Effect of sleep deprivation and shift work on metabolic and cardiovascular function

by

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University of Surrey

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ABSTRACT

Alterations in metabolism and heart rate variability (HRV) and endothelial dysfunction as well as sleep deprivation and shift work have been associated with an increased risk for cardiovascular disease. The aim of the current study was to investigate the effect of one night of total sleep deprivation (TSD) (as a proxy for the first night of shift work) and recovery sleep on metabolic and cardiovascular function and alertness and mood under controlled laboratory conditions in experienced shift workers compared with non-shift workers.

Eleven shift workers (SW) (shift work ≥ 5 years) and 14 non-shift workers (NSW), matched for age, BMI and cholesterol, followed a 7-day regular sleep-wake cycle prior to the laboratory session, consisting of adaptation sleep, baseline sleep, TSD (30.5 h), a 4-h nap and recovery sleep. All interventions were relative to wake up time (body posture, food intake and light controlled throughout).

After TSD, basal plasma triacylglycerol (TAG) levels and postprandial glucose clearance decreased and the morning peak in HRV variance increased. After recovery sleep, TAG and insulin responses increased and non-esterified fatty acid (NEFA) responses and HRV variance decreased. These data suggest shifts in insulin sensitivity and sympathetic/parasympathetic balance after TSD and recovery sleep. Mood and alertness deteriorated after TSD but the recovery periods provided effective recuperation. There were no differences in screening parameters and circadian phase between SW and NSW. However, SW felt more alert, cheerful, elated and calmer and showed a lower HRV variance and higher sympathetic activity as well as a trend for lower endothelial function throughout the study and a smaller increase in insulin and decrease in NEFA responses after recovery sleep. Further research is needed to unravel the mechanisms underlying these group differences and to clarify why some changes in metabolic and cardiovascular function take place after TSD while others are observed following recovery sleep.
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I would also like to thank all 25 subjects who participated in this study and without whose perseverance during sleepless nights and demanding conditions, the data described here would not be present.

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STATEMENT OF ORIGINALITY

This thesis and the work to which it refers are the results of my own efforts. Any ideas, data, images or text resulting from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to their originator in the text or bibliography. This thesis has not been submitted in whole or in part for any other academic degree or professional qualification.

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ABBREVIATIONS

(LF/HF) BPV  (low/high frequency modulation of) blood pressure variation
1/RT  mean reciprocal reaction time
Ach  acetylcholine
ACTH  adrenocorticotropic hormone
ADA  adenosine deaminase
AIRg  insulin response
ALT  alanine amino transferase
AP  arterial pressure
Apo  apolipoprotein
AST  amino transferase
AUC  area under the curve
BDI  Beck Depression Inventory
BMI  body mass index
BP  blood pressure
bpm  beats per minute
BRS  baroreflex sensitivity
CAD  coronary artery disease
CBT  core body temperature
CHD  coronary heart disease
C  see chol
chol  cholesterol
CM  chylomicron
cort  cortisol
CR  constant routine protocol
CV  coefficient of variation
CVD  cardiovascular disease
DBP  diastolic blood pressure
DI  disposition index
DLMO  dim light melatonin onset
DN  data not available
ECG  electrocardiogram
EDHF  endothelium-derived hyperpolarizing factor
EEG  electroencephalogram
EGP  endogenous glucose production
EMG  electromyogram
eNOS  endothelial nitric oxide (NO) synthase
EOG  electrooculogram
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<td>ESS</td>
<td>Epworth Sleepiness Scale</td>
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<tr>
<td>F</td>
<td>female</td>
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<tr>
<td>FA</td>
<td>fatty acid</td>
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<td>FD</td>
<td>forced desynchrony protocol</td>
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<td>FMD</td>
<td>flow-mediated dilatation</td>
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<td>FRT</td>
<td>fastest 10% reaction times</td>
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<tr>
<td>GCR</td>
<td>glucose clearance rate</td>
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<td>GE</td>
<td>glucose effectiveness</td>
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<td>GH</td>
<td>growth hormone</td>
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<td>GIR</td>
<td>glucose infusion rate</td>
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<td>gluc</td>
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<td>HDL</td>
<td>high-density lipoprotein</td>
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<td>HEC</td>
<td>hyperinsulineic euglycemic clamp</td>
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<td>HF norm</td>
<td>normalised high frequency power in the ECG (HF/(LF+HF) in the current study)</td>
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<td>high frequency power in the ECG (0.15 – 0.4 Hz in the current study)</td>
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<td>hypoglycaemic clamp</td>
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<td>hepatic lipase</td>
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<td>Horne-Östberg questionnaire</td>
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<td>HOMA</td>
<td>homeostatic model assessment</td>
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<td>HPA</td>
<td>hypothalamus pituitary axis</td>
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<td>HR</td>
<td>heart rate</td>
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<td>HRV</td>
<td>heart rate variability</td>
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<td>hsCRP</td>
<td>high sensitivity c-reactive protein</td>
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<td>HSL</td>
<td>hormone sensitive lipase</td>
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<tr>
<td>IAUC</td>
<td>incremental area under the curve (basal level subtracted)</td>
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<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
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<td>IDL</td>
<td>VLDL remnant</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
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<td>IHD</td>
<td>ischaemic heart disease</td>
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<td>interleukin- β</td>
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<td>interleukin-6</td>
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<td>ISR</td>
<td>insulin secretion rate</td>
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<td>insulin suppression test</td>
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<td>IVGTT</td>
<td>intravenous glucose tolerance test</td>
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<tr>
<td>Kc</td>
<td>calcium-activated potassium channel</td>
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<td>Kcal</td>
<td>kilocalories</td>
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<td>Kₙ</td>
<td>glucose tolerance</td>
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<td>KSS</td>
<td>Karolinska Sleepiness Scale</td>
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Abbreviations

LCAT lecithin cholesterol acetyltransferase
LDL low-density lipoprotein
LF norm normalised low frequency power in the ECG (LF/(LF+HF) in the current study)
LF low frequency power (0.04 – 0.15 Hz in the current study) in the ECG
LF/HF low frequency power divided by high frequency power in the ECG
LPL lipoprotein lipase
M male
MAP mean arterial blood pressure
MI myocardial infarction
n.u. normalised units
NEFA non-esterified fatty acid
NN interval normal-to-normal interval (intervals between QRS complexes) in the ECG
NN50 NN intervals longer than 50 ms in the ECG
NO nitric oxide
nREM non-rapid eye movement sleep
NSW non-shift worker(s)
OGTT oral glucose tolerance test
OR odds ratio
PER3 PERIOD3 gene
PGI$_2$ prostacycline
pNN50 percentage of NN intervals longer than 50 ms in the ECG
PSD partial sleep deprivation
PSG polysomnography
PSQI Pittsburgh Sleep Quality Index
PVT psychomotor vigilance test
QC quality control
Rd rate of glucose disposal
rec recovery
REM rapid eye movement sleep
RMR resting metabolic rate
RMSSD square root of the mean of the sum of the squares of differences between NN intervals in the ECG
rNN autocorrelation coefficient of NN intervals
ROI region of interest
RR interval see NN interval
RRT mean reciprocal reaction time, 1/RT
RT reaction time
SBP systolic blood pressure
SCN suprachiasmatic nuclei
SD standard deviation
<table>
<thead>
<tr>
<th>Abbr.</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDNN index</td>
<td>standard deviation of all NN intervals in the ECG</td>
</tr>
<tr>
<td>SDNN</td>
<td>standard deviation of the NN intervals (in a 5-min epoch) in the ECG</td>
</tr>
<tr>
<td>SDSD</td>
<td>standard deviation of differences between NN intervals in the ECG</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>$S_g$</td>
<td>glucose effectiveness</td>
</tr>
<tr>
<td>SI</td>
<td>insulin sensitivity</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
</tr>
<tr>
<td>SOL</td>
<td>sleep onset latency</td>
</tr>
<tr>
<td>SRT</td>
<td>slowest 10% reaction times</td>
</tr>
<tr>
<td>SSG</td>
<td>steady state glucose</td>
</tr>
<tr>
<td>SSI</td>
<td>Standard Shift work Index</td>
</tr>
<tr>
<td>SSS</td>
<td>Stanford sleepiness scale</td>
</tr>
<tr>
<td>SW</td>
<td>shift worker(s)</td>
</tr>
<tr>
<td>SWS</td>
<td>slow wave sleep</td>
</tr>
<tr>
<td>T3</td>
<td>triiodothyronine;</td>
</tr>
<tr>
<td>TAG</td>
<td>triacylglycerol</td>
</tr>
<tr>
<td>TAUC</td>
<td>total area under the curve</td>
</tr>
<tr>
<td>TG</td>
<td>see TAG</td>
</tr>
<tr>
<td>TIB</td>
<td>time in bed</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TP</td>
<td>total power in the ECG</td>
</tr>
<tr>
<td>TSD</td>
<td>total sleep deprivation</td>
</tr>
<tr>
<td>TSH</td>
<td>thyrotropin.</td>
</tr>
<tr>
<td>TST</td>
<td>total sleep time</td>
</tr>
<tr>
<td>VAS</td>
<td>visual analogue scale</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
</tr>
<tr>
<td>VLF</td>
<td>very low frequency power (0.003 – 0.04 Hz in the current study)</td>
</tr>
<tr>
<td>WHR</td>
<td>waist-hip ratio</td>
</tr>
</tbody>
</table>
CHAPTER 1 INTRODUCTION

1.1 Metabolic syndrome and cardiovascular disease

Cardiovascular disease (CVD) is the primary cause of morbidity and mortality nowadays; nearly half of all deaths (49%) in Europe are from CVD; 55% of the deaths in women and 43% of the deaths in men (British Heart Foundation Statistics 2009). Therefore, it is not surprising that this is a major research area.

CVD has traditionally been seen as a disease of lipid metabolism. The metabolic parameters reported to be associated with CVD are clustered in the ‘metabolic syndrome’. Depending on the guidelines applied (from the US, Europe or global), different combinations of the following criteria have to be met to be diagnosed with metabolic syndrome: glucose intolerance/insulin resistance, central obesity, hypertension, elevated triacylglycerol (TAG) levels, decreased high-density lipoprotein (HDL) levels (Grundy et al. 2004; Shaw et al. 2005) (Table 1.1) and increased cholesterol levels (above 5 mmol/L) (Betteridge et al. 1993; British Cardiac Society et al. 2005). Glucose intolerance and insulin resistance, required to be present by the World Health Organisation (WHO) and European Group for the study of Insulin Resistance (EGIR), are subsequent phases preceding diabetes. The time course of diabetes development is characterised by maintenance of normal to mildly elevated glucose levels due to increased insulin secretion (glucose intolerance/insulin insensitivity or resistance), followed by failure of the β-cells to produce more insulin to keep glucose levels within the homeostatic range (diabetes) (DeFronzo 1988; Reaven 2002) (Figure 1.1). Other authors suggest lower cut off values than 1.7 or 2 mmol/L for TAGs and report that an elevation above TAG levels of 1.5 mmol/L is associated with smaller and denser low-density lipoprotein (LDL), which may increase the risk for CVD (Griffin et al. 1994). Some of the risk factors for the metabolic syndrome also fall into another cluster of risk factors, such as the atherogenic lipoprotein phenotype, characterised by abnormal TAGs, HDL cholesterol and the presence of small dense LDL (Austin et al. 1988).

A downstream marker which would functionally integrate the above markers of CVD is endothelial function. Indeed, rhythmicity in endothelial function may well explain several processes occurring in CVD (Walters et al. 2003).
Table 1.1. Definitions of the metabolic syndrome: global, US and Europe.

<table>
<thead>
<tr>
<th>NCEP ATP III</th>
<th>WHO</th>
<th>EGIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three of the following:</td>
<td>Diabetes or impaired glucose tolerance or insulin resistance (under clamp conditions) and two of the following:</td>
<td>(Definition for non-diabetics only) Insulin resistance or fasting hyperinsulinaemia and two of the following:</td>
</tr>
<tr>
<td>1. Fasting plasma glucose ≥6·1 mmol/l (≥110 mg/l)</td>
<td>1. Microalbuminuria Urinary albumin excretion rate ≥20 μg/min or Albumin:creatinine ≥30 mg/g</td>
<td>1. Fasting plasma glucose ≥6·1 mmol/l</td>
</tr>
<tr>
<td>2. Central obesity Waist circumference &gt;1020 mm (men), &gt;880 mm (women)</td>
<td>2. Central obesity BMI &gt;30 kg/m² and/or WHR &gt;0·9 (men), &gt;0·85 (women)</td>
<td>2. Central obesity Waist circumference ≥940 mm (men), ≥800 mm (women)</td>
</tr>
<tr>
<td>3. Hypertension Blood pressure ≥130/85 mmHg</td>
<td>3. Hypertension Blood pressure ≥140/90 mmHg</td>
<td>3. Hypertension Blood pressure ≥140/90 mmHg and/or Medication</td>
</tr>
<tr>
<td>4. Hypertriglyceridaemia Triacylglycerols ≥1·7 mmol/l HDL &lt;1·0 mmol/l (men), &lt;1·3 mmol/l (women)</td>
<td>4. Dyslipidaemia Triacylglycerols ≥1·7 mmol/l and/or HDL &lt;0·9 mmol/l (men), &lt;1·0 mmol/l (women)</td>
<td>4. Dyslipidaemia Triacylglycerols ≥2·0 mmol/l or HDL &lt;1·0 mmol/l or Treated for dyslipidaemia</td>
</tr>
</tbody>
</table>

BMI, body mass index; EGIR, European Group for the Study of Insulin Resistance; HDL, high-density lipoprotein; NCEP ATP III, National Cholesterol Education Programme Adult Treatment Panel III; WHR, waist hip ratio. Adapted from: Shaw et al. (2005).

Figure 1.1. Progression of blood glucose and insulin levels in patients with type II diabetes.
Adapted from Champe et al. (2008).
A fairly new, non-invasive technique to assess endothelial function, measurement of flow-mediated dilatation (FMD) (section 1.5.2.1) by ultrasound recordings (Celermajer et al. 1992), has been used within our research group (Hampton et al. 2010; Walters et al. 2006) and others groups (e.g. Bots et al. 2005; Charakida et al. 2005; Clarkson et al. 1997; Donald et al. 2006; Fathi et al. 2004; Sorensen et al. 1995). The assessment of FMD compares the diameter of the brachial artery at rest and at the peak during shear stress-induced dilatation (e.g. Charakida et al. 2005; Clarkson et al. 1997; Fathi et al. 2004; Sorensen et al. 1995). It has been shown that FMD is lower in populations with diabetes or coronary heart disease (CHD) than in healthy populations but that there is a large overlap between these groups (Bots et al. 2005). Moens et al. (2005) suggested that a FMD in healthy subjects is above 7%, whereas in patients with CVD the FMD is 0-5%. However, this technique requires a defined protocol to achieve optimal reproducibility (Donald et al. 2006).

Another functional integrator of cardiovascular function is heart rate variability (HRV) (section 1.5.3.1), defined as ‘the oscillation in the interval between consecutive heart beats as well as the oscillations between consecutive instantaneous heart rates’ (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996). It is commonly assessed by time domain measures derived from an electrocardiogram (ECG), e.g. the SDNN, the standard deviation (SD) of the normal-to-normal (NN) interval between QRS complexes. Mainly sympathetic, but possibly also parasympathetic modulation, is thought to be reflected in the low frequency (LF) power in the frequency domain, while parasympathetic activity is the major component of high frequency (HF) power. The balance between these autonomic systems is reflected by the LF/HF ratio. A lower variance (e.g. lower SDNN) in the ECG and in some studies a shift towards higher sympathetic activation have been associated with CVD (Bucchelletti et al. 2009; Koskinen et al. 2009; Manfrini et al. 2008; Perciaccante et al. 2006; Rabbone et al. 2009; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996; Thayer et al. 2010).
1.2 General epidemiology of sleep duration and metabolic and cardiovascular function

1.2.1 Sleep duration

The first obvious question to ask in a study on sleep deprivation is: how many people are sleep deprived? Some statistics have shown that over 15% of Americans sleep less than 6 h a night (Kripke et al. 2002). In agreement with this, another poll revealed that 15% of respondents (18 years and older) slept less than 6 h on weekdays and 10% slept less than 6 h on weekends over the past year (The National Sleep Foundation 2002). Although these percentages provide interesting information about the distribution of sleep durations, they do not necessarily give an indication about the average sleep duration in a population. The first study reported a modal sleep duration of 8 h and almost half of the sample slept more than 7.5 h (Kripke et al. 2002). The second poll showed that, on average, the respondents slept 6.9 h on weekdays and 7.5 h on weekends (The National Sleep Foundation 2002).

Whereas many studies on sleep duration use subjective self-reported sleep parameters, a few more objective studies have been carried out. As part of the Coronary Artery Risk Development in Young Adults (CARDIA) study, wrist activity monitoring was used in 669 participants as an objective and non-invasive way to assess various sleep parameters (Lauderdale et al. 2006). Although mean time in bed was 7.5 h, average sleep duration was only 6.1 h. Sleep duration (and other parameters) showed race-gender differences, even after adjustment for factors such as socioeconomic status (Lauderdale et al. 2006). The downside of this study, however, was that sleep parameters were only measured during 2 weekdays and 1 weekend day. Therefore, it cannot be assumed that the results reflect subjects' normal sleep patterns.

It may also be of interest to look at sleep need. Percentages of reported insufficient sleep seem to be high in adolescents (15% of the 13 year olds to 25% of the 17 year olds) and again there are differences depending on gender and ethnicity (Dorofaeff & Denny 2006). However, insufficient sleep is something else than sleep duration as these adolescents had a much longer sleep duration that the adults in the studies above, with an average of 8 h and 40 min during the week and 9 h and 23 min during the weekend (Dorofaeff & Denny 2006). Another study on adolescents, showed similar numbers, but also stated that 91.6% of the 12-18 year old students slept less than (but still) 9.2 h per night and that this was related to the amount of extracurricular activities of these
students (Loessl et al. 2008). These studies indicate that it is not possible to simply define sleep deprivation as a set number of hours sleep, but that it is likely to depend on individual characteristics, such as age.

Furthermore, it has been suggested that we nowadays sleep less and less. It has been reported that the percentage of Americans of 18 years and older sleeping less than 6 h in a 24 h period increased over the years, with roughly 15-25% in 1984 and 20-35% in 2004 (Centers for Disease Control and Prevention 2005). However, a British study conducted in 2003 using face-to-face interviews in adults aged 16 years and older in the general population via NOP Omnibus Surveys, showed an average sleep duration of 7.04 h (Groeger et al. 2004). The authors state that there are not many good historical data for comparison but the reliable data suggest ‘that we are not sleeping less than we did but, perhaps, less than we now need’. In agreement with this, the British Time Use Survey using Office for National Statistics Omnibus Surveys, investigated time spent on main activities in 2005 in those over 16 years and showed that on average people slept 8 h on weekdays and 8.8 h during the weekend or 8.2 h on average (Lader et al. 2006). In agreement with this, The American Time Use Survey 2003-2005 in respondents older than 14 years using phone interviews showed that Americans slept (including napping) on average 8.3 h on weekdays and 9.3 h in weekends (Basner et al. 2007). Other authors also suggest that we may not need more sleep, but more free time (Anderson & Horne 2008).

