, Nandurkar HH, Koentgen F, Begley CG. Infertility in female mice lacking the receptor for interleukin 11 is due to a defective uterine response to implantation. Nat Med. 1998;4(3):303-8.
372. Huang HY, Wen Y, Kruessel JS, Raga F, Soong YK, Polan ML. Interleukin (IL)-1beta regulation of IL-1beta and IL-1 receptor antagonist expression in cultured human endometrial stromal cells. J Clin Endocrinol Metab. 2001;86(3):1387-93.
373. Bourdiec A, Martel V, Akoum A. Synchronous regulation of the determinants of endometrial receptivity to interleukin 1 at key stages of early embryo implantation in vivo. Fertil Steril. 2014;101(4):1183-93.
374. Sharkey AM, Smith SK. The endometrium as a cause of implantation failure. Best Pract Res Clin Obstet Gynaecol. 2003;17(2):289-307.
375. Dimitriadis E, White CA, Jones RL, Salamonsen LA. Cytokines, chemokines and growth factors in endometrium related to implantation. Hum Reprod Update. 2005;11(6):613-30.
376. Jana B, Andronowska A. Expression of nerve growth factor and its receptors in the uterus of gilts with endometritis induced by infection with Escherichia coli. J Comp Pathol. 2012;147(4):522-32.
377. Zheng Y, Liu X, Guo SW. Therapeutic potential of andrographolide for treating endometriosis. Hum Reprod. 2012;27(5):1300-13.
378. Lalitkumar S, Boggavarapu NR, Menezes J, Dimitriadis E, Zhang JG, Nicola NA, et al. Polyethylene glycated leukemia inhibitory factor antagonist inhibits human blastocyst implantation and triggers apoptosis by down-regulating embryonic AKT. Fertil Steril. 2013;100(4):1160-9.
379. Cheng JG, Rodriguez CI, Stewart CL. Control of uterine receptivity and embryo implantation by steroid hormone regulation of LIF production and LIF receptor activity: towards a molecular understanding of "the window of implantation". Rev Endocr Metab Disord. 2002;3(2):119-26.
380. Zhao J, Zhang Q, Wang Y, Li Y. Uterine Infusion With Bone Marrow Mesenchymal Stem Cells Improves Endometrium Thickness in a Rat Model of Thin Endometrium. Reprod Sci. 2014.
381. Grasso E, Paparini D, Aguero M, Mor G, Perez Leiros C, Ramhorst R. VIP contribution to the decidualization program: regulatory T cell recruitment. J Endocrinol. 2014;221(1):121-31.

382. Evans J, Salamonsen LA. Decidualized human endometrial stromal cells are sensors of hormone withdrawal in the menstrual inflammatory cascade. Biol Reprod. 2014;90(1):14.
383. Russo N, Russo M, Daino D, Freschi L, Fiore L, Merlini S, et al. Evaluation of brain-derived neurotrophic factor in menstrual blood and its identification in human endometrium. Gynecol Endocrinol. 2012;28(6):492-5.
384. Wessels JM, Wu L, Leyland NA, Wang H, Foster WG. The brain-uterus connection: brain derived neurotrophic factor (BDNF) and its receptor (Ntrk2) are conserved in the mammalian uterus. PLoS One. 2014;9(4):e94036.
385. Schwenke M, Knofler M, Velicky P, Weimar CH, Kruse M, Samalecos A, et al. Control of human endometrial stromal cell motility by PDGF-BB, HB-EGF and trophoblast-secreted factors. PLoS One. 2013;8(1):e54336.
386. Lash GE, Innes BA, Drury JA, Robson SC, Quenby S, Bulmer JN. Localization of angiogenic growth factors and their receptors in the human endometrium throughout the menstrual cycle and in recurrent miscarriage. Hum Reprod. 2012;27(1):183-95.
387. Mitsunari M, Harada T, Tanikawa M, Iwabe T, Taniguchi F, Terakawa N. The potential role of stem cell factor and its receptor c-kit in the mouse blastocyst implantation. Mol Hum Reprod. 1999;5(9):874-9.
388. Chan RW, Schwab KE, Gargett CE. Clonogenicity of human endometrial epithelial and stromal cells. Biol Reprod. 2004;70(6):1738-50.
389. Attarchi M, Darkhi H, Khodarahmian M, Dolati M, Kashanian M, Ghaffari M, et al. Characteristics of menstrual cycle in shift workers. Glob J Health Sci. 2013;5(3):163-72.
390. Ahlborg G, Jr., Axelsson G, Bodin L. Shift work, nitrous oxide exposure and subfertility among Swedish midwives. Int J Epidemiol. 1996;25(4):783-90.