In summary, different studies show different results on the average sleep duration in a population. This can be due to differences in the populations studied e.g. age, gender and ethnicity (see also section 1.8) and prevalence of sleep disorders and diseases. It could also be related to the specific questions asked such as sleep on weekdays compared to sleep on weekend days.

1.2.2 Sleep duration, metabolic and cardiovascular function

The previous section gave an idea about the prevalence of different sleep durations in the population. However, for how many people is short sleep duration associated with adverse effects on cardiovascular and metabolic function? When answering this question, specific conditions such as obesity, hypertension or diabetes can be considered as subsets of risk factors for CVD. The metabolic syndrome, for instance, is
one of the well known clusters of symptoms associated with increased risk for CVD (Shaw et al. 2005) (see section 1.1).

Indeed, recently, various reviews have elaborated on the effects of sleep duration on metabolism, obesity, diabetes and CVD, although the majority of these reviews come from the same group of researchers (Copinschi 2005; Crispim et al. 2007; Knutson et al. 2007; Martins et al. 2008; Mullington et al. 2009; Schultes et al. 2005; Spiegel et al. 2005; Spiegel et al. 2009; Trenell et al. 2007; Van Cauter et al. 2007; Van Cauter & Knutson 2008; Wolk et al. 2005). Generally taken, it is thought that there is a U-shaped association between sleep duration and all-cause mortality. This U-shaped relationship is also found in most, if not all, studies on sleep deprivation and metabolic/cardiovascular function (Alvarez & Ayas 2004; Gangwisch et al. 2006; Sabanayagam & Shankar 2010; Taheri et al. 2004). Sleep deprivation, per se or alongside a medical condition or sleep disorder such as sleep apnoea, is indeed associated with metabolic syndrome and CVD (Spiegel et al. 2009).

In a population of men and women with and without sleep apnea/hypopnea in The Sleep Heart Health Study it was shown that those with a habitual sleep duration of less than 6 h were at higher risk for diabetes mellitus and an impaired glucose tolerance test (Gottlieb et al. 2005). Other aspects of cardiovascular function have also been investigated, for example in the same study but in a larger subject group, it was shown that in those sleeping less than 7 h, the prevalence of hypertension was increased after adjustments were made, also for the prevalence of diabetes mellitus and CVD (Gottlieb et al. 2006). In agreement with this, a recent national health and nutrition study in a population without sleep disorders has shown that sleeping 5 h or less increased the risk for hypertension but only in those aged 32-59 years, not in the oldest age group, even after strict adjustments for factors like alcohol consumption, obesity and diabetes (Gangwisch et al. 2006). Recent results from the National Health Interview Survey in over 31,000 American adults showed that a self-reported sleep duration of < 7 h (or < 5 h in the multivariable model) was associated with a higher risk for CVD even when multivariable adjusted (Sabanayagam & Shankar 2010).

As is the case for the studies above, most epidemiological data include self-reported sleep duration, creating another type of limitation of these studies. The only study to my knowledge using polysomnography (PSG) as an objective method to assess sleep duration showed that amongst people with an habitual sleep of less than 8 h, shorter sleep was associated with a higher body mass index (BMI) and if sleep was less than 5
h, with low leptin levels (Taheri et al. 2004). Unfortunately, PSG was only carried out for 1 overnight and showed higher grehlin levels in those who slept less than 5 h on this night. Both leptin and grehlin results were adjusted for age, sex and BMI. The observation that there may be an independent link between weight gain and short sleep was also shown by a recent meta-analysis (Patel & Hu 2008). However, the association was less clear in adults than in children. A study on the heritability of sleep duration and obesity indicated that self-imposed sleep restriction is associated with elevated BMI, independent of genetics and a common living environment (Watson et al. 2010).

Despite the outcomes from these large studies, some researchers still doubt whether this statistical significance actually implies a clinical risk as very short sleep (5 h) is not so common and most short sleepers are not obese and do not suffer from the metabolic syndrome (Horne 2008). Moreover, many epidemiological studies assess cardiovascular and metabolic function in short sleepers with sleep (associated) disorders which makes it difficult to assess the effects of sleep deprivation per se, even after adjustments have been made. Another factor which compromises interpretation of the results is the already mentioned U-shaped association between sleep duration and various risks. This indicates that sleeping more than 8 or 9 h might also be associated with negative health effects (Youngstedt & Kripke 2004). Indeed some of the previously cited studies on sleep deprivation show aversive health effects of sleeping longer than 8 or 9 h (Gottlieb et al. 2005; Gottlieb et al. 2006; Sabanayagam & Shankar 2010; Taheri et al. 2004). This emphasises that it is very difficult to tease out the various interactions and cause-consequence relationships in epidemiological studies.

There are no epidemiological studies on the association between sleep deprivation and FMD and HRV, apart from studies on the relation between sleep problems and cardiovascular function (e.g. primary insomnia is associated with lower variance within HRV (Spiegelhalder et al. 2010).

1.3 General epidemiology of shift work and metabolic and cardiovascular function

1.3.1 Shift work

One of the reasons for the short sleep duration reported in previous sections may be the demand for shift work. In today's 24/7 society sleep is under high pressure. Self-selected
as well as imposed work-related and social requirements put a heavy burden on sleep timing, duration and quality. The high demand for shift work is illustrated by national statistics showing that in 2009 14.5% of the UK working population (2,892,5000) (ONS Labour Force Survey 2010) was involved in some type of shift work (4,206,000) (ONS Labour Force Survey 2009) (see Table 1.2).

An EU wide study showed slightly higher numbers in 2000 (Boisard et al. 2002). The survey on working conditions carried out in 15 EU countries showed that almost half of the working population was engaged in 'evening or night shifts', 'shift work' or 'alternating shifts' and that 18.8% of the employees had at least 1 night shift a month.

Not only the general definition of shift work may vary, but also within shift work it is difficult to make subgroups as there is an infinite number of combinations of shifts and shift schedules (Costa 2003; Foster & Wulff 2005) also indicated by the fairly large 'other type of shift work' group in Table 1.2. For instance, in 126 train drivers and 104 traffic controllers, 4 combinations of schedules ending in a night shift and 4 ending in a morning shift were possible within 48 hours of irregular shift work (Sallinen et al. 2003). Obviously, these shift schedules strongly influence sleep (see also section 1.7) and napping schedules. One study found that sleep duration and napping before the shift were dependent on the combination and the timing of the shifts (Sallinen et al. 2003). In agreement with this, the timing of the shifts and speed of shift rotation affected sleep length and sleepiness in counter clockwise and clockwise rotating shift schedules (Cruz et al. 2003; Harma et al. 1998; Pilcher et al. 2000). Sleep before a shift was usually significantly shortened, while the occurrence and length of napping before or after a shift was highly dependent on the shift work pattern and other circumstances (Akerstedt 2003). In addition, a wide range of shift work durations has been studied (section 1.3.2).

1.3.2 Shift work, metabolic and cardiovascular function

Previous research has suggested that shift work might not be without risk and it is thought to have consequences for mental and physical health (Costa, 2003). A major concern has been the association of shift work with CVD, which is the primary cause of morbidity and causality nowadays (section 1.1). This is association is supported by animal research showing that 'chronic circadian desynchronisation (reversal of the light-dark cycle) decreased survival by 11% in hamsters with cardiomyopathic heart disease' (Penev et al. 1998).
Table 1.2. Shift patterns\(^1\) for people in employment*  

<table>
<thead>
<tr>
<th></th>
<th>1999</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three-shift working</td>
<td>677</td>
<td>599</td>
<td>542</td>
<td>501</td>
<td>543</td>
<td>501</td>
<td>504</td>
<td>480</td>
<td>454</td>
<td>415</td>
<td>400</td>
</tr>
<tr>
<td>Continental shifts</td>
<td>154</td>
<td>148</td>
<td>164</td>
<td>136</td>
<td>136</td>
<td>124</td>
<td>106</td>
<td>108</td>
<td>100</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>Two-shift system early/late-double day</td>
<td>1,351</td>
<td>1,338</td>
<td>1,249</td>
<td>1,265</td>
<td>1,284</td>
<td>1,265</td>
<td>1,168</td>
<td>1,229</td>
<td>1,242</td>
<td>1,188</td>
<td>1,178</td>
</tr>
<tr>
<td>Sometimes nights sometimes days</td>
<td>525</td>
<td>561</td>
<td>549</td>
<td>551</td>
<td>516</td>
<td>551</td>
<td>561</td>
<td>624</td>
<td>646</td>
<td>625</td>
<td>557</td>
</tr>
<tr>
<td>Split shifts</td>
<td>162</td>
<td>159</td>
<td>163</td>
<td>165</td>
<td>151</td>
<td>165</td>
<td>188</td>
<td>173</td>
<td>182</td>
<td>186</td>
<td>160</td>
</tr>
<tr>
<td>Morning shifts</td>
<td>108</td>
<td>99</td>
<td>97</td>
<td>106</td>
<td>115</td>
<td>106</td>
<td>125</td>
<td>114</td>
<td>123</td>
<td>116</td>
<td>111</td>
</tr>
<tr>
<td>Evening or twilight shifts</td>
<td>243</td>
<td>258</td>
<td>241</td>
<td>256</td>
<td>226</td>
<td>256</td>
<td>250</td>
<td>223</td>
<td>242</td>
<td>227</td>
<td>211</td>
</tr>
<tr>
<td>Night shifts</td>
<td>440</td>
<td>445</td>
<td>430</td>
<td>428</td>
<td>418</td>
<td>428</td>
<td>412</td>
<td>422</td>
<td>386</td>
<td>388</td>
<td>349</td>
</tr>
<tr>
<td>Weekend shifts</td>
<td>47</td>
<td>40</td>
<td>47</td>
<td>74</td>
<td>54</td>
<td>74</td>
<td>67</td>
<td>59</td>
<td>73</td>
<td>78</td>
<td>70</td>
</tr>
<tr>
<td>Other type of shift work</td>
<td>837</td>
<td>951</td>
<td>977</td>
<td>1,009</td>
<td>987</td>
<td>1,009</td>
<td>960</td>
<td>1,012</td>
<td>993</td>
<td>1,022</td>
<td>1,071</td>
</tr>
<tr>
<td><strong>Total shift work</strong></td>
<td><strong>4,546</strong></td>
<td><strong>4,600</strong></td>
<td><strong>4,461</strong></td>
<td><strong>4,493</strong></td>
<td><strong>4,532</strong></td>
<td><strong>4,493</strong></td>
<td><strong>4,361</strong></td>
<td><strong>4,444</strong></td>
<td><strong>4,451</strong></td>
<td><strong>4,350</strong></td>
<td><strong>4,206</strong></td>
</tr>
</tbody>
</table>

* Applies to all respondents in employment who may do shift work. Values are numbers of people in thousands.

Three-shift working, day divided into three working periods: morning, afternoon and night; continental shifts, continuous three-shift system that rotates rapidly; split shifts, full shifts divided into two distinct parts with a gap of several hours in between; morning shift, if full-time: most commonly 0:600-14:00 h; evening shift, if full-time: most commonly 15:00-24:00 h; night shift, if full-time: most commonly 18:00-06:00 h, and usually continuing after midnight; weekend shift, work during Fridays, Saturdays, Sundays (06:00-18:00 h), when there is no other work; other type of shift work, only when none of the above apply.

Adapted from: ONS Labour Force Survey (2009).

A comprehensive meta analysis has shown that shift work is associated with a broad range of changes in metabolic and cardiovascular factors; especially higher levels of TAGs and total cholesterol tended to be more prominent in studies of better quality (Boggild & Knutsson 1999). The authors concluded that SW have an estimated increased risk of CVD of 40%. Because of the completeness of this analysis, only later studies confirming these data or adding some new perspectives will be discussed hereafter.

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\(^1\) In addition to the definitions in Table 1.2 more details on the shift patterns are as follows: three-shift working, this kind of shift work usually, but not always, involves one or more weeks of mornings, followed by one or more weeks of afternoons, followed by one or more weeks of nights; continental shifts, e.g. three mornings, then two afternoons, then two nights; two-shift system, early and late or double day shifts. Usually there is a break between shift changes; two-shift system, normally two shifts of eight hours each, e.g. 06:00-14:00 h and 14:00-22:00 h. Shifts are usually alternated weekly or over longer intervals; split shifts, used in industries where peak demands are met at different times of the day e.g. catering, passenger transport and service industries; morning shift, this code is used if the morning shift is the only shift worked or worked part time during the morning; evening shift, also used for a part-time shift 17:00-21:00 h or 18:00-22:00 h. Part-time evening shifts are usually called twilight shifts; night shift, this code is used only for permanent night work.
Chapter 1  Introduction

CHD was indeed significantly higher in a recent cohort of rotating SW (13.5%) compared with daytime workers (7.1%) (on average almost 15 years of work before the diagnosis) (Ellingsen et al. 2007). Prevalence of coronary artery disease (CAD)/myocardial infarction (MI) or hypertension/brain strokes was about twice as high in SW than in NSW. When investigating CVD risk factors, a later population based cross-sectional study assessing health at 30, 40, 50 and 60 years of age again indicated a higher prevalence of decreased HDL-cholesterol (< 0.9 mmol/L in men, < 1.0 mmol/L in women) (at 30 years of age) in SW, increased TAGs (> 1.7 mmol/L) (in 40 year old men and women and in 60 year old women), but also increased BMI (≥ 30) (in almost all groups) compared to day workers (Karlsson et al. 2001). Female SW had higher total cholesterol levels (except for women who were 30 years old) and impaired glucose tolerance (at 60 years). Other cross-sectional data from a sub-population within the work, lipids and fibrinogen (WOLF) study showed significantly higher fasting insulin (8.2 mU/L compared to 7.5 mU/L in day workers) (~ 49.2 pmol/l compared to 45 pmol/L), a higher percentage of hypertriglyceridaemia (> 1.7 mmol/L), increased waist-hip ratio (WHR) (> 0.9) and lower HDL-cholesterol (< 0.9 mmol/L), but similar prevalence of hyperglycaemia (> 7 mmol/L) amongst SW (Karlsson et al. 2003). These results were presented for all age groups (and both genders) together but there appeared to be some differences between those older than 50 years compared to the younger group. Shift work duration was not reported, only that subjects had to be permanently employed. In addition, the study did not assess at what circadian time blood samples were taken, but that they were taken earlier in SW than in day workers. This is important as differences in circadian phase and not shift work itself may result in changes in the parameters (see section 1.4). Other population-based cross-sectional data showed some additional changes in SW, such as increased BMI (27.1), WHR (0.95), diastolic blood pressure (78 mmHg) and morning blood concentrations of fasting insulin (65.5 pmol/L), HOMA (2.1), TAGs (1.7 mmol/L) and uric acid and a higher leukocyte count (independent of other factors) (Sookoian et al. 2007). Like in many other studies on SW, it was not stated what the history of shift work was and when these blood samples were taken in relation to the shifts.

A cross-sectional study which actually included the duration of shift work, also compared male SW in manufacturing industry (average age 29.1 years) and female nurses (average age 28.5 years) to day workers and assessed shift work duration (Ha & Park 2005). A longer shift duration resulted in increased blood pressure in men, but decreased diastolic blood pressure in women, increased cholesterol in men but decreased in women and an increased WHR in women. There were no effects on fasting blood glucose levels. The authors proposed that the difference between male and
females could be due to the so-called 'healthy-worker effect' in nurses; i.e. those who are healthier can cope for longer with aversive conditions and therefore measurements taken in these subjects are 'healthier'. Gender differences could also be related to a sex-hormone effect.

In a very recent cross-sectional study, a well-defined cohort of rotating SW (on a schedule with 1 or 2 mornings, afternoons and nights and 3 or 4 days off) were compared to day workers (both groups had worked according to their schedules for 10 or more years) (Esquirol et al. 2009). Blood samples were taken from 198 men, at the start of a morning shift in the rotating SW and during the morning in day workers. Significantly higher TAGs (167.07 mg/dL ~ 1.9 mmol/L), free fatty acids (FFAs) (15.64 mg/dL ~ 0.55 mmol/L), fasting glucose (91.7 mg/dL ~ 5 mmol/L) and HDL cholesterol (47.84 mg/dL ~ 1.2 mmol/L) were reported in SW compared to day workers.

Prospective studies also show that the effect of shift work is not universal. At baseline 309 male current rotating SW were at higher risk for CVD compared to 1220 NSW but they were not suffering from the metabolic syndrome (De Bacquer et al. 2009). However after a mean follow up time of 6.6 years and separated by age and years of rotating shift work, it appeared that rotating SW older than 45 years (n = 205) were at risk for the metabolic syndrome (OR 1.36 - 1.82), whereas this only held for SW younger than 45 years if they had worked rotating shifts for more than 10 years (n = 67, OR = 1.61). This study showed that SW were at higher risk for CVD even after adjustment for initial confounding factors. However, the authors did not provide information about the shift work history especially before the baseline measurement. In addition, the group of SW assessed at follow up was not the same as the SW at baseline as some SW had left their jobs which may indicate the 'healthy worker effect'. In agreement with this, a prospective study in a cohort of nurses showed that those nurses who slept 5 h or less, had an increased risk of CHD, non-fatal MI or fatal CHD, of around 90%, even after adjustment for smoking, BMI and other confounders (Ayas et al. 2003b). In the same cohort, the authors also observed an increased risk of diabetes of around 50% for short sleepers (Ayas et al. 2003a). This association remained after adjustment for various factors but the relation to short sleep disappeared after controlling for BMI. However, when severe diabetes was focussed on, the risk remained significant.

By contrast a recent meta-analysis concluded that 'there was limited epidemiological evidence for a causal relation between shift work and ischaemic heart disease (IHD)' (Frost et al. 2009). The authors reviewed 7 papers on death from IHD, 6 on the incidence
and 1 which reported both. Two mortality studies showed an increased risk, whereas 6 studies found a risk around 1. Six out of 7 studies including both fatal and non-fatal incidence data showed a slightly higher risk. However, not all studies were of high quality and had properly described exposure to shift work or controlled for confounders. In addition, in a recent study investigating the effect of shift work on hypercholesterolaemia (> 5.7 mmol/L), the authors concluded that they did not observe a consistent link between shift work and cholesterol levels (Dochi et al. 2008). The relationship was only significant after adjustment for many factors but not after adjustment for age or a few other factors. In agreement with this, a cohort study showed that SW had a significantly higher BMI and WHR than day workers but total cholesterol, LDL and LDL/HDL were only non-significantly higher in the SW. Working shifts for one year, showed only an aversive effect on smoking, whereas BMI and the LDL/HDL ratio decreased in SW, which was a significantly different change compared to day workers (van Amelsvoort et al. 2004).

HRV has been assessed in SW (Togo & Takahashi 2009), but not in many epidemiological studies. Some studies with larger subject numbers observed a lower mean SDNN for all 5 min segments in the recordings (SDNN index) during sleep and work in 103 SW compared to 32 day workers (van Amelsvoort et al. 2000) and a higher normalised low frequency power (LF norm, (LF/(LF+HF) or LF/(total power)) when night shift days were compared to morning shifts days in 65 SW especially in irregular shift systems (Van Amelsvoort et al. 2001a). LF norm and LF/HF were higher after 2 day shifts in rotating nurses compared to nurses who had been on day shifts (Ishii et al. 2005) or on day shifts for at least a year (Ishii et al. 2004). However, van Amelsvoort et al. (2001b) did not find any significant differences in HR, SDNN index and HF when they compared 49 SW with 22 day workers (partly from the same population) after a 1 year follow up. In addition, another study observed no significant differences in LFnorm and LF/HF between 153 male SW and 87 male day workers (Murata et al. 2005). There is only one study to date assessing FMD in SW, but this is a smaller scale field study and will therefore be discussed together with other studies on HRV in Section 1.7.

In summary, the above studies have shown some inconsistent results. This indicates that caution should be taken when drawing conclusions about the risks of shift work as the effects may depend on various factors. From most studies it is not entirely clear what the best predictor for CVD risk is, namely duration, type or timing of shift work. This results largely from the fact that most studies simply do not report these aspects in much detail.
or mention wide shift work ranges or average shift work durations. To illustrate this, the studies on FMD and HRV mentioned in section 1.3.2 and 1.7 included subjects with shift work duration ranges from 0.5 – 37 years (Ishii et al. 2005; Mitani et al. 2006; Munakata et al. 2001) and averages from 3 – 17 years (Amir et al. 2004; Chung et al. 2009; Furlan et al. 2000; Murata et al. 2005; Su et al. 2008). Such ranges and differences in shift work durations were also found in the data on metabolic syndrome and CVD (De Bacquer et al. 2009; Ha & Park 2005; van Amelsvoort et al. 2004) summarised in this section. It is, therefore, unclear after how many years changes in metabolic and cardiovascular function would start to become overt. Some studies suggest no relationship with the duration of shift work at all while others indicate a linear relationship (Boggild & Knutsson 1999). This statement was also reinforced by a recent editorial of Boggild (2009), who proposes that future epidemiological studies should accurately describe the type of shift work, shift work schedules, amount of shift work per month or year and number of years in shift work. He also suggested that differences between studies could be due to the control group used for comparison, selection into, out of shift work, the ‘healthy-worker effect’ (Knutsson 2004) and confusion about the difference between confounding variables (which are not affected by shift work and can be adjusted for) and mediating factors (which can be affected by shift work and do not necessarily have to be adjusted for, e.g. cholesterol). This decision is not always easy to make as some factors, such as smoking, can fall into either of the groups (Boggild 2009; Nabe-Nielsen et al. 2008).

1.4 Sleep and circadian rhythms

1.4.1 Regulation of sleep

There are many brain areas involved in the regulation of sleep and wakefulness, but the core components are thought to be located in the hypothalamus. According to the widely accepted “flip-flop switch” model, this is where the actual transition between sleep and wakefulness takes place (Saper et al. 2005). Briefly summarised, the model proposes a mutual inhibition between monoaminergic cell groups in the brain stem, which are part of the ascending arousal system and the ventrolateral preoptic nucleus (VLPO) in the hypothalamus, promoting sleep. During wakefulness, monoaminergic cells inhibit the VLPO, thereby removing their own inhibition as well as suppression of the orexin neurons, which results in the prevention of sleep. During sleep, however, the VLPO inhibits the orexin neurons and the monoaminergic cells which in turn, causes disinhibition of the VLPO cells.
For a long time it had been thought that sleep was a simple, uniform process. However in the 1980's, it was proposed that sleep is regulated by 2 separate but interacting processes; the 2 process model (Beersma 1998; Borbely 1982; Daan et al. 1984) (Figure 1.2). This model proposes that sleep is driven by a homeostatic component, inducing higher sleep pressure after prolonged wakefulness (process S), as well as an endogenous oscillator with a clear day/night rhythm (process C). The homeostatic component (sleep or process S in the model) is usually compared to an hour glass. As wakefulness is extended, sleep pressure builds up, but at some point, the turning point, sleep occurs and the sleep pressure dissipates again.

Figure 1.2. Schematic representation of the two-process model of sleep regulation. Process S (solid line) and process C (dotted line) interact to regulate sleep (S) and wakefulness (W). Adapted from Beersma (1998).

1.4.2 Regulation of circadian rhythms

The endogenously generated circadian rhythms have a period around (circa) a day (dian). Circadian rhythms are generated by ‘an internal clock’, located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Ralph et al. 1990) and are present even in the absence of external time cues or ‘zeitgebers’. The most important cue for the human circadian system is light (Duffy & Wright 2005), which synchronises the internal rhythms to the 24 h day. Circadian rhythms are most frequently described in melatonin, cortisol, body temperature and sleep/wakefulness (Czeisler et al. 1999) but they also drive the cycles of many other processes in the body, such as metabolic activity and cardiovascular function (section 1.5). This is important to bear in mind since for many functions assessed, the outcome will be (partly) dependent on the time with respect to the internal clock (i.e. circadian time or phase). Although the clock located in the SCN is regarded as the ‘master clock’ it is also known that there are oscillators in peripheral
tissues, so-called peripheral clocks. These are thought to be synchronised to each other and to the master clock (Maywood et al. 2007) and play an important role in metabolic function (section 1.5.1.2).

A way to assess the timing of the clock in the SCN is by assessing the timing of a characteristic point within a circadian rhythm, a circadian phase marker. The most reliable phase marker is thought to be melatonin (Arendt 2003; Klerman et al. 2002), a hormone which is produced by the pineal gland in the brain in response to signals from the SCN. It can be measured in plasma and saliva, of which the latter was done in this project (section 5.2.1). Assessment of melatonin profiles has to be performed in controlled conditions and the collection of samples and analysis of the data requires time. Therefore, an a priori circadian phase assessment can simply be done by looking at wake up time. In volunteers with a regular sleep-wake cycle wake up time is closely related to a reproducible circadian phase assessed by melatonin (Burgess et al. 2003; Revell et al. 2005).

Circadian rhythms are only reliably investigated under certain conditions. The first type of protocol discussed in following sections is a constant routine (CR) protocol first proposed by Mills et al. (1978). In this protocol external factors known to influence the clock are minimised (i.e. no sleep, constant posture, dim light and regular or constant nutrition (Duffy & Dijk 2002)). The second type is a forced desynchrony (FD) protocol (Kleitman & Kleitman 1953) where subjects are usually put on a sleep-wake (or more correct: rest-activity) schedule with a cycle length longer (around 28 h) or shorter (around 20 h) than 24 h but still with 1/3 of the cycle spent asleep. This imposed sleep-wake cycle has a period which deviates too much from the endogenous period (which is a little longer than 24 h in most people (Czeisler et al. 1999)) to remain entrained and will cause circadian rhythms to ‘free-run’ (Blatter & Cajochen 2007; Hiddinga et al. 1997; Wright et al. 2001). Both methods have an ‘unmasking effect’; a CR protocol (high sleep pressure) allows assessment of phase while a FD protocol (low sleep pressure) was originally designed for assessment of endogenous period. A FD protocol has the extra advantage that processes can be evaluated at different circadian phases but with the same time elapsed awake (and for this reason does not induce sleep deprivation).

Therefore, only studies using these CR and FD protocols are taken into consideration when assessing circadian rhythmicity of all parameters in section 1.5.
1.4.3 Output pathways of sleep and circadian rhythms

The above sections described how sleep and circadian rhythms originate and interact. This section focuses on how they control various processes in the body. It is suggested that the SCN regulates its output to the periphery via a dual control mechanism (Kalsbeek et al. 2006a). In essence, this means that signals from the SCN are conveyed both via (neuro)endocrine and neuronal (autonomic) (and intermediate) pathways. These separated, but integrated polysynaptic pathways control multiple outputs such as the classical examples of cortisol, melatonin and body temperature, but likely also metabolic and cardiovascular function via balancing inhibitory (GABA) and excitatory (glutamate) pathways (Buijs et al. 2006; Kalsbeek et al. 2006a; Kalsbeek et al. 2006b; Scheer et al. 2003; Scheer et al. 2001) (Figure 1.3).

Figure 1.3. Review scheme of the various SCN outputs.
Three main types of SCN outputs can be discerned: connections with endocrine, preautonomic, and intermediate neurons. From: Kalsbeek et al. (2006a).

Misalignment of those endogenous biological rhythms with externally imposed schedules (e.g. in night shift work where individuals are often active during the rising phase of melatonin and eat during decreased metabolic activity) is one of the explanations for the above mentioned observations in SW (section 1.3.2) (Boggild & Knutsson 1999; Knutsson & Boggild 2000).
Indeed, it has been shown that SW have a different dietary intake, with a higher energy intake in SW older than 30 years, especially in those with midnight shifts, compared to day workers (Morikawa et al. 2008). In addition, rotating shift workers have been shown to have a higher meal frequency, fewer calories at breakfast and more at the intermediate meals (Esquirol et al. 2009). Moreover, a high energy intake at dinner (time not specified) appeared to contribute to metabolic syndrome. However, after the adjustment for dietary intake the association with shift work remained present. In addition, altered metabolic responses have been observed when the same meal was provided during the night compared to during the day in simulated night shifts and non-adapted SW (section 1.7). Secondly, lifestyle factors such as smoking and alcohol are said to be involved in the changes in metabolic and cardiovascular function observed in SW. And thirdly, disturbances in the homeostatic component of sleep (increased sleep pressure after prolonged wakefulness) may contribute to the observed alterations (Knutsson & Boggild 2000).

Although, there are some proposals as to how the SCN transfers messages to the periphery (e.g. via autonomic innervation), it is less clear how sleep affects the rest of the body. Some mechanistic links have been proposed between sleep and energy balance, but these mainly focus on feeding and appetite (Horne 2009; Tsujino & Sakurai 2009; Vanitallie 2006). As the function of the autonomic nervous system is all about the balance between activity and rest, an interaction between sleep and the autonomic nervous system is logical.

The autonomic nervous system is involved in involuntary actions, thereby affecting cardiovascular and metabolic function in various ways. It controls blood vessel diameter, heart rate, digestion and pancreatic, hepatic and intestinal function (Figure 1.4). The output is determined by the balance between parasympathetic and the sympathetic innervations. The parasympathetic system is active during periods of quiescence ('rest and digest') whereas the sympathetic nervous system is associated with active periods ('fight-or-flight reaction') (Hall & Nicolelis 2001). There are several central networks thought to coordinate these autonomic functions, of which the hypothalamus is a particularly important one. It is thought to 'integrate autonomic response and endocrine function with behaviour' (Iversen et al. 2000). In addition, autonomic outputs may affect each other, e.g. decreased insulin sensitivity and an increased insulin response have been linked to a higher heart rate (Facchini et al. 1996).
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Figure 1.4. The sympathetic and parasympathetic branches of the autonomic nervous system regulate metabolic and cardiovascular function.

As mentioned earlier, it is likely that there are interactions between sleep and the autonomic nervous system. Therefore, it is not surprising that brain areas involved in sleep are close to those regulating autonomic processes such as cardiovascular and metabolic function. For example, the hypothalamus contains the key components regulating the transition between sleep and wakefulness (1.4.1) but it also integrates
autonomic responses. The preoptic area of the hypothalamus for instance, regulates blood pressure, while the tuberomamillary nucleus in the posterior part of the hypothalamus contains histamine producing cells involved in wakefulness and arousal (Iversen et al. 2000). Other anatomical studies have shown that neurons with differential activity during sleep and wakefulness have projections from the lamina terminalis and ventrolateral preoptic area to the lateral hypothalamic area (LHA) and paraventricular nucleus (PVN), which are regions involved in sympathetic activity during arousal (Ushakov et al. 2006).

The wake promoting factor orexin/hypocretin (Saper et al. 2005) in the lateral hypothalamus is possibly the most logical candidate for a common substrate for both sleep and metabolism, also mentioned by others (Horne 2009; Tsujino & Sakurai 2009; Vanitallie 2006). Neurons from the hypothalamus project to fat tissue, the liver and the pancreas (Kreier et al. 2006) and orexin has been shown to stimulate sympathetic neurons innervating these tissues (van den Top et al. 2003), which would lead to, for example, increased glucose mobilisation and altered insulin sensitivity (Shiuchi et al. 2009; Yi et al. 2009). In addition, orexin has been suggested to stimulate sympathetic neurons in the raphe nuclei in the medulla innervating brown adipose tissue resulting in thermogenesis (Berthoud et al. 2005). Orexin containing neurons have also been shown to project to thyrotropin releasing hormone expressing neurons which are involved in vagally mediated gastrointestinal, pancreatic and hepatic functions and to extend into the ventral medulla thereby affecting heart rate (Berthoud et al. 2005).

An increase in cerebrospinal fluid orexin levels in both squirrel monkeys and rats as well as hypothalamic prepro-orexin mRNA levels in rats was regulated by circadian inputs as well as sleep pressure after sleep deprivation (Deboer et al. 2004; Martins et al. 2010; Zeitzer et al. 2007). In addition, the orexin system showed synaptic potentiation after prolonged wakefulness (Tononi & Cirelli 2007). Although the exact mechanism has not yet been defined, these studies indicate that an altered expression of and response to orexin during and after sleep deprivation may affect the balance between sympathetic and parasympathetic innervations, resulting in alterations in metabolic and cardiovascular function.

The interaction between sleep and metabolic and cardiovascular function may also occur via other neurotransmitters or neuromodulators such as adenosine. Adenosine accumulates during prolonged wakefulness in the basal forebrain and promotes the transition from wakefulness to slow wave sleep (SWS), but is also involved in brain
energy metabolism (Porkka-Heiskanen et al. 2003). In addition, elevated levels of adenosine inhibit cholinergic neurons in the preoptic nucleus (Arrigoni et al. 2006) and impair performance on the rat psychomotor vigilance test (PVT) (Christie et al. 2008). However, it is not clear what role adenosine would play in transferring messages from the brain to the periphery.

1.5 Assessed parameters

1.5.1 Metabolism

1.5.1.1 Normal physiology of metabolism

The body takes up fuels in the form of macronutrients: carbohydrate, fat and protein, which are either oxidised or stored (Frayn & Akanji 2003). In the next sections the normal physiology of some subtypes of some metabolites such as glucose, TAGs and NEFAs, and their relation to lipoproteins, cholesterol and insulin will be described. Although these components will be discussed separately, they cannot be seen as independent, as their functioning is highly inter-linked. The connection between the tissues is mainly regulated by nervous and hormone systems. The tissues themselves are innervated by parasympathetic (from the prevertebral ganglia), sympathetic nerves (from the dorsal vagal nucleus) and enteric nerves (Iversen et al. 2000).

So called ‘substrate fluxes’ from and to the tissues are different in the fasting and postprandial states (Figure 1.5). Below, mainly the postprandial processes are described although the general mechanisms hold for both conditions (Frayn & Akanji 2003).

1.5.1.1.1 Glucose

The body obtains glucose as part of a meal and can produce glucose endogenously from stores in the liver. The blood concentrations of glucose depend on the nutritional state but are strictly regulated. During an overnight (10 h) fast (the post-absorptive state), circulating glucose is mainly the result of glycogen break down (glycogenolysis) and gluconeogenesis (from lactate, pyruvate and glycerol), in the liver. The steady state fasting concentration is 3 - 6 mmol/L (Frayn & Akanji 2003; Nussey & Whitehead 2001) in healthy subjects but will vary with age and other factors such as weight.
When a meal is eaten, the glucose concentration rises but is tightly regulated and returns to fasting levels fairly quickly. Glucose absorbed from the diet is first converted (a process termed glycolysis) to lactate in non-hepatic tissue (for example, the gut) and, in the liver, is converted to pyruvate. This end-product (and the intermediates) can be synthesized back into glucose (glyconeogenesis), for example by using lactate as a substrate. After a meal, some of the circulating glucose is immediately used by the splanchnic (hepatic plus gastrointestinal) tissues and muscle and adipose tissue. Most of the circulating glucose, however, is stored as glycogen in muscle and liver or converted to TAGs in adipocytes (DeFronzo 1988; Nussey & Whitehead 2001; Randall et al. 1996).

1.5.1.1.2 TAGs, NEFAs and lipoproteins

TAGs belong to the category of simple lipids (esters of fatty acids with various alcohols), while NEFAs are derived lipids (hydrolysis products of simple or compound lipids). TAGs are primarily used as energy for the body rather than as structural components and are the major source of energy (and the principal component of dietary lipids) in a Western diet (Frayn & Akanji 2003; Griffin 2009). There are various processes in the digestion of
fats, composing a chain of reception, emulsification, lipolysis, solubilisation and absorption of lipids (Griffin 2009) (Figure 1.6). The major processes in lipid homeostasis are lipogenesis and lipolysis. Lipogenesis is the synthesis of lipids (e.g. TAGs) from their constituent free fatty acids (FFAs) or NEFAs; lipolysis results in the production of NEFAs from lipids (Griffin 2009; Vernon et al. 1999).

After an overnight fast, NEFAs are produced by lipolysis of TAGs in adipose tissue and their concentration is around 0.5 – 1 mmol/L. They can be used by tissues such as liver and skeletal muscle as a source of energy via beta oxidation or taken up by the liver and adipocytes to be converted into TAGs. In the fasting state TAGs are again secreted from the liver as very low-density lipoprotein (VLDL) with a typical concentration of 0.5 – 1.5 mmol/L (Frayn & Akanji 2003) (Figure 1.6). The rate of production of VLDL is determined by the delivery of fatty acids (FAs) to the liver, mainly from the circulation in the form of NEFAs (Griffin 2009).

Following ingestion of dietary fat, short and medium chain length FFAs go via the portal circulation (bound to albumins) to the liver. However, long-chain FAs, such as TAGs, and cholesterol have to be solubilised in order to be transported in watery medium like blood. Therefore, lipids and cholesterol associate with mixed micelles and are taken up by the enterocytes where re-esterification into TAGs takes place (Figure 1.6). Subsequently, the water soluble apolipoproteins are formed. TAG-rich apolipoproteins (chylomicrons (CMs), of which the size depends on the fat content of the meal), are released via lymphatic vessels into the blood, resulting in postprandial lipaemia. When measuring serum TAG, in fact TAG associated with TAG-rich lipoproteins are measured. In normal subjects, in the morning, these peak between 3 and 4 hours postprandially and return to basal levels after 5-6 hours. Sometimes, a TAG peak can appear within 30 minutes after ingestion of fat. This is thought to be preformed lipid from a previous meal, released into the circulation by the new arrival of fat. This is why day time postprandial responses can be bi- or polyphasic, whereas those after an overnight fast are usually monophasic (Griffin 2009).

Subsequently, TAG in TAG-rich CM from the intestines and in VLDL from the liver (to a lesser extend in postprandial conditions since chylomicron TAG is preferred substrate) can be lipolysed into FFAs by endothelial lipoprotein lipase (LPL) (in peripheral tissues such as adipose tissue and skeletal muscle) or hepatic lipase (HL) (Frayn & Akanji 2003;
Moreover, hormone sensitive lipase (HSL) is suppressed by insulin resulting in a decreased release of NEFAs from the adipocytes (Frayn & Akanji 2003). In addition, both liver and fat tissues can synthesise FFA *de novo* from acetyl-CoA resulting from catabolism of carbohydrates (Nussey & Whitehead 2001; Vernon *et al.* 1999). However, this process has only a small contribution for humans on a Western diet (Griffin 2009).

Not only chylomicrons and VLDL, but also LDL and HDL belong to the group of lipoproteins, whose function is to produce, transport and release TAGs and remove cholesterol from the circulation (Figure 1.6).

**Figure 1.6. Metabolism of TAGs, NEFAs and lipoproteins.**
The main lipids in lipoproteins are free and esterified cholesterol (C) and triglyceride (TG or TAG). The metabolism of TAG, low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) is shown. In TAG metabolism, hydrolysed dietary fats enter intestinal cells (enterocytes) via fatty acid (FA) transporters. Reconstituted TAG is packaged with C ester into chylomicrons (CMs). CMs circulate until they interact with lipoprotein lipase (LPL). The released free FAs (or NEFAs) enter peripheral cells and are resynthesised into TAG in adipocytes. TAG can in turn be hydrolysed by hormone sensitive lipase (HSL). In liver cells (hepatocytes), TAG is packaged with cholesterol into very low-density lipoprotein (VLDL). The TAG contained in VLDL is also hydrolysed by LPL, releasing FAs and VLDL remnants (IDL) that are hydrolysed by hepatic lipase (HL), thereby yielding LDL. LDL transports cholesterol from the liver (where cholesterol is recycled or synthesized *de novo*) to the periphery. HDL mediates reverse cholesterol transport by uptake of cholesterol and then enters hepatocytes. Adapted from Hegele (2009).
All lipoproteins, apart from HDL, are derived from TAG-rich particles, which deliver FFAs lipolysed from TAGs to the tissues (Griffin 2009; Nussey & Whitehead 2001). This happens by means of a delipidation cascade in which TAG-rich chylomicrons and VLDL undergo lipolysis, resulting in the formation of cholesterol rich remnants and ultimately LDL (Figure 1.6). The more lipid is lost, the smaller, but heavier and denser the particle (Frayn & Akanji 2003; Griffin 2009). HDL particles are produced by the liver and the gut and increase their size when they obtain cholesterol from TAG-rich proteins and peripheral tissues. HDLs are the only particles which can remove cholesterol from the circulation. This is done in the form of cholesterol esters (CEs) in HDL which is transported to the liver. Intra-cellular cholesterol can either be synthesised by the liver cells or obtained by uptake and degradation of LDL particles. LDL is mainly taken up by the LDL-receptors of cells and uptake decreases with a higher intracellular cholesterol level (Griffin 2009).

Thus, the levels of serum TAGs and lipoprotein function are highly interlinked. When serum TAG levels and thus TAG-rich lipoprotein concentrations are increased, a net transfer of TAGs into HDL and LDL takes place. The high TAG concentration makes them sensitive to HL resulting in smaller, denser particles. Small, dense HDL is rapidly catabolised by the liver, leading to lower serum HDL levels and impaired CE transport (lower HDL cholesterol). Small, dense LDL is removed less effectively by its receptor and is more likely to be oxidised in the arterial wall (Griffin 2009). These observations in TAGs, LDL and HDL cholesterol are known as the atherogenic lipoprotein phenotype (ALP) (see also section 1.1) (Griffin 2009).

1.5.1.1.3 Insulin

Insulin forms an important link within this interconnected system as it plays a role in the synthesis and removal of TAGs, NEFAs and glucose. Insulin is secreted by the β cells of the islets of Langerhans in the pancreas. The major stimulus for this secretion is an elevation in blood glucose concentration, for example after consumption of a meal. The main target tissues for insulin are liver, muscle, adipose tissue and to a minor extent the kidneys (Evans et al. 2004; Frayn & Akanji 2003; Randall et al. 1996) (Figure 1.7).

Insulin mainly has an anabolic function. It controls glucose homeostasis through suppression of endogenous glucose production and glycogen breakdown and stimulation
of glycogen synthesis and glycolysis by the liver as well as stimulation of glucose uptake by the splanchnic tissues, muscle and adipose tissue (DeFronzo 1988; Frayn & Akanji 2003; Randall et al. 1996). This process is influenced by feedback from adipocytokines such as adiponectin, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6).

![Figure 1.7. The role of insulin and its target tissues in glucose and lipid homeostasis.](image)

Glucose derived from diet or endogenous sources stimulates insulin secretion. Insulin promotes glucose uptake by skeletal muscle and fat, opposes hepatic glycogenolysis and gluconeogenesis, and inhibits fat lipolysis. Free fatty acids liberated from adipose tissue contribute to insulin resistance in skeletal muscle and liver. Additional fat-derived signals, including TNF-α, resistin and adiponectin, modulate insulin sensitivity and fatty acid metabolism in muscle and liver. Adapted from Evans et al. (2004).

Tissues which are sensitive to insulin are the liver and adipose tissue. Insulin inhibits intracellular lipolysis of stored TAG in adipose tissue by inhibiting HSL and oxidation of FAs in the liver (Evans et al. 2004; Frayn & Akanji 2003; Griffin 2009) and it stimulates LPL in adipose tissue, thereby activating removal of TAGs from the plasma (Frayn & Akanji 2003; Griffin 2009; Moustaid et al. 1996). It also stimulates TAG synthesis by the liver and adipose tissue and de novo lipogenesis by the liver (Frayn & Akanji 2003). In addition, insulin suppresses VLDL release from the liver (also due to a decreased NEFA release) and stimulates cholesterol synthesis. Therefore, in cases of insulin resistance,
lipoysis of TAG-rich particles is impaired and mobilisation of NEFAs and VLDL release are less suppressed, contributing to postprandial lipidaemia (Griffin 2009).

Other factors that affect the regulation of macronutrient metabolism are glucagon, catecholamines (epinephrine and norepinephrine), cortisol, growth hormone (GH), insulin-like growth factors, thyroid hormones and leptin (Frayn & Akanji 2003).

1.5.1.2 Metabolism, sleep and circadian rhythms

As discussed in section 1.4.3, circadian rhythms underlie cyclic processes in many bodily functions. This is also the case for metabolic function (Green et al. 2008; Laposky et al. 2008). Other evidence indicating that the SCN and circadian rhythms are linked to metabolism comes from polymorphisms in clock genes that have been associated with alterations in the endogenous rhythmic regulation of metabolism in mice (Turek et al. 2005) and humans (Monteleone et al. 2008; Scott et al. 2008; Sookoian et al. 2008; Woon et al. 2007). Metabolic activity has indeed been shown to have both a circadian and sleep-homeostatic component, but the extent to which either of these contribute may vary depending on the parameter and study looked at (Laposky et al. 2008; Morgan et al. 2003).

A controlled study in eight healthy men, with constant dextrose infusion during nocturnal sleep, nocturnal sleep deprivation and diurnal sleep, showed 3 acrophases for glucose during these 3 parts of the protocol (2.5 to 5 h after sleep onset) but lower elevation during sleep restriction suggesting that circadian and sleep influences are superimposed (Van Cauter et al. 1991). The insulin secretion rate (ISR) and insulin levels paralleled these changes. Although ISR levels at the acrophase were equally high during sleep and sleep deprivation, no consistent acrophase for insulin was observed during sleep deprivation. The authors suggested that constant nocturnal insulin concentrations despite a rise in the secretion rate indicated circadian variation in insulin clearance, with a higher insulin disposal rate at night. Results of this study are in agreement with a later study which also showed a stable circadian rhythm in glucose levels during a CR but a more variable, though significant, rhythm in insulin (Shea et al. 2005). Both parameters showed a peak around the normal time of awakening. A study using a FD protocol with CRs before and after FD and hourly nutrition, showed that glucose and TAGs (and insulin non-significantly) are modulated by sleep and the endogenous clock (Morgan et
al. 1998). NEFAs were too variable to assess. Other studies have indicated diurnal rhythms in insulin sensitivity. For instance a study by the same group, although not a CR protocol, demonstrated lower postprandial LPL activity around 23:30 h than 11:30 h suggesting decreased sensitivity of lipid clearance to insulin in the evening (Arasaradnam et al. 2002). Furthermore, these researchers showed that glucose levels after an insulin tolerance test (ITT) decreased slower at 20:30 h than at 8:30 h (Morgan et al. 1999). This coincided with higher fasting NEFA levels but a steeper decline in post-ITT NEFA levels in the evening than in the morning. The authors suggested that the glucose data are indicative of peripheral insulin insensitivity. By contrast the post-ITT NEFA levels suggest an increased sensitivity of HSL to insulin in the evening, suppressing the release of NEFAs. The authors propose that the combination of these data indicates a dissociation between the diurnal effects of insulin on carbohydrate and lipid metabolism and a NEFA-induced insulin resistance in the evening.

The presence of circadian rhythms (also in other aspects of metabolism such as energy expenditure or carbohydrate oxidation) does not only depend on the study and investigated parameter but also on the exact composition of the meal and pre-meals. When men where provided with 6 isocaloric meals across a 24 day, either high in fat or high in carbohydrates, circadian patterns were observed in glucose and NEFAs, but in TAGs only with the high fat diet (Holmbak et al. 2002). Therefore, the possible effect of circadian rhythms in metabolic activity will also play a role, but to what extent remains unclear.

1.5.2 Endothelial function

1.5.2.1 Normal physiology of endothelial function

The endothelium is the layer of cells in the tunica intima, lining the lumen of all blood vessels. In order to deliver the appropriate amount of blood to a tissue, arteries have to be able to dilate and constrict. Arteries expand in response to various stimuli such as hormones, carbon dioxide, lactic acid and nerve impulses (Chiras 1999). The latter have a parasympathetic and sympathetic component. The sympathetic nervous system increases peripheral resistance by constricting small arterioles (increasing blood pressure), whereas parasympathetic stimulation has a smaller contribution and induces vasodilatation by mediators such as nitric oxide (NO) (Iversen et al. 2000).
Arteries can also dilate simply due to the flow of blood itself (Nussey & Whitehead 2001) (Figure 1.8). This is called FMD and is an endothelium-dependent process. It is mainly brought about by shear-induced endothelial NO production, due to a temporary increase in blood flow: reactive hyperaemia (Fathi et al. 2004; Moens et al. 2005). A lower FMD is associated with deteriorated endothelial function (section 1.1).

**Figure 1.8. The genesis of FMD, in response to different changes in shear stress.**

* very short-term changes; ** changes taking place over slightly longer periods (minutes); *** changes taking place over a longer time (many minutes or hours).

EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide (NO) synthase; Kc, calcium-activated potassium channel; PGI₂, prostacycline. Adapted from Moens et al. (2005).

1.5.2.2 Endothelial function, sleep and circadian rhythms

As endothelial function is considered a predictor for CVD (section 1.1), changes in rhythms in endothelial function were assessed in relation to cardiovascular risk (Shaw et al. 2001). In addition, most studies investigated diurnal variation in factors linked to endothelial function other than FMD, such as thrombotic factors (Walters et al. 2003). Not much is known about circadian rhythms in endothelial function measured by FMD.
There is only one study to date indicating that endothelial function assessed by FMD in CR conditions was lower in the morning and higher in the evening (Walters et al. 2006). FMD was also measured without CR conditions at 21:00 h, 6:00 h and 11:00 h but in supine position and after at least 4 h fast. (Otto et al., 2004). In agreement with the data from Walters et al. (2006) this protocol showed that FMD was decreased in the early morning compared to 11:00 and 21:00 h. Endogenously generated rhythms in vascular function might result directly from the activity of the SCN i.e. due to cycles in parasympathetic and sympathetic activity or indirectly via circadian rhythms in other factors which influence endothelial function. Examples of such interactions are melatonin acting on vascular melatonin receptors or increased blood flow, insulin and endothelin-1 stimulating vasoactive mediators like NO and endothelium-derived hyperpolarizing factor (EDHF) which affect platelet aggregation, vessel relaxation and cell adhesion molecules like E-selectin (adapted from Walters et al. 2003).

1.5.3 Heart rate variability

1.5.3.1 Normal physiology of heart rate variability

HRV is defined as 'the oscillation in the interval between consecutive heart beats as well as the oscillations between consecutive instantaneous heart rates' (section 1.1) (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996). The heart beat originates from the sinoatrial node (SA node), which is capable of spontaneous depolarisation, called the pacemaker potential. The generated action potential spreads through the atria via gap junctions and travels through the atroioventricular node (AV node) via the bundle of His into the Purkinje fibers in the ventricular walls. This causes the ventricles to contract and blood to be pumped into the circulation (Fox 1996). Heart rate and force of contraction are influenced by the balance between activity of parasympathetic nerves (from the ventrolateral nucleus ambiguus) and sympathetic nerves (from the cervical ganglia), mainly through stimulation by (nor)epinephrine and inhibition by acetylcholine (Iversen et al. 2000).

The depolarisation and repolarisation of all myocardial cells combined produce a potential difference between two electrodes placed on the surface of the heart (or anywhere else on the body) which can be displayed in an ECG. The different phases of the cardiac cycle are known as P, QRS and T waves (Figure 1.9) (Levick 2003). The exact shape of the ECG depends on the orientation of the heart, the position of the electrodes and any other conditions such as individual physiology or medical conditions
In order to assess HRV, it is common practice as a first step to detect QRS complexes from the ECG, which mainly reflect ventricular depolarisation. Subsequently, various HRV parameters can be derived of which the most commonly used ones are SDNN and LF/HF (section 1.1).

**Figure 1.9. The basic pattern of electrical activity across the heart including the schematic representation of a QRS complex.**

From: Ashley and Niebauer (2004).

### 1.5.3.2 Heart rate variability, sleep and circadian rhythms

Biological rhythms are found in cardiovascular function (Walters *et al.* 2003) (section 1.5.2.2). However, it is less clear what the relative contribution of circadian rhythms and exogenous factors (e.g. changing body posture) is. In rats there is a clear circadian rhythm in heart rate that is not caused by activity (Scheer *et al.* 2001). This rhythmicity is only present when the SCN is intact and it is thought to be regulated by a multisynaptic pathway containing neurons projecting from the SCN to the PVN and on to preganglionic sympathetic and parasympathetic nuclei of the heart (Scheer *et al.* 2003; Scheer *et al.* 2001). In agreement with this, studies in humans have shown that circadian rhythms are likely to be present in some of the HRV parameters. A circadian rhythm was shown in SDNN with a peak between 4:45 and 7:00 h (Bonnemeier *et al.* 2003; Vandewalle *et al.* 2007; Viola *et al.* 2002) although the study of Bonnemeier did not use a CR protocol. In addition, peaks were seen at 6:15 h for LF and 5:16 h for HF (Vandewalle *et al.* 2007),
but not in other HRV parameters such as LF/HF (Vandewalle et al. 2007; Viola et al. 2002).

### 1.5.4 Sleep, alertness and mood

For a long time, it has been thought that sleep is a simple, uniform process. However, after the introduction of the electroencephalogram (EEG) in the 1920's which allowed visualisation of electrical activity of the cerebral cortex (Saper 2000) by recording voltage differences between electrodes on the scalp, it became clear that sleep is comprised of different stages characterised by differences in the frequencies and amplitudes of the voltage differences in the EEG. Later, other measurements such as respiratory effort, the ECG, eye movements in the electrooculogram (EOG) and muscle tone in the electromyogram (EMG) were included in polysomnography (PSG), which was given its name in 1974 (Dement 2005). Initiation, depth and termination of sleep are regulated both by circadian rhythms and sleep pressure (section 1.4.1). Although the function of sleep is still largely unknown, inadequate quality, quantity and timing of sleep can have consequences for metabolic and cardiovascular function (sections 1.2.2 and 1.3.2). In addition, both alertness and mood are influenced by sleep; section 1.6 discusses that sleep deprivation has a large impact, especially on alertness. As assessment of the effects of sleep deprivation and shift work on mood and alertness are not the main focus of this thesis, these will only be briefly discussed in this and following sections.

The alert state is defined as ‘the change that occurs when a person awakens or when a person who could not respond to commands becomes able to respond to commands.’ Furthermore, ‘alertness is thought to be a family of states that differ by the degree of alertness (heightened attention, indifference, inattention, sleepiness)’ (Kandel 2000a). More specifically, alertness is referred to as ‘selective and sustained attention’, whereas the term sleepiness may be used for ‘the desire to sleep’ (Roehrs et al. 2005). Performance, a term which is often mentioned when discussing alertness and sleepiness, refers to ‘cognitive functions ranging in complexity from psychomotor vigilance and working memory to logical reasoning and complex thought’. There are numerous ways of assessing objective and subjective alertness (Roehrs et al. 2005), of which the subjective Karolinska Sleepiness Scale (KSS) (Akerstedt & Gillberg 1990) and 9-digit rating scale and objective psychomotor vigilance test (PVT) (Dinges & Powell 1985) were used in this project.
The neural basis of alertness lies, generally speaking, in activation of the thalamus and cortex by neurons of the brain stem and its reticular formation. The major neurotransmitter systems, e.g. cholinergic, dopaminergic, serotonergic and noradrenergic neurons are involved in the regulation of alertness (Kandel 2000a). In accordance with this, good performance on the PVT involves the sustained-attention network and cortical and subcortical motor areas, whereas slow reaction times (RTs) have been associated with higher activation of 'default mode' brain areas, indicating inattention (Drummond et al. 2005). A positron emission tomography (PET) study in sleep deprived subjects also indicated that the thalamus and prefrontal and posterior parietal cortices may play a role in alertness and cognitive performance (Thomas et al. 2000).

Mood, 'in clinical descriptions of emotional states ... refers to a sustained emotional state lasting weeks or more, while the term affect (or affective response) refers to the immediate or momentary state of a person' (Kandel 2000b). In this thesis these terms will, although not strictly correct, be used interchangeably. Amongst the normal affective responses are some parameters assessed in this project, such as elation and depression (Kandel 2000b) Calmness and cheerfulness may possibly strictly speaking not be affective responses but are here regarded as aspects of mood. As holds for alertness, aspects of mood have a neural and physiological basis. In the mood disorder familial unipolar and bipolar depression, for example, activity of the subgenual region of the frontal cortex is affected and antidepressants act on the serotonergic and noradrenergic pathways. In severe depression there are disturbances in the hypothalamus-pituitary-adrenal (HPA) axis with an excessive secretion of adrenocorticotropic hormone (ACTH) having extensive physiological consequences (Kandel 2000b).

1.5.4.1 Sleep, alertness, mood and circadian rhythms

One of the earliest studies into circadian rhythms in alertness used a CR protocol (40 h wakefulness) to assess this phenomenon and a FD protocol (9.33 h sleep, 18.67 h wakefulness for approximately 4 weeks) (Dijk et al. 1992) to separate the effects of circadian modulation from prior wakefulness (section 1.4.2). Both protocols showed that subjective alertness (by visual analogues scale (VAS)) and calculation performance closely followed the rhythm in core body temperature (CBT). Both alertness and performance increased for the first 3 h after waking, then remained stable for 13-14 h.
and subsequently decreased with minimum alertness and performance around or just after, respectively, the CBT minimum usually approximately 2 h before habitual wake up time. The data also showed that the effect of prior wakefulness was strongest around the CBT minimum and weakest around the CBT maximum. A later study using a FD protocol for only 12 days showed similar results (Wright et al. 2002). Effects of time awake and circadian phase were shown on subjective alertness (VAS) and most of the PVT parameters: slowest 10% reaction time (SRT), fastest 10% reaction time (FRT) and lapses (RT > 500 ms) but not median reaction time (median RT). Lowest alertness and performance levels were observed around the CBT minimum. A FD protocol with lower sleep pressure (shorter rest-activity cycle of 20 h for 4 weeks) also showed an effect of time awake on and circadian modulation of median RT, lapses and KSS (Wyatt et al. 1999). In addition, circadian modulation increased with more hours of prior wakefulness. These findings were also confirmed in a CR protocol, which demonstrated that subjective alertness (KSS) and PVT performance (mean reaction time (RT), median RT, SRT and FRT) were constant during the first 16 h after waking but then deteriorated and were worst 2-4 h after the melatonin peak (which is around 4 h before habitual wake up time) (Cajochen et al. 1999). Largest changes were revealed by SRT and the smallest differences by FRT. A stable performance during approximately 16 h of wakefulness and a deterioration after that was also seen in young and postmenopausal women (Urrila et al. 2007).

In agreement with these observations, a longer FD protocol (6 weeks) investigating objective alertness showed that all 3 assessed PVT parameters, mean RT, lapses and SRT were regulated in a circadian manner (Lee et al. 2009). The protocol also showed effects of time awake on the PVT parameters. In addition, mean RT and lapses showed a stronger circadian modulation of the effect of wakefulness at 60°, just after the CBT minimum at 0°. In contrast to the previous studies, mean RT and SRT increased from 6 h awake, not 16 h, whereas lapses increased continuously. A worsening PVT performance across the 6 weeks, even when returning to ‘normal’ circadian phase, indicated a build up of sleep debt despite a normal sleep-wake ratio.

Also by using a ‘nap protocol’ (10 cycles of 75 min scheduled sleep and 150 min scheduled wakefulness) in order to create identical time elapsed awake between measurements in addition to elimination of the effects of sleep deprivation, it was shown that subjective sleepiness (KSS) displayed a circadian rhythm (Cajochen et al. 2001). Maximum sleepiness was observed just after the CBT minimum. When objective alertness in the nap protocol was compared with a CR protocol, it was confirmed that
lapses, SRT and FRT showed circadian modulation and that lapses and SRT were affected by prolonged wakefulness (they increased between 23.75 and 35/38.75 h of sleep deprivation in the CR protocol) (Graw et al. 2004). However, FRT appeared not to be sensitive to sleep deprivation.

Another nap or ultra short sleep/wake protocol (around 18 cycles of 1 h sleep, 2 h wake) also showed circadian rhythms in sleepiness (Stanford Sleepiness Scale (SSS)) and PVT parameters (mean RT, median RT, FRT and 1/SRT (SRRT)) as well as in mood (Profile of Mood States Questionnaire (POMS), and total mood disturbance (TMD) (Kline et al. 2010)). In accordance with previous findings, a trough in PVT performance and, in this study in, mood was observed between 0° (intra-aural body temperature minimum) and 30° and largest sleepiness around the temperature minimum (330 - 60°). FD protocols also showed a circadian rhythm in mood (VAS scales for cheerfulness and happiness) but the authors state that 'the time at which the circadian variation of mood reached its trough depended on the duration of prior wakefulness' (Boivin et al. 1997).

These studies indicate that mood and both subjective and objective alertness/sleepiness are modulated by the circadian as well as the homeostatic component. It appears that during the first 16 h of wakefulness subjective and objective alertness are fairly stable but deteriorate if wakefulness is prolonged beyond this, resulting in an alertness dip around the minimum body temperature and worst performance just after. The circadian dip in alertness has been shown to be worsened as wakefulness is more prolonged and sleep deprivation gets more severe.

1.5.4.2 Correlation between subjective and objective sleepiness

As people generally feel they are slower and less accurate when they are sleepy, it is striking that measures of subjective and objective sleepiness do not correlate as well as expected. When a dose-response relationship between hours of sleep in the previous night and either sleepiness (SSS) or PVT performance was investigated, it also appeared that although both improved in a saturating exponential manner with more sleep, subjective sleepiness improved more slowly than PVT performance (Jewett et al. 1999). Another study found significant differences between subjects in the number of lapses during 40 h sleep deprivation whereas sleepiness levels according to the SSS were not different (Van Dongen et al. 2004). These results indicated that there is no clear correlation between subjective and objective sleepiness. Similarly, sleep onset latency
on the multiple wakefulness test for objective alertness decreased substantially in the worst performers whereas their SSS scores were not affected (Frey et al. 2004). In agreement with this, Fanzen et al. (2008) also observed independence between lapses and speed on one hand and subjective sleepiness on the other hand (Franzen et al. 2008). In addition, the VAS for global vigor score has been shown not to correlate with computerised RT and lapses under CR conditions (Leproult et al. 2003).

### 1.6 Laboratory studies on sleep deprivation

Whereas the previous sections described epidemiological studies on sleep duration, this section describes laboratory studies on healthy individuals experimentally subjected to sleep deprivation. Only human studies assessing the same parameters as the current study are included in Table 1.3 and Table 1.4 and are discussed in the subsequent sections. Studies on alertness and mood are not included in the tables as this was not a major topic within this thesis. In addition, processes occurring during sleep deprivation are not discussed here as they are discussed in the sleep/circadian sections of each parameter.

#### 1.6.1 Total Sleep Deprivation (TSD)

In studies on TSD, subjects are kept awake for at least one complete night. The main studies on the effects of TSD on metabolic and cardiovascular function are summarised in Table 1.3.

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Protocol</th>
<th>Parameters measured</th>
<th>Changes after TSD</th>
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</thead>
<tbody>
<tr>
<td>Ewing (1991)</td>
<td>8 M</td>
<td>DN</td>
<td>48 h</td>
<td>Baseline TSD Recovery</td>
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<tr>
<td>Gonzaliz-Ortiz et al.</td>
<td>28, 14 exp and</td>
<td>DN</td>
<td>24 h</td>
<td>no</td>
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Table 1.3. Overview of laboratory studies on the effects of total sleep deprivation on cardiovascular and metabolic function.
<table>
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<tr>
<th>Study</th>
<th>Subjects</th>
<th>Protocol</th>
<th>Parameters measured</th>
<th>Changes after TSD</th>
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<td>Base line</td>
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<td>Recovery</td>
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<td>no</td>
<td>60 h</td>
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<td>Ilan et al. (1992)</td>
<td>64 M</td>
<td>7 h / 1 night</td>
<td>76-80 h</td>
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<td>Pagani et al. (2009)</td>
<td>12 F, 12 M</td>
<td>7</td>
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<tr>
<td>Sauvet et al. (2009)</td>
<td>12 M</td>
<td>8 h / 2 nights</td>
<td>40h</td>
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### Study Subjects

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<th>Changes after TSD</th>
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<td>Base line TSD Recovery</td>
<td>gluc, -glucagon</td>
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<td></td>
<td></td>
<td>C-peptide, (nor)epinephrine</td>
<td>-glucagon: basal and HGC</td>
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<td></td>
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<td>-ACTH, -GH, lactate</td>
<td>but stronger increase</td>
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<td>-β-hydroxybutyrate</td>
<td>during end of HGC when</td>
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<td></td>
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<td>-NEFAs, -hunger, -PSG</td>
<td>normalised to basal</td>
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<td>-basal cort ↓</td>
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<td>-normalised HGC GH ↑</td>
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<td>-overall NEFA ↓, sign at end of HGC</td>
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<td>-basal hunger ↑</td>
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<td>-C-peptide, -cort</td>
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<td>-ISR</td>
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<td>10 M DN 60 h 7 h / 1 night</td>
<td>OGGTT</td>
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<tr>
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<td>7 M DN h/3 nights 120 h DN h / 4 nights</td>
<td>muscle total chol, free chol, non-collagen protein, LCAT</td>
<td>-rhythm in plasma TAG dampened</td>
</tr>
<tr>
<td></td>
<td></td>
<td>serum total chol,</td>
<td>-serum chol had no rhythm but level ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAGs, total protein,</td>
<td>-LCAT ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>haematocrit</td>
<td>-muscle total and free chol ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>All reversed by rec</td>
</tr>
<tr>
<td>Zhong et al. (2005)</td>
<td>19 M, 2 no 12, 14 and 36 h</td>
<td>SBP</td>
<td>-Changes in physiology</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DBP</td>
<td>dependent on supine or seated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAP</td>
<td>position and on amount of TSD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HR</td>
<td>-RT ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-(LF/HF) HRV</td>
<td>ESS ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-(LF/HF) BPV</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BRS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NN</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ESS</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations and symbols used:** ↓ = decreased or smaller, ↑ = increased or larger, Ach, acetylcholine; ACTH, adrenocorticotropic hormone; AP, arterial pressure; apo, apolipoprotein; AST, amino transferase; ALT, alanine amino transferase; BP, blood pressure; BRS, baroreflex sensitivity; chol, cholesterol; cort, cortisol; DBP, diastolic blood pressure; DN, data not available; EOG, electrooculogram; ESS, Epworth Sleepiness Scale; F, female; GH, growth hormone; gluc, glucose; HDL, high-density lipoprotein; HF norm, normalised high frequency power (HF/(LF+HF)) in the electrocardiogram (ECG); HGC, hypoglycaemic clamp; HR, heart rate; HRV, heart rate variability; hs-CRP, high-sensitivity c-reactive protein; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin-6; IL- β, interleukin- β; ins, insulin; ISR, insulin secretion rate; IST, insulin
Chapter 1 Introduction

suppression test; KSS, Karolinska Sleepiness Scale; LCAT, lecithin cholesterol acetyltransferase; HRV LF/HF, low frequency power/high frequency power in the ECG; (LF/HF) BPV, (low/high frequency modulation of) blood pressure variation; LF norm, normalised low frequency power (LF/(LF+HF)) in the ECG; M, male; MAP, mean arterial blood pressure; NEFA, non-esterified fatty acid; NN interval, normal-to-normal interval (between adjacent QRS complexes in the ECG); NN50, NN intervals longer than 50 ms in the ECG; OGGTT, oral glucose tolerance test; PSG, polysomnography; rec, recovery; RT, reaction time; SBP, systolic blood pressure; SNP, sodium nitroprusside; SSG, steady state glucose; T3, triiodothyronine; TNF-α, tumor necrosis factor-α; TSD, total sleep deprivation; TAG, triacylglycerol; BRS, baroreflex sensitivity; VCAM-1, vascular cell adhesion molecule-1; VLDL, very low-density lipoprotein. 'no' for the baseline nights indicates that no adaptation / baseline nights were spent in the laboratory; this does not mean that regular sleep/wake cycles at home have not been adopted prior to the laboratory study.

1.6.1.1 Metabolic and cardiovascular function

The effects of TSD on metabolic and cardiovascular function are summarised in Table 1.3. An early protocol with isocaloric meals every 3 hours showed that 3 to 5 days of TSD resulted in a significant dampening of the TAG rhythm (Vondra et al. 1986). This dampening was reversed on day 3 of the recovery period (but no data were provided for the recovery day 1 and 2). Serum total cholesterol did not show a rhythm but the levels were significantly lower during day 4 and 5 of sleep deprivation. During recovery sleep, levels remained lower but not significantly so. In agreement with this, another study showed that 76-80 h TSD resulted in a significant decrease of TAG levels from 165 ± 12 to 143 ± 14 mg/dL (~1.9 to 1.6 mmol/L), but a significant increase in HDL from 46.4 ± 4.4 to 54.6 ± 4.2 mg/dL (~1.2 to 1.4 mmol/L) (Han et al. 1992). In this study, however, food intake was ad libitum and body posture was not controlled.

Although there are differences in glucose levels during nocturnal sleep and wakefulness (section 1.5.1.2), mean glucose levels between 8:00 and 11:00 in the morning were not different after a TSD night compared with a night with sleep (Van Cauter et al. 1991). In addition, the total plasma glucose response to an oral glucose tolerance test (OGTT) was not different but the insulin response was higher after 60 h of TSD (VanHelder et al. 1993). On the other hand, steady state glucose levels assessed by a modified insulin suppression test (IST) were 18% higher after 24 h TSD compared to after a night of normal sleep within the same subjects, while metabolic characteristics such as TAGs and cholesterol were not different between the sleep deprived and the control group (Gonzalez-Ortiz et al. 2000). However, the timing and duration of 'normal sleep' in this
study were not described and subjects were allowed to move freely. Another study did not find any effects on glucose and insulin levels but observed an overall decrease in NEFA levels at hypoglycaemic clamp (HGC) after sleep deprivation compared to 7 h sleep (Schmid et al. 2007).

The effects on cardiovascular function highly depend on whether the subjects were supine or seated. In both conditions, NN intervals decreased over the course of sleep deprivation (Zhong et al. 2005). Heart rate decreased after 12 and 36 hours in a supine position but increased after 24 hours in a seated position. LF and HF did not change in the supine position while in a seated position, LF increased after 12 and 24 hours, HF decreased after 12 hours and the LF/HF ratio increased after 12 hours (Zhong et al. 2005). These assessments were made within subjects and not compared to a baseline night, so they may be due to circadian rhythms in HR and HRV (section 1.5.3.2). However the authors state that no changes were observed in 6 control subjects who underwent the same measurements, but then slept at home (Zhong et al. 2005). Others have observed a lower NN50 during 48 h sleep deprivation (Ewing et al. 1991) and a longer NN interval after one night TSD (Pagani et al. 2009). Another study also showed increased LFnorm but decreased HF norm (normalised high frequency power (HF/(LF+HF)) after 40 hours of total sleep deprivation in 12 men, but only at 15:00 h and 18:00 h compared to the same time on the day before (after normal sleep) (Sauvet et al. 2009). By contrast, one study reported no difference in LF/HF throughout a 60 h sleep deprivation period (Gould et al. 2009). Sauvet and coworkers (2009) also assessed microvascular reactivity by endothelium-independent vasodilatation (skin blood flow measurements after sodium nitroprusside solution administration) and endothelium-dependent vasodilatation (skin blood flow measurements after acetylcholine administration). Endothelial function by endothelium-dependent vasodilatation was lower at 12:00 h after sleep deprivation (and lower at 12:00 h compared to 18:00 h both after baseline sleep and TSD). Endothelial function by endothelium-independent dilatation was lower after sleep deprivation at both times.

The data suggest that one night of total sleep deprivation is sufficient to impair metabolic and cardiovascular function. However, the effects appear to depend on the parameter measured and the time at which the parameter was assessed throughout the course of and after TSD.
1.6.1.2 Alertness and mood

As the relationship between sleep deprivation and alertness and mood has not been addressed in sections 1.2.2 and 1.3.2, this will be introduced briefly in this section. Studies into the circadian and homeostatic modulation of alertness and mood in discussed in section 1.5.4.1 have already shown that alertness and mood are affected by prolonged wakefulness. The studies in this section and section 1.6.2 focus on the sleep-deprived versus non-sleep deprived condition and the effect of recovery sleep. There is ample research outside CR and FD settings showing that sleep deprivation affects alertness (Rogers et al. 2003). Amongst the various aspects which are covered by the term alertness, vigilant attention is commonly investigated as it is thought to be important for all other cognitive tasks and is very sensitive to sleep deprivation (Lim & Dinges 2008). A meta-analysis by the same authors showed that the largest effects of 24 – 48 h TSD were observed on simple attention, such as lapses and RT often assessed by the PVT developed by their group (Lim & Dinges 2010).

Although it is clear that sleep deprivation results in decreased alertness, the impact on mood seems to be bidirectional. There are not that many studies on the effects of restricted sleep on mood in healthy volunteers but in most people, sleep deprivation has been shown to increase negative mood (Pilcher & Huffcutt 1996). On the other hand, sleep deprivation has been reported to be a very effective treatment for depression (Giedke & Schwarzler 2002; Hemmeter et al. 2010; Ringel & Szuba 2001).

The following studies are some examples to illustrate what has been stated in these reviews. An experiment which compared sleep deprived to non-sleep deprived subjects observed a negative effect of 1 night of TSD on subjective mood and alertness (by VAS and Positive and Negative Affect Schedule (PANAS)) (but no effect on negative affect on the PANAS) (Franzen et al. 2008). One night TSD also decreased objective physiological alertness and increased the harmonic mean RT and frequency of lapses (Franzen et al. 2008) as well as simple RT and sleepiness on a 0-10 point scale or the Epworth Sleepiness Scale (ESS) (Pagani et al. 2009; Zhong et al. 2005). Furthermore, deterioration in subjective sleepiness (VAS) and RT (PVT) following 40 h sleep deprivation did return to baseline after 1 night with 9 h recovery sleep but not after 5 nights 6 h sleep (Jay et al. 2007). Additional findings from this group showed that 2 TSD nights resulted in a recovery for SSS after 1 night of 9 h recovery sleep but PVT performance did not even recover after 2 nights (Lamond et al. 2007). Moreover, 2 or more nights of TSD in healthy individuals increased sleepiness and induced changes in
some aspects of mood, such as anxiety and depression but not in others (Gould et al. 2009; Kahn-Greene et al. 2007). Taken overall, these studies indicate that TSD impairs alertness and some aspects of mood.

1.6.2 Partial Sleep Deprivation (PSD)

While during TSD, volunteers do not sleep at all, during partial sleep deprivation (PSD) volunteers are kept awake only part of the night or for several consecutive nights. Table 1.4 summarises the most relevant studies on PSD.

Table 1.4. Overview of laboratory studies on the effects of partial sleep deprivation on cardiovascular and metabolic function.

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Protocol</th>
<th>Parameters measured</th>
<th>Change after PSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Base line</td>
<td>PSD</td>
<td>Recovery</td>
</tr>
<tr>
<td>Buxton et al. (2010)</td>
<td>20 M</td>
<td>10 h / 3 nights</td>
<td>5 h / 7 nights, 10 h / 1 night</td>
<td>IVGTT (SI, AIG, DI, KG, So), HEC (ins resistance), RMR, cort, -(nor)epinephrine, KSS, lapses</td>
</tr>
<tr>
<td>Donga et al. (2010)</td>
<td>5 M / 6 F</td>
<td>8.5 h</td>
<td>8.5 h compared to 4 h (cross over)</td>
<td>gluc, NEFA, ins, glucagon and cort during basal and clamped state, HEC, PSG</td>
</tr>
<tr>
<td>Kerkhofs et al. (2007)</td>
<td>10 F</td>
<td>1 night</td>
<td>4 h / 3 nights, 8 h / 1 night</td>
<td>leukocytes, total chol, LDL, HDL, TAG, Apo A and B, hsCRP, more</td>
</tr>
<tr>
<td>Muenster et al. (2000)</td>
<td>6 M / 4 F</td>
<td>4 h / 4 nights</td>
<td>no</td>
<td>RMSSD, baroreflex, SBP, HR, orthostatic control</td>
</tr>
<tr>
<td>Nedeltcheva et al. (2009)</td>
<td>5 F / 5 M</td>
<td>5.5 h</td>
<td>no</td>
<td>weight, OGTT, IVGTT</td>
</tr>
<tr>
<td>Study</td>
<td>Subjects</td>
<td>Protocol</td>
<td>Parameters measured</td>
<td>Change after PSD</td>
</tr>
<tr>
<td>------------------</td>
<td>----------</td>
<td>----------</td>
<td>---------------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Schmid <em>et al.</em> (2009)</td>
<td>10 M</td>
<td>4.5 h comp compared to 7 h (cross over)</td>
<td>-PSG Basal and under HGC: -gluc -ins -C-peptide -glucagon -epinephrine -ACTH -cort -GH</td>
<td>-reduced ins sensitivity and gluc effectiveness at basal ins -dampened and delayed cort rhythm when looking at best fit curve -lower GH during 1st 4 h of night, only in M -overall epinephrine ↑, night time (nor)epinephrine ↑ -5 h 11 min sleep</td>
</tr>
<tr>
<td>Spiegel <em>et al.</em> (1999)</td>
<td>11 M</td>
<td>4 h / 6 nights 12 h / 7 nights</td>
<td>-sleep -GCR (after IVGTT) ~ R₄ -GE ~ S₀ -ins resistance (after IVGTT) -gluc and ins after meals -LF/HF -thyrotropin -thyroxin index -cort -SSS Compared to rec but not to baseline</td>
<td>-3 h 49 min sleep, awakenings and REM, nREM ↑ -GCR ↓ -GE ↓ -ins response to gluc (IVGTT) ↓ -gluc and ins after breakfast ↑ -LF/HF ↑ -mean thyrotropin ↓ -thyroxin index ↑ -cort in afternoon and evening ↑ and ↓ of free cort slower -SSS sleepiness</td>
</tr>
<tr>
<td>Spiegel <em>et al.</em> (2004)</td>
<td>11 M</td>
<td>8 h / 3 nights (8 h / 7 nights 1 year later)</td>
<td>-sleep -leptin -rNN -cort -TSH -gluc -ins -HOMA -subjective stress Compared to recovery but not to baseline</td>
<td>-sleep, awakenings and REM, nREM ↑ -leptin mean, max and amplitude ↓ -rNN ↑ -cort decline ↓, acrophase value lower, evening levels ↑, nadir later, elevation faster -TSH mean and amplitude ↓ -gluc after breakfast ↑ -ins after breakfast slightly ↑ -HOMA after breakfast ↑ -leptin, cort and HOMA profiles from 8h sleep were intermediate between 4h and 12h</td>
</tr>
</tbody>
</table>
Table 1.4. Effects of partial sleep deprivation on metabolic function and heart rate variability.

<table>
<thead>
<tr>
<th>Study</th>
<th>Protocol</th>
<th>Parameters measured</th>
<th>Change after PSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van Leeuwen et al. (2010)</td>
<td>8 M control / 15 M in exp</td>
<td>8 h / 2 nights</td>
<td>Change after recovery gluc, IGF-1 in experimental group</td>
</tr>
<tr>
<td></td>
<td>4 h in cont, exp / 5 nights</td>
<td>-gluc</td>
<td>-ins, -IGF-1, -leptin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 h / 2 nights</td>
<td>-ins/gluc, -leptin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>after recovery gluc, IGF-1</td>
</tr>
</tbody>
</table>

Abbreviations and symbols used: ↓ = decreased or smaller, ↑ = increased or larger, ACTH, adrenocorticotrophic hormone; AIRq, insulin response; apo, apolipoprotein; AUC, area under the curve; chol, cholesterol; cort, cortisol; DI, disposition index; EGP, endogenous glucose production; F, female; GCR, glucose clearance rate; GE, glucose effectiveness; GH, growth hormone; GIR, glucose infusion rate; gluc, glucose; HDL, high-density lipoprotein; HEC, hyperinsulinemic euglycemic clamp; HGC, hypoglycaemic clamp; HOMA, homeostatic model assessment; HR, heart rate; hsCRP, high sensitivity c-reactive protein; IGF-1, insulin-like growth factor-1; ins, insulin; IVGTT, intravenous glucose tolerance test; KSS, Karolinska Sleepiness Scale; Kq, glucose tolerance; LDL, low-density lipoprotein; LF/HF, low frequency power/high frequency power in the electrocardiogram (ECG); M, male; NEFA, non-esterified fatty acid; NN interval, normal-to-normal interval (between adjacent QRS complexes in the ECG); nREM, non-rapid eye movement sleep; OGTT, oral glucose tolerance test; PSG, polysomnography; Rd, rate of glucose disposal; rec, recovery; REM, rapid eye movement sleep; RMR, resting metabolic rate; RMSSD, square root of the mean of the squares of differences between NN intervals in the ECG; rNN, autocorrelation coefficient of NN intervals; SBP, systolic blood pressure; S0, glucose effectiveness; SI, insulin sensitivity; SSG, steady state glucose; SSS, Stanford Sleepiness Scale; SWS, slow wave sleep; PSD, partial sleep deprivation; TAG, triacylglycerol; TSH, thyrotropin. ‘no’ for the baseline nights indicates that no adaptation / baseline nights were spent in the laboratory; this does not mean that regular sleep/wake cycles at home have not been adopted prior to the laboratory study.

1.6.2.1 Metabolic function and heart rate variability

The effects of PSD on metabolic and cardiocasual function are summarised in Table 1.4. There is only a small number of published studies which have investigated the effects of PSD on metabolic function. Spiegel et al. (1999) studied 11 young men who had 3 baseline nights (bl 1-3) with 8 h time in bed, 6 nights with 4 h (centred around the normal midpoint of sleep, PSD 1-6), followed by 7 recovery nights with 12 h in bed (rec1-7). Subjects had to stay in bed in the clinic during the last 2 days of each of the 3
conditions and they consumed carbohydrate rich (62%) meals every 5 h (i.e. at 9:00, 14:00 and 19:00 h). On PSD5 and rec5, an intravenous glucose tolerance test (IVGTT) was carried out at 9:00 h (and thus the breakfast was omitted) and on PSD6 and rec6 24 hour profiles of glucose and hormone concentrations were obtained. NN intervals and sleepiness were also recorded. Glucose clearance after the IVGTT was 40% slower and the acute insulin response to glucose was 30% lower after sleep deprivation compared to recovery sleep. After the breakfast, the glucose response was higher despite a slightly higher insulin response after sleep deprivation compared to recovery sleep. According to Nedeltcheva et al. (2009) this indicates that insulin secretion is affected but not insulin sensitivity. There were no differences in the responses after lunch and dinner (Spiegel et al. 1999). The LF/HF ratio was higher after PSD than after baseline sleep and recovery sleep which, according to the authors, could explain the impaired glucose breakdown after sleep deprivation. A downside of the study was the fact that the IVGTT and response to breakfast were not reported after baseline sleep, so these implications cannot be tested. A later study by the same group using the same protocol (and possibly the same subjects but this is not clear from the methods section) showed very similar results indicating that leptin, cortisol and homeostatic model assessment profiles after baseline sleep were intermediate between those after sleep deprivation and recovery sleep (Spiegel et al. 2004). Another study did not find significant effects of PSD (4 h sleep a night for 4 nights) on HRV as assessed by the root mean square of sequential differences of NN intervals (RMSSD) (Muenter et al. 2000). A similar recent study found comparable results to those of Spiegel and colleagues (1999) in 15 young men when they were subjected to 2 baseline nights (8 h), 5 PSD nights (4 h time in bed) and 3 recovery nights (8 h) and compared to a control group who spent 10 h in bed across the 10 days (van Leeuwen et al. 2010). In the experimental group but not in the control group, higher basal insulin levels and a higher basal insulin/glucose ratio were observed after sleep deprivation (insulin: 8.6 ± 7 mU/L ~ 51.5 pmol/L, ratio: 1.8 ± 1.3) compared to baseline sleep (insulin: 6 ± 2.9 mU/L ~ 36 pmol/L, ratio: 1.2 ± 0.57). Basal glucose levels were lower after recovery sleep (4.7 ± 0.17 mmol/L) compared to baseline sleep (4.9 ± 0.24 mmol/L) in the experimental group. However, physical activity was not restricted, food intake was 50 kcal higher in the experimental group and the paired t-tests done between either PSD or recovery sleep and baseline sleep are not appropriate. In agreement with these findings, IVGTT and hyperinsulinemic euglycemic clamp in an inpatient study in 20 men showed decreased insulin sensitivity (20%), disposition rate and glucose tolerance after 7 nights of 5 h in bed compared to 3 nights of 10 h in bed (Buxton et al. 2010). In addition, fasting glucose and insulin levels and insulin secretion were not changed. Another recent study, investigated 6 women and 5 men (39.5 ± 5
years) when they were on a 14 day schedule of either 5.5 h or 8.5 h time in bed (randomised cross over), with a sedentary life style and ad libitum food intake (apart from the last days of each condition when energy intake was controlled) (Nedeltcheva et al. 2009). During both conditions their weight increased by 2 kg. The 5.5 h time in bed condition resulted in increased glucose values 2 h after the oral glucose bolus and an increased area under the curve (AUC) after the OGTT. This was not the case for insulin. Indices for insulin sensitivity (SI) were reduced and glucose effectiveness (Sg) at basal insulin levels was increased. These results suggest, that despite a similar weight gain (and ad libitum food intake), sleep restriction results in a decreased glucose tolerance and insulin sensitivity.

In addition, Kerkhofs and colleagues observed significant increases in cholesterol and LDL after 3 days PSD (4 h sleep per night) but no difference in TAGs and HDL (Kerkhofs et al. 2007).

Other authors have aimed to investigate the effects of a single night of PSD (4 h time in bed (TIB)) compared to 8.5 h TIB on insulin sensitivity by hyperinsulinaemic euglycemic clamp study (Donga et al. 2010). PSD did not affect basal glucose (during labelled glucose infusion), NEFAs, insulin, glucagon, cortisol or endogenous glucose production. During the hyperinsulinaemic clamp study, steady state glucose and insulin did not differ between the sleep conditions but endogenous glucose production increased, the rate of glucose disposal decreased, the required glucose infusion rate decreased and plasma NEFA levels increased after PSD, all parameters with a magnitude around 20%, indicating decreased insulin sensitivity after PSD.

The only study that did not show any effects of sleep deprivation on glucose tolerance and insulin sensitivity was one by Schmid et al. (2009). A night of PSD affected neither basal glucose and insulin levels nor their response during hypoglacaemic clamp.

As there is also debate as to whether sleeping too long is as harmful as sleeping too little, one study investigated the effect of TIB restriction (Zielinski et al. 2008). Volunteers (n = 42) aged 50-70 years olds with an average sleep duration of ≥ 8.5 h were asked to reduce their TIB by 90 minutes for 8 weeks. Fasting glucose was only 1% higher after reducing TIB and fasting insulin, glucose tolerance, postprandial insulin and insulin sensitivity did not change.
Other recent studies have investigated the role of different sleep stages and nocturnal (micro)arousals rather than sleep as a whole on cardiovascular and metabolic function. One study, for instance, showed that suppression of SWS, without a reduction in total sleep time (TST), resulted in decreased insulin sensitivity which was not compensated by an increase in insulin release (Tasali et al. 2008). Glucose tolerance was also decreased. The authors suggested that this was due to a relative increase in sympathetic activity (assessed by HRV), rather than HPA axis activity (assessed by cortisol). In subjects working in IT, it was shown that those with a higher arousal frequency during the night, had higher morning cortisol, HR, DBP, total cholesterol, LDL and LDL/HDL ratio (Ekstedt et al. 2004). SBP and HDL were not so clearly affected.

1.6.2.2 Endothelial function

Endothelial function has been measured in groups of people with different types of cardiovascular problems but not in many populations with different kinds of sleep patterns. Evidence for the effect of sleep deprivation on endothelial function (either after TSD or PSD and either with ultrasound recordings or another method) is lacking. There are only 2, poorly controlled field studies. In 30 male college students FMD was significantly decreased from 7.4 ± 3 to 3.7 ± 2.3% after 4 weeks of chronic sleep deprivation (sleep < 80% of the normal amount) during exam preparations (Takase et al. 2004). However, this period was also considered as chronic stress, which may confound the results. A study on patients with obstructive sleep apnoea showed that FMD was significantly lower in sleep apnoea patients than in controls both in the morning (6.04 ± 3.18% vs 10.9 ± 2.6%) and in the afternoon (10.38 ± 4.23% vs 13.9 ± 2.32%) (Ofiaz et al. 2006). However, actual sleep was not mentioned in these patients. Within the group of OSAS patients, there was no difference between those with or without metabolic syndrome (Amra et al. 2009).

1.6.2.3 Alertness and mood

Alertness and mood are not only affected by TSD (section 1.6.1.2) but also by PSD. PSD (5 h a night, 7 nights) resulted in an increase in sleepiness on the SSS and in most of the subscales of the profile of mood states (POMS) but not depression (Dinges et al. 1997). Subjective mood and alertness worsened after the first night of PSD compared to
baseline sleep and returned to near baseline levels after 1 night of recovery sleep (10 h in bed) and complete recovery after 2 nights of sleep. FRT decreased across PSD whereas the number of lapses increased. Both FRT and lapses only changed after 2 nights of PSD whereas SRRT already changed after 1 night. As for the subjective measures, it took 2 days of recovery sleep for PVT performance to return to baseline levels. PSD only affected VAS ratings alert-sleepy and mental exhaustion and calmness but not others such as happy-unhappy. A similar protocol that also allowed 5 h TIB for 7 nights (Buxton et al. 2010) showed an increase in PVT lapses and KSS sleepiness after the first PSD night. Sleepiness on the SSS also increased after 4 h sleep for 6 nights (Spiegel et al. 1999). Another study showed as well that PVT lapses and KSS sleepiness deteriorated significantly throughout 5 days of sleep restriction (4 h sleep per night) compared to a control group who slept 8 h each night (Haavisto et al. 2010). Lapses and KSS were not significantly different between the groups after the first 8 h recovery night, but it took 2 nights of 8 h recovery sleep for lapses to fully return to baseline levels. In agreement with this, a semi-laboratory study (subjects were allowed to leave the laboratory for part of the protocol) showed an increase in KSS sleepiness and in RT and lapses after the first night of PSD (4 h sleep a night for 5 nights) which did not return to baseline levels after 1 night of recovery sleep and lapses did not even recover after 7 days (Axelsson et al. 2008). Similarly, another semi-laboratory protocol which allowed subjects to go home between sleep and assessment periods also observed an increase in lapses, median RT, FRT and SRT after PSD (6 h sleep for 8 nights) (Vgontzas et al. 2004)). PSD (4 h sleep per night for 12 nights) did not only increase fatigue but also decreased physical well-being and other aspects of emotional well-being such as optimism-sociability (Haack & Mullington 2005).

Another type of FD protocol than mentioned in sections 1.4.2 and 1.5.4.1 (with a cycle of 42.85 hours with 32.85 h of extended scheduled wakefulness and 10 h of scheduled sleep), induced both acute (in contrast to previous FD protocols) and chronic sleep deprivation. This study showed that acute sleep loss was dissipated during the 10 h time in bed while the build up of chronic sleep loss resulted in a faster deterioration of median RT across the hours awake (Cohen et al. 2010). Moreover, in support of the previous findings, the circadian rhythm in performance (with a dip between 0° (melatonin peak) and 60° corresponding to 3:00 - 7:00 h) was stronger the longer subjects had been awake and in the chronic sleep deprivation condition. Similar time of day influences on the effects of sleep deprivation have also been shown by another recent study as the decrement in PVT lapses and subjective sleepiness was most affected around 8:00 h but less between 16:00 and 20:00 h (Mollicone et al. 2010).
Another study showed decreased alertness (KSS) and multitask performance after one night with 2 h of sleep compared to a night with 8 h sleep (Sallinen et al. 2008). Both did not fully recovery after 1 night of recovery sleep. A 10 min rest improved multitasking and KSS but this was not more effective in the sleep debt condition. Subjective sleepiness (SSS) was significantly higher after a night of 2 h in bed and reversed to baseline levels after a nap plus 8 h recovery sleep or 10 h recovery sleep. However, 8 h recovery sleep was only sufficient at 13:00 but not 15:00 h on this day (Faraut et al. 2010).

These studies indicate that mood and alertness are negatively affected in partially sleep deprived conditions and that the length and distribution of recovery sleep influences the extent of reversal of mood and alertness levels to baseline. The findings suggest that subjective alertness may recover faster than objective alertness.

1.7 Smaller scale studies on shift work

There are several ways of assessing what shift work does to cardiovascular and metabolic function; either in the field in 'real' SW, via simulated shift work, or by a combination of the two methods.

1.7.1 Field studies of shift work

1.7.1.1 Metabolism

The effects of 'real shift work' were studied in SW on Antartica who were on a schedule of 7 day shifts (time in bed 00:00-8:00 h), 7 night shifts (time in bed 8:00-16:00 h) and again 7 day shifts. Acrophase determined by urinary 6-sulphatoxymelatonin showed that subjects delayed into the shift protocol (Lund et al. 2001). However, the delay in acrophase did not reach the 8 h by which sleep was delayed by the end of the night shifts and did not advance again into day shifts, causing the SW to be constantly out of phase from day 9 onwards. Blood samples were taken before and after a test meal at 13:30 h on day 1 and day 16 and at 1:30 h on day 9. Fasting glucose, insulin, TAG and NEFA levels were similar on all study days. However, postprandial glucose, insulin and TAG levels (by AUC) were increased on the second night of night shifts while NEFA AUC was smaller on the second night shift. As the authors propose, this was possibly due to decreased glucose tolerance and insulin sensitivity at night (section 1.5.1.2). The
increased insulin response may be responsible for the suppressed NEFA response (section 1.5.1.1). Whereas glucose and insulin levels decreased to normal levels upon returning to day work, TAG levels remained higher and NEFA levels remained lower. The authors suggest that these data show that altered postprandial responses take place in those who are maladapted. Furthermore, glucose and insulin responses had returned back to normal after returning to day work while the delay in melatonin acrophase was reaching significance then, reflecting an even larger desynchronisation. According to the authors this may be due to time awake overriding clock-related changes since for some parameters the clock component may be stronger than for others (see also section 1.4).

1.7.1.2 Heart rate variability

When data on cardiac function were compared at the same time of day (suggestive of similar circadian phase), LF norm and LF/HF were higher and RMSSD and the number of NN intervals longer than 50 ms (NN50) (means across 24 h or only the night) were lower when SW were on night duty compared to when they were off duty and asleep at night (Ewing et al. 1991; Furlan et al. 2000; Ito et al. 2001; Rauchenzauner et al. 2009; Su et al. 2008). LF/HF was also significantly higher and HF norm was lower during sleep from 3:00 – 5:00 h on 24 h duty than at the same time when off duty in ambulance men (Mitani et al. 2006). Even on days off LF and LF/HF were higher during night time sleep in permanent night nurses compared to day nurses when body posture and work were controlled (Chung et al. 2009). By contrast, other authors did not observe differences in variance, LF norm and LF/HF between 24 h recordings including either a night shift or a day shift (Freitas et al. 1997).

1.7.1.3 Endothelial function

Endothelial function in SW has been investigated only in a single study (Amir et al. 2004). In this study, 30 physicians with a shift work history of 5 ± 3 years (range 0.5 to 15), had one FMD assessment between 8:00 and 10:00 h on a working day without previous night shift (baseline day) and a second assessment between 8:00 and 10:00 h after a continuous workday of 24 h, including a night shift. Baseline FMD was lower in those who had worked shifts for more than 3 years (8.6 ± 3.4%) than in those with a shorter shift work history (14.3 ± 4.1%). FMD was also significantly smaller after a night shift (6.7 ± 4.8%) compared to the baseline day (10.5 ± 4.5%). Multivariate regression showed that a larger decrease in FMD after shift work was associated with longer shift
work duration, less sleep during the shift, higher baseline FMD and younger age (Amir et al. 2004). Although these results indicate that shift work may cause endothelial dysfunction, caution has to be taken when drawing this conclusion. The authors did not provide details about the shift schedules and whether the measurements after a night shift were always taken after 1 night shift or after more consecutive night shifts. This also makes it difficult to assess whether both the baseline FMD measurement and the measurement after the night shift have been taken at the same circadian phase.

Indirectly related to this, Hattori and Azami (2001) state that the von-Willebrand factor is an early marker of endothelial injury. Both this factor and blood glucose 3 h after the last meal were higher the morning after night work compared to the morning after a day shift, whereas systolic blood pressure was lower (Hattori & Azami 2001). Other differences depended on what time the shift ended. The authors claim that this demonstrates endothelial injury in taxi drivers. However, the study was poorly controlled, e.g. food intake and movement were not comparable between day and night shifts.

1.7.1.4 Alertness and mood

Ample research over the last decades has consistently shown that shift work including night shifts affects sleep, sleepiness and performance (Akerstedt 1998, 2003; Akerstedt & Wright 2009; Harma et al. 1998). Although earlier studies indicated that shift length and rotation have little effect on sleep and mental and physical health (Smith et al. 1998; Tucker et al. 2000), more recent studies suggest that the sleep obtained in SW may be highly dependent on the combination and order of shifts and length of the working day and week (Harma et al. 1998; Sallinen et al. 2003; Sallinen & Kecklund). In irregular SW, for example, sleepiness is highest on morning shifts and night shifts. Important factors affecting sleepiness were shift length and duration of the main sleep period (Harma et al. 2002). In accordance with this, subjective alertness and RT (by assessments other than KSS, VAS and PVT) was lower at 3:00 than at 15:00 and 19:00 h in rotating SW (Galy et al. 2008). Sleep and sleepiness are also affected in other conditions similar to shift work, such as jet lag. In airline pilots, for example, self-rated fatigue was predicted both by prior sleep and the type of intercontinental flight (Petrilli et al. 2006).

By contrast, there are not many comprehensive studies on the effects of shift work on mood. A recent study showed that rotating SW have a higher odds ratio for depression, even after adjustment for several factors such as age and educational level. However,
the association was no longer present when corrected for work-related issues such as support and job demand (Driesen et al. 2010). These findings indicate that it may be mainly the flexibility aspect of shift work that influences job satisfaction, irritability and anxiety (Costa et al. 2006).

1.7.2 Simulated shift work studies

1.7.2.1 Metabolism

A simulated shift work semi-laboratory study was conducted by Ribeiro et al. (1998). Twelve volunteers were subjected to a protocol consisting of 4 days regular sleep/wake cycle, 5 days of gradual advance and 6 days back to the regular sleep/wake cycle. Blood samples were taken before and after a test meal at 13:30 h (postprandial) during the baseline period, immediately after the gradual advance and after the return to a regular sleep/wake cycle, achieving sampling at different times of the body clock. No differences were observed in insulin and glucose. However, lower basal and postprandial NEFA levels were found immediately after the phase shift compared to before and 2 days after. In addition, basal TAG levels were lower but postprandial TAG levels (with basal levels subtracted) were higher after the phase shift than before and 2 days after, depending on the time points of the postprandial response considered. Both NEFAs and TAGs also returned to basal levels slower after the phase shift. The authors suggest that the increased TAG levels may be due to a lower LPL activity. The results from this protocol were different from those emerging from a previous, very similar, study also from the Surrey laboratory (Hampton et al. 1996), which compared postprandial response to a test meal at 13:30 h after baseline sleep and after a simulated phase shifting period (after approximately 25.5 h of wakefulness). In this study an increased postprandial glucose and insulin response and a higher insulin/glucose ratio were observed after the simulated phase shift suggestive of insulin insensitivity. A delayed postprandial TAG response was demonstrated, again indicative of lower LPL activity; the delayed NEFA response implied an increased suppression of HSL by the hyperinsulinaemia. The authors mentioned that the different fat content of the pre-meal (before the test meal) or elapsed time awake could account for the discrepancy between the two studies. The study by Ribeiro et al. (1998) used a low-fat (4 %), high carbohydrate (92 %) pre-meal (as opposed to 27.5 % fat in the study by Lund (2001) and 49 % fat in the study by Hampton (1996)), which may have prevented the shift-induced elevation in glucose and insulin responses. Nevertheless, both data sets show that the
response to a meal depends on the internal body clock time. Differences in postprandial responses were also observed when a snack was included at 16:00/4:00 h next to main meals at 13:30/1:30 h and 19:00/7:00 h, in subjects that were entrained to day shifts (Al-Naimi et al. 2004). The TAG response was significantly higher at night, with the largest difference after the snack. The insulin response was larger after the last meal but smaller after the first meal. Glucose and NEFA responses were not significantly different at night compared to during the day.

In another protocol, using FD to investigate various processes at different circadian phases, subjects were given breakfast, lunch, dinner and a snack and blood was sampled hourly (Scheer et al. 2009). This study showed that, when subjects were 12 hours out of phase (on the 4th day of the FD) they had higher glucose levels (especially 3 h after breakfast) even though their insulin was also raised. They also had increased mean arterial pressure but there was no effect on heart rate or HRV.

1.7.2.2 Alertness and mood

In support of the findings from field studies, a simulated shift work study showed that reaction times (median RT, 25th and 75th percentile RT) were slower, lapses (> 90th percentile of the daytime baseline) were more frequent and the VAS score of sleepiness was higher on the first night shift compared to a day shift and subsequent night shifts (Santhi et al. 2007). These parameters also significantly deteriorated across a night shift but not during a day shift.

1.7.3 Simulated shift work combined with field studies of shift work

To my knowledge there is only one study, comparing SW and NSW in controlled laboratory conditions (Simon et al. 2000). In this study 24 h rhythms in glucose and ISR (under continuous enteral nutrition and recumbent position) in 8 night workers (6 nights a week for at least 2 years) were compared to 8 NSW with nocturnal sleep on one occasion and 8-h shifted sleep on the second occasion. The data showed that mean 24-h glucose and ISR were the same in the 3 conditions and that they were mainly regulated by sleep but also by a circadian component. The night shift workers were partly adapted as the glucose and ISR peaks shifted with 8 hours as did their sleep so that the peaks remained at mid-sleep and the glucose and ISR peak and nadir levels were not different from NSW. However, the adaptation was not complete as the sleep
associated rise in glucose had only shifted by 6 hours and the ISR was prolonged. The night shift workers also showed some differences in ultradian oscillations.

In a study on shift work and sleepiness, SW followed simulated shift schedules (Eriksen et al. 2006). In the trained navigators in a 6 h on/6 h off sea watch simulator, sleep was reduced and sleepiness increased during the night and early morning shifts and on days off.

1.8 Interindividual differences in the tolerance to sleep deprivation and shift work

As for all biological processes, it is likely that there are interindividual differences in the tolerance to sleep deprivation and shift work. Previous sections have shown that the effects of sleep deprivation may depend on factors such as age, gender and ethnicity (section 1.2.2 and 1.3.2). Other studies have shown interindividual differences in neurobehavioural aspects such as variation in the effects of sleep deprivation on alertness and performance, not necessarily related to these factors (Van Dongen & Belenky 2009). Some studies (section 1.5.4.2) found significant differences between subjects in the number of lapses during 40 h sleep deprivation, with some subjects being very affected whereas others were not (Van Dongen et al. 2004). Similarly, when subjects were divided into resistant or vulnerable to sleep deprivation according to their increase in SRT during the selection sleep night, it appeared that during a subsequent sleep deprivation night, resistant subjects were able to maintain a fairly stable RT whereas vulnerable subjects showed an increase in RT, while both groups felt equally sleepy (KSS) (Galliaud et al. 2008). These interindividual differences in performance persisted even within a group of F-117 pilots self-selected to be sleep deprived as a result of their jobs (Van Dongen et al. 2006).

Furthermore, when subjects were divided into morning and evening types (Horne & Östberg 1976) evening people were likely to be less sensitive to sleep debt (Volk et al. 1994). In accordance with this, evening types accumulated more sleep debt during the working week but did not feel sleepier than morning types (Taillard et al. 1999). This might be due to a difference in the regulation of homeostatic sleep as morning types started their night with a higher level of slow wave activity in the EEG, indicating a higher sleep pressure at the end of the waking day (Mongrain et al. 2006b). In addition, an early study indicated that diurnal type affected the sleep length in rotating SW, with less sleep
after afternoon and night shifts in morning types, although this could be related to the positive correlation between age and morningness (Akerstedt & Torsvall 1981). These inter individual differences in shift work tolerance have also been described in a recent systematic review (Saksvik et al. 2010). Mood in morning and evening types was differentially affected by TSD and PSD (Selvi et al. 2007). After PSD mood of morning types was not affected while depression in evening types decreased. After TSD depression increased in morning types but decreased in evening types.

Similar to morning types, individuals homozygous for the 5/5 genotype of the PERIOD 3 (PER3) VNTR polymorphism had higher slow wave activity at the start of their baseline sleep than 4/4 genotypes (PER3^{4/4}) and the deterioration of cognitive performance in response to TSD was greater (Viola et al., 2007). During PSD, slow wave energy was also higher in PER3^{5/5} but, by contrast, the genotypes were not different at baseline and PER3^{5/5} subjects even had a slightly better cognitive throughput during PSD (Goel et al. 2009). One of the first studies showing interindividual differences in physiology during sleep and sleep deprivation indicated changes in HRV (mainly an increased sympathetic activity) in PER3^{5/5} genotypes, particularly during baseline sleep (Viola et al. 2008). In addition, others suggest that polymorphisms in the clock genes hCLOCK, PER2 and PER3 are associated with an individuals ability to adapt to shift work in terms of changes in body temperature, reproductive hormones and metabolites thought to be involved in the development of cancer (Davis & Mirick 2006).

Furthermore, variation in the adenosinergic system, which is important for the regulation of sleep (Porkka-Heiskanen et al. 2003), may affect sleep and vulnerability to the effects of sleep loss in humans (Landolt 2008). As for the PER3 genotypes, genetic substrates might show comparable phenotypes in homeostatic sleep regulation. An example is the G/A genotype of the adenosine deaminase enzyme (ADA) which is linked to fewer awakenings, more SWS and deeper sleep than the G/G genotype (Retey et al., 2005).

These studies have shown that there are interindividual differences in the regulation of sleep and the response to sleep deprivation and that there may be a genetic basis for this. It will be of interest to explore whether it is also possible to detect such interindividual differences between NSW and SW.
1.9 Rationale and aims

Both epidemiological studies (sections 1.2.2 and 1.3.2) and laboratory studies (sections 1.6 and 1.7) have shown that sleep deprivation and shift work affect cardiovascular and metabolic function and alertness and mood. However, these studies have often been confounded by factors such as circadian phase, physical activity, body posture, food intake and stress levels. Therefore, it is unclear whether the observations can be attributed purely to sleep deprivation and shift work or whether they are (partly) a result of these confounding factors.

Moreover, the effects of TSD on postprandial metabolic and insulin responses after a standard breakfast have not been investigated. Endothelial function following TSD has rarely been researched and it has not been assessed by the ultrasound method identical to the one used in our research group. In addition, the effects of sleep deprivation on HRV parameters in the time domain have not been reported widely.

Finally, it is still not known what aspects of shift work are causative. Interpretation of studies is often limited due to a poor description of the shift work population and lack of an appropriate control group. It is also unclear how many years of shift work are needed before changes in metabolic and cardiovascular function start to take place. Moreover, to date, few, if any, studies have compared SW directly to NSW in identical laboratory settings.

Therefore, the aim of the present study was to investigate the effect of a night of TSD, as a proxy for the first night of shift work, on metabolic and endothelial function and heart rate variability in controlled laboratory conditions. In addition, the effect of TSD on subjective and objective alertness as well as subjective mood was assessed. The effect of a recovery nap and recovery sleep following TSD was also investigated. Finally, experienced SW were compared to NSW in identical settings to assess the effects of chronic exposure to shift work, rather than the acute effects due to circadian misalignment, on the subject's response to sleep deprivation and recovery sleep.
1.9.1 Hypotheses

It was hypothesised that:

- One night of TSD in a well controlled environment will adversely affect metabolic and endothelial function, heart rate variability and mood and alertness,
- Recovery sleep after one night of TSD will restore any disturbances in the measured parameters so that values after recovery sleep will return to or approach levels measured prior to TSD,
- There will be no difference in circadian phase or phase angle between SW and NSW,
- SW will have similar levels of CVD risk factors as matched controls on the baseline day of the study as they will be entrained and matched with NSW for age, cholesterol and BMI during screening,
- Following TSD, SW will have different responses in metabolic and endothelial function and heart rate variability compared to NSW due to previous chronic exposure to sleep deprivation and circadian misalignment,
- SW will show differential responses in the neurobehavioural effects of TSD compared to NSW.
CHAPTER 2 METHODS

2.1 Procedures

The University of Surrey gave a favourable ethical opinion for all aspects of this study (EC/2007/43/SBMS; appendix A). All volunteer information was kept coded and held in the strictest confidence in compliance with the Data Protection Act (1998). All participants gave written informed consent stating that they agreed to all assessments, were aware of any potential risks and discomfort and understood that they were free to withdraw from the study at any given time.

2.2 Recruitment and screening

Male SW and NSW between 25 and 45 years of age were mainly recruited via advertisements in local newspapers and on local websites. In addition, volunteers were recruited via posters at the University and the University email list. A University press release was also distributed which resulted in a local newspaper editorial and 3 recorded radio interviews. Finally, some volunteers were informed about the study by advertising at their work place or by word of mouth.

Pre-study screening and the laboratory part of the study occurred in the Clinical Investigation Unit (CIU) of the Faculty of Health and Medical Sciences (FHMS), at the University of Surrey. An extensive screening procedure was applied. In the first stage applicants were provided with a brief overview of the study, either via email or on the telephone. If they were interested, they were asked some initial questions about their health, nicotine, alcohol and caffeine intake (categorical or in actual units/day) and shift work history (appendix B). If applicants appeared suitable, they were invited to visit the CIU to discuss the study in more detail, provide a blood sample for a standard haematological and biochemical screen (Royal Surrey County Hospitals NHS Trusts, Partnership Pathology Services) (appendix C), a urine sample for drugs of abuse (Epsom and St. Helier University Hospitals NHS Trust, Pathology Services) (appendix C), measure waist and hip circumference ((van der Kooy & Seidell 1993) appendix D) and answer a general health questionnaire and 4 validated questionnaires (appendix E): Horne-Östberg (HÖ) questionnaire (Horne & Östberg 1976), Pittsburgh Sleep Quality Index (PSQI) (Buysse et al. 1989), Beck Depression Inventory (BDI) (Beck &
Beamesderfer 1974; Beck et al. 1974) and Epworth Sleepiness Scale (ESS) (Johns 1991, 1992). As PSQI, BDI and ESS appeared to be dependent on the current shift pattern in SW, second assessments were carried out for some of the subjects during the week prior to the study or on the adaptation night. Subjects were then asked to keep only days off or day shifts in mind when completing the questionnaires. SW were also asked to fill out the Standard Shift Work Index (SSI) (Barton et al. 1995) which was mainly used to determine the total shift work duration and the shift pattern of their last job (appendix E). Finally written consent was obtained from the subject’s GP confirming that there was no known reason why the applicant would be unsuitable to participate in the study and stating dose, frequency and last administration of (recently) prescribed medication.

2.3 Subjects

If applicants were found suitable they were informed of their eligibility to participate in the study. For inclusion and exclusion criteria see Table 2.1. Moreover, SW had to have worked shifts continuously, with at least three night shifts per month to be eligible. Twelve SW and 14 NSW matched for age, BMI and cholesterol participated in the study. However, one shift worker withdrew from the study after the adaptation night and was not included in any further analyses. The results for the screening for all participants can be found in Table 3.1 and appendix F. Shift work schedules are summarised in appendix G. Although some subjects exceeded the official cut off values, they were included in the study. This will be discussed in section 6.1.4. The self-reported pre-study questionnaires confirmed that the subjects neither had a history of medical conditions or medication thought to affect cardiovascular, metabolic, gastrointestinal or immune function nor suffered from sleep or mental disorders at the time of recruitment and during the study.

2.4 Prior to the laboratory session

In order to maintain or establish entrained circadian rhythms (i.e. for the SW to be adapted to nocturnal sleep prior to the laboratory session) and to minimise sleep debt, volunteers were asked to keep a self-selected regular sleep-wake cycle (Revell et al. 2005) (or, strictly speaking, go to bed - get up cycle), with a sleep duration of 7.5 or 8 h the week prior to the study, (with bed times of 23.3 ± 0.5 h, 22 - 24 h (average ± SD, range, n = 25), wake up times of 7.2 ± 0.5, 5.5 - 8 h and a sleep duration 7.9 ± 0.2, 7.5 - 8 h (for individual times, see appendix F). They were instructed to start this schedule 8 evenings prior to the laboratory study and to try to sleep in a dark, quiet room without
distraction (and not to deviate in their bed and get up times by more than 15 minutes). Volunteers were given a 4 hour window in the afternoon (centred 12 hours away from the midpoint of their night-time sleep) in which they were permitted to nap if they chose to do so. Naps scheduled this way are thought not to phase shift circadian rhythms (Buxton et al. 2000).

Table 2.1. Inclusion and exclusion criteria.

Participants must:
- be between 25 and 45 years of age,
- be male,
- in the case of SW: have done shift work for an accumulated duration of at least 5 years,
- be able and willing to give informed oral and informed written consent,
- have authorisation from their medical practitioner,
- complete and meet the defined criteria of pre-study questionnaires,
- agree to keep a constant sleep/wake cycle with a self-selected sleep duration of 7.5 or 8 hours (from which can not be deviated by more than 15 minutes) for one week prior to the lab study,
- obtain 15 minutes of sunlight within 1.5 hours of waking up and agree to nap only within a 4 h designated nap window for one week prior to the lab study,
- allow confirmation of compliance to these instructions by reporting wake up and sleep times to the lab voicemail, wearing Actiwatches (AWL) continuously and complete daily sleep and event diaries for one week before the study session,
- agree to refrain from alcohol, caffeine, heavy exercise and certain food components two days before the study session,
- agree to refrain from prescribed, ‘over the counter’ medication and food supplements for a wash-out period determined by the research team.

Participants will be excluded if they:
- are taking regular medication (also non-prescribed) or food supplements (e.g vitamins, minerals, fish oil, antioxidant tablets) from which it is not possible to refrain, known to influence:
  - sleep/alertness/the circadian timing system (e.g. β-blockers, barbituates, antidepressants, benzodiazepines, melatonin, ritalin, modafinil, soporifics, St John's Wort),
any of the metabolic functions (e.g. affecting thyroid, kidney, liver or gastrointestinal function)
o any of the inflammatory markers (e.g. aspirin, ibuprofen, antibiotics, hay fever medication, medication for sore throats and colds)
o and/or any of the endothelial markers (e.g. ACE inhibitors and angiotensin (receptor) blockers, diuretics, β-blockers, anti-thrombosis medication),

- have a history of any circadian or sleep disorder or metabolic, cardiovascular or chronic infectious / inflammatory disease as confirmed by the GP or the pre-study questionnaires,
- have a history of psychiatric or neurological disease or drug and alcohol abuse,
- have a positive drug screen. A urine screen will be carried out to ensure that the participants are negative for drugs of abuse,
- have donated over 400 ml of blood in the three months preceding the study,
- are anaemic, confirmed by a haematology screen,
- have travelled across more than two time zones within two weeks of the study,
- in the case of NSW: have participated in shift work for more than 6 months,
- in the case of the SW, have been involved in shifts other than day shifts the week prior to the study,
- do not keep a regular sleep-wake cycle
- do not refrain from alcohol, caffeine containing drinks (e.g. coffee, coke, tea, redbull), heavy exercise and certain foods (e.g. those high antioxidants and nitrates/nitrates) two days before the laboratory session

To confirm this regular sleep-wake cycle participants were asked to call the laboratory’s voicemail within 10 min before going to bed and after getting up. They were also provided with two L-actiwatchs (AWL) (Cambridge Neurotechnology, Cambridge, UK), one around the neck and one around the non-dominant wrist, both recording motor activity and light exposure via a light sensor on the upper side. The volunteers were also asked to complete a daily sleep diary, recording bed and get up times, estimates of sleep onset latency and awakings, a rating of the sleep quality on a 9-digit scale and whether they would have liked to sleep longer or nap (Lockley et al. 1999). For the 7 days prior to and on the morning of the laboratory study participants were asked to obtain 15 min exposure to outdoor natural light (without wearing sunglasses) within the 90 min after waking up to strengthen the regularity of the circadian rhythms (Revell et al. 2005). This was complemented by recording in a light diary when participants went outdoors during the light phase and whether they were wearing sunglasses and/or riding in a car/bus or train. In addition, volunteers were asked to complete an event diary, recording when,
what and how much they ate and drank and when and how long they exercised and what type of exercise they did.

Participants were also asked to inform the research team of any changes in their medical condition and to take only paracetamol instead of non-steroidal anti-inflammatory drugs (NSAIDs) for minor aches. Moreover, it was emphasised that recreational drugs were prohibited. Finally, 24 out of 26 volunteers visited the CIU for an ‘actiwatch download appointment’ halfway through this week to confirm the actiwatches were working and to check whether subjects were adhering to the regular sleep/wake cycle.

The 2 days prior to the laboratory session, participants were asked to refrain from heavy exercise, smoking (although none of them were current smokers), alcohol, caffeine, foods high in anti-oxidants (Hampton et al. 2010) and nitrites/nitrates (Walters et al. 2006) (appendix H). On the day the subjects entered the laboratory they were asked to refrain from foods and drinks other than water for the 6 h before the dinner provided in the laboratory.

2.4.1 Genotyping

Buccal swabs (Epicentre® Biotechnologies, supplied by Cambio Ltd., Cambridge, UK) were collected from all participants prior to the study and they were genotyped for PER3 according to an established protocol (Ebisawa et al. 2001), with some modifications (Vandewalle et al. 2009) (appendix I). The results can be found in Table 3.1 and appendix F.

2.5 The laboratory session

Volunteers spent 4 nights and 4 days in the CIU (Figure 2.1). They were not allowed to leave the CIU or to have visitors, but were allowed to know clock time and to make phone calls, watch television or DVDs, listen to music, read or use their laptop (with the brightness of the laptop screen reduced) when they were not doing the mood and alertness tests.

Light was kept constant at < 8 lux in the direction of gaze (immediately following the electrode attachment for the PSG recordings was finished until after the last alertness and mood assessment, apart from darkness during the sleep periods).
Figure 2.1. Laboratory study protocol.
The diagram shows the 5 study days for a subject with a habitual sleep from 23:00 to 7:00 h. Interventions (see key) were all relative to the subject’s wake up time (see relevant sections). HRV, heart rate variability.
This was regularly checked with a calibrated lux meter (Edmund Optics, York, UK). Temperature was set around 20°C and both light and temperature were continuously recorded with Hobos (Tempcon, Arundel, UK). There were at least 3 Hobos placed above the subjects head at the end of each bed and a random point in the room. For some sessions there were 2 additional Hobos at the end of each subject's bed.

In order to control for inter-individual differences in circadian phase, all interventions and measurements were scheduled relative to each subject's self-selected wake up time (appendix F) which can be used as a proxy for circadian phase (Burgess et al. 2003). During each session, only 2 volunteers were present because of the duration and frequency of the measurements. Their wake up times were scheduled 30 min apart to allow time to take all the measurements. The volunteers were in the same ward but could draw a curtain around their beds to allow for some privacy.

On the first afternoon subjects entered the laboratory around 11 h after wake up time. They were allowed some time to settle in and told again briefly what the study involved. On the adaptation, baseline and recovery night they were asked to try to sleep in the dark at the same times which were already agreed with them for the schedule the week prior to the study. They were provided with eye masks (and ear plugs if needed). From waking up after the baseline night, subjects were asked to stay awake for 30.5 h (as a proxy for the first night shift), followed by a 4 h recovery nap and recovery sleep, which was equal in length to adaptation and baseline sleep. During wake time subjects were monitored by staff in order to ensure they did not fall asleep, which was evaluated post hoc with polysomnography (section 2.5.4).

Timing of other interventions and measurements will be described in the relevant sections.

### 2.5.1 Body posture

As many of the measurements (e.g. cardiovascular parameters) are affected by body posture, this was strictly controlled throughout the study. During assigned periods, subjects were asked to remain in a semi-recumbent posture (with the bed at approximately 45°). During these periods they were not allowed to get out of bed. If they needed the toilet they were provided with a urinal. The semi-recumbent periods were the sleep periods, the sleep deprivation night (from habitual bed time to wake up time) and 4
h periods each afternoon from 6.5 to 10.5 h after wake up time. Initially, this was done to establish controlled conditions mainly for the HRV assessments but the data during these periods were not analysed for this thesis due to the combined effect of sleep deprivation and the nap which makes the afternoons less comparable across days.

Physical activity was further restricted during blood and saliva sampling which was performed in a seated position. Subjects were allowed to use the toilet during these periods but were instructed to be seated 20 minutes before each sample as saliva melatonin concentrations may be influenced by changes in body posture (Deacon & Arendt 1994). Mood and alertness assessments were also conducted in a seated position. In addition, all subjects were allowed to have a shower after the last mood and alertness test before the sleep deprivation night. Finally, ultrasound recordings were taken in supine position.

Practically, this meant that subjects were in bed all the time and only got out of bed to use the bathroom. This ensured that body movement and energy expenditure were similar for all volunteers.

2.5.2 Food intake

All subjects were provided with the same standard breakfast, lunch, dinner and evening snack (Table 2.2) (appendix J) at 1.75, 4.75, 12 and 15 h after wake up time, respectively. These meals were provided from the dinner on the first evening until the dinner on the last evening included. Between these meals subjects were not allowed any other food or drinks, except for water ad libitum.

Table 2.2. Percentage protein, fat and carbohydrates and energy for each of the meals.

<table>
<thead>
<tr>
<th></th>
<th>Protein (%)</th>
<th>Fat (%)</th>
<th>Carbohydrate (%)</th>
<th>Energy (kcal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakfast</td>
<td>7.8</td>
<td>39.7</td>
<td>53.3</td>
<td>967.5</td>
</tr>
<tr>
<td>Lunch</td>
<td>14.1</td>
<td>53.6</td>
<td>32.2</td>
<td>996.9</td>
</tr>
<tr>
<td>Dinner</td>
<td>13.3</td>
<td>43.9</td>
<td>42.5</td>
<td>950.0</td>
</tr>
<tr>
<td>Evening snack</td>
<td>14.9</td>
<td>25.4</td>
<td>59.1</td>
<td>300.0</td>
</tr>
<tr>
<td>Overall composition</td>
<td>12.0</td>
<td>43.9</td>
<td>44.1</td>
<td>3214.4</td>
</tr>
</tbody>
</table>

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Chapter 2 Methods

The breakfast had a relatively high fat and sugar content in order to elicit a well-defined postprandial response. The snack was included in the protocol to reduce feelings of hunger and irritation during sleep deprivation.

### 2.5.3 Blood sampling

On the first evening a blood sample was taken prior to the evening snack. On the adaptation day, 4 blood samples were taken prior to breakfast, lunch, dinner and the evening snack. Samples taken before breakfast are from here onwards referred to as ‘basal’ samples. The 5 blood samples from the adaptation and baseline day were taken by venepuncturing by a qualified phlebotomist. On the baseline day, a cannula was inserted into the left arm by a qualified nurse, which allowed for frequent blood samples to be taken. After baseline sleep, sleep deprivation and recovery sleep, blood samples were taken 15 and 0 minutes prior to and 15, 30, 45, 60, 90, 120, 180 and 240 minutes after breakfast and again prior to the dinner and evening snack. The first sample on these 3 days was taken 1.5 h after wake up time (following a 10.5 h fast). This resulted in a total of 41 blood samples per subject over the course of the experiment. The samples taken after adaptation sleep and before the dinner and the snack each day were initially collected to be able to assess the daily pattern of metabolites, insulin and inflammatory markers across the days, but these data are not described in this thesis.

Each blood sample (with a total volume of 12 ml) was collected into 3 different collection tubes. One ml was collected into a 1 ml fluoride oxalate tube (Teklab Ltd., Sacriston, UK), 3 ml into a 5 ml tripotassium EDTA tube (Teklab Ltd.) and 8 ml into a 10 ml lithium heparin tube (Teklab Ltd.). The tubes were centrifuged at 1750 g, 4°C for 10 min and the resulting supernatant was aliquoted into 1.5 ml microcentrifuge tubes (Apex® plus, Alpha Laboratories Ltd., Eastleigh, UK) and stored at -20°C until analysis. Directly after each blood sample the cannula was flushed with 0.9% w/v sodium chloride (MacoPharma Ltd., Twickenham, UK) to keep the cannula patent.

### 2.5.4 Polysomnographic (PSG) recordings

Participants were prepared for PSG after their dinner on the first night of the laboratory session (see sections 4.2.3 and 5.2.2) (HRV and sleep) for further details). The PSG included EEG/EOG/EMG (section 5.2.2), ECG (section 4.2.3) and respiratory effort. To record respiratory effort a thoracic and abdominal band (Nicolet/Viasys Healthcare,
Warwick, UK) were put around the chest (at the height of the nipple line) and around the abdomen (at the belly button). PSG was then performed continuously throughout the laboratory session until the last alertness and mood test on the recovery day, with the exception of periods with data loss due to logistical and technical issues (e.g. to allow the subjects to take a shower, network connection problems, battery replacement, etc.). All electrodes were connected to an ambulatory system (Siesta EEG/PSG recorder, Compumedics Ltd, Abbotsford, Victoria, Australia) and signals were continuously recorded at 256 Hz with ProFusion PSG 2 (version 2.1 build 134, Compumedics Ltd). Impedance was checked regularly and if it was too high (> 5 kΩ or > 15 kΩ for ECG and EMG) electrodes were removed, the site was cleaned, new paste applied and the electrode reattached. The acquired data were converted into European Data Format (EDF) and imported into Somnologica Studio (version 5.0.1 build 1662, Embla, supplied by Stowood Scientific Instruments Ltd., Oxford, UK) for further analysis.

2.6 General statistics

Statistics were carried out in SPSS 14, 16 and 17 (SPSS Inc., IBM company, Chicago, Illinois, USA), Graphpad Prism 4 and 5 (GraphPad Software Inc., La Jolla, San Diego, California, USA) and Statistica (release 5.1 – '97 edition, StatSoft Inc., Tulsa, Oklahoma, USA). The 3 programs yield similar results as long as exactly the same tests and adjustments were used. However, all programs had their limitations and therefore they were used alongside each other. For example, SPPS could not calculate post hoc tests for some main effects such as ‘day’ and ‘time’ whereas Statistica had this option. Tests for sphericity and Greenhouse-Geisser adjusted P-values (see next sections) for some effects and interactions were not calculated by Statistica but were available in SPSS. Finally, areas under the curve were calculated in Graphpad Prism (see next sections).

2.6.1 Normality

Almost all tests described in the next chapters are parametric tests. It is recommended that normality of the data is tested for before using parametric tests. Normality tests were carried out preferably per group, day and time point as each of these can be seen as an ‘intervention’ and may cause the data not to be normally distributed. Although data were not normally distributed in some of the cases, parametric tests were proceeded with (according to advice from University statisticians) as the normality tests were thought not to be of major importance, due to the design of the study and the small sample size.
2.6.2 Analyses of variance (ANOVAs) and Mauchly’s test of sphericity

The lay outs of the data sets in this thesis were suitable for testing with ANOVAs. The most complex design used was the three-factor ANOVA with repeated measures for 2 within subjects factors (‘day’, having as many levels as the number of days compared and ‘time’, having as many levels as the time points within a day) and 1 between subjects factor (‘group’, which was defined by being a shift worker or non-shift worker).

When carrying out the ANOVAs, firstly, the data were tested for sphericity. According to SPSS results coach “Mauchly derived a test that verifies the variance-covariance matrix structure by performing a test of sphericity on the orthonormalised transformed dependent variables. Mauchly’s test of sphericity tests the null hypothesis that the error covariance matrix of the orthonormalised transformed dependent variables is proportional to an identity matrix. The null hypothesis is rejected if the significance value is less than 0.05.” Therefore, when the null hypothesis was rejected, sphericity could not be assumed and one of the adjusted (usually higher,) significance values had to be chosen, which most commonly was the Greenhouse-Geisser adjusted value. As carrying out tests for sphericity and displaying adjusted values was not in the default settings of Statistica, these settings had to be customised. Furthermore, sphericity is assumed by GraphPad Prism and it is not possible to obtain adjusted values. Therefore, the areas under the curve (AUCs) (3.2.5.4) calculated by GraphPad Prism had to also be analysed in Statistica.

2.6.3 ANOVA post hoc tests

If a significant main effect was found in an ANOVA with repeated measures, there are two ways to locate the differences between groups, days and time points; either by contrast analysis or by post hoc tests. While contrast analysis only allows testing significance of a priori specified comparisons (e.g. 2 comparisons for 3 days), post hoc tests allow multiple comparisons (e.g. between SW and NSW on different days). The latter is more likely to reveal more significant differences as Statistica help states that it is based on the assumption that for a comparison the most extreme (different) means (e.g. 2 means) are chosen out of a number of total means (e.g. 20 means with a sample size of 5 each), whereas contrast analysis would test the difference between a number of samples from the original pool (e.g. 2 samples out of 100). It is an individual choice which of these methods to use.
For this thesis it was decided to use Tukey honest significance difference (HSD) post hoc tests as this allowed all comparisons of \textit{a priori} interest. As SPSS allows only contrast analyses but no post hoc tests for three-factor ANOVAs, the analyses were carried out in Statistica. If no significant interactions were found in the within subjects main effects, the post hoc tests were only used to locate differences within each main effect separately (e.g. between days). On the other hand, if significant interactions were found (e.g. for ‘day’ by ‘group’) both factors were combined to locate differences with the post hoc tests (e.g. between days within SW).

\textbf{2.6.4 Mixed-model analyses}

As ANOVA cannot be carried out on data sets with missing values, these had to be replaced or subjects had to be excluded. After the data in \textit{CHAPTER 3} were analysed with ANOVA a University Statistician (Mr Peter Williams) designed a software code that could analyse the FMD data set (which also had missing values) and then this was modified to analyse the HRV and alertness and mood data. This mixed-model regression analysis was conducted in SAS 9.1 (TS level 1M3, SAS Institute Inc., Cary, NC, USA). Similarly to the ANOVA, the model determined main effects (group, day and time) and interactions between these main effects, with repeated