391. Barzilai-Pesach V, Sheiner EK, Sheiner E, Potashnik G, Shoham-Vardi I. The effect of women's occupational psychologic stress on outcome of fertility treatments. Journal of occupational and environmental medicine / American College of Occupational and Environmental Medicine. 2006;48(1):56-62.
392. Bryant HE, Love EJ. Effect of employment and its correlates on spontaneous abortion risk. Soc Sci Med. 1991;33(7):795-800.
393. El-Metwalli AG, Badawy AM, El-Baghdadi LA, El-Wehady A. Occupational physical activity and pregnancy outcome. European journal of obstetrics, gynecology, and reproductive biology. 2001;100(1):41-5.
394. Hjollund NH, Kold Jensen T, Bonde JP, Henriksen TB, Kolstad HA, Andersson AM, et al. Job strain and time to pregnancy. Scandinavian journal of work, environment & health. 1998;24(5):344-50.
395. Infante-Rivard C, David M, Gauthier R, Rivard GE. Pregnancy loss and work schedule during pregnancy. Epidemiology. 1993;4(1):73-5.
396. Labyak S, Lava S, Turek F, Zee P. Effects of shiftwork on sleep and menstrual function in nurses. Health care for women international. 2002;23(6-7):703-14.
397. Lohstroh PN, Chen J, Ba J, Ryan LM, Xu X, Overstreet JW, et al. Bone resorption is affected by follicular phase length in female rotating shift workers. Environmental health perspectives. 2003;111(4):618-22.
398. McDonald AD, Armstrong B, Cherry NM, Delorme C, Diodati-Nolin A, McDonald JC, et al. Spontaneous abortion and occupation. Journal of occupational medicine : official publication of the Industrial Medical Association. 1986;28(12):1232-8.
399. McDonald AD, McDonald JC, Armstrong B, Cherry NM, Cote R, Lavoie J, et al. Fetal death and work in pregnancy. Br J Ind Med. 1988;45(3):148-57.
400. Messing K, Saurel-Cubizolles MJ, Bourgine M, Kaminski M. Menstrual-cycle characteristics and work conditions of workers in poultry slaughterhouses and

canneries. Scandinavian journal of work, environment & health. 1992;18(5):302-9.
401. Swan SH, Beaumont JJ, Hammond SK, VonBehren J, Green RS, Hallock MF, et al. Historical cohort study of spontaneous abortion among fabrication workers in the Semiconductor Health Study: agent-level analysis. American journal of industrial medicine. 1995;28(6):751-69.
402. Uehata T, Sasakawa N. The fatigue and maternity disturbances of night workwomen. J Hum Ergol (Tokyo). 1982;11 Suppl:465-74.
403. Carpen JD, Archer SN, Skene DJ, Smits M, von Schantz M. A single-nucleotide polymorphism in the 5'-untranslated region of the hPER2 gene is associated with diurnal preference. Journal of sleep research. 2005;14(3):293-7.
404. Panda S, Hogenesch JB, Kay SA. Circadian rhythms from flies to human. Nature. 2002;417(6886):329-35.
405. Xu Y, Padiath QS, Shapiro RE, Jones CR, Wu SC, Saigoh N, et al. Functional consequences of a CKIdelta mutation causing familial advanced sleep phase syndrome. Nature. 2005;434(7033):640-4.
406. Chang AM, Buch AM, Bradstreet DS, Klements DJ, Duffy JF. Human diurnal preference and circadian rhythmicity are not associated with the CLOCK 3111C/T gene polymorphism. Journal of biological rhythms. 2011;26(3):276-9.
407. Mishima K, Tozawa T, Satoh K, Saitoh H, Mishima Y. The 3111T/C polymorphism of hClock is associated with evening preference and delayed sleep timing in a Japanese population sample. American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics. 2005;133B(1):101-4.
408. Roenneberg T, Kuehnle T, Juda M, Kantermann T, Allebrandt K, Gordijn M, et al. Epidemiology of the human circadian clock. Sleep medicine reviews. 2007;11(6):429-38.
409. Katzenberg D, Young T, Finn L, Lin L, King DP, Takahashi JS, et al. A CLOCK polymorphism associated with human diurnal preference. Sleep. 1998;21(6):569-76.
410. Shi S, Hida A, McGuinness OP, Wasserman DH, Yamazaki S, Johnson CH. Circadian clock gene Bmal1 is not essential; functional replacement with its paralog, Bmal2. Current biology : CB. 2010;20(4):316-21.
411. Etchegaray JP, Lee C, Wade PA, Reppert SM. Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. Nature. 2003;421(6919):177-82.
412. Ripperger JA, Schibler U. Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. Nat Genet. 2006;38(3):369-74.
413. Buhr ED, Takahashi JS. Molecular components of the Mammalian circadian clock. Handb Exp Pharmacol. 2013(217):3-27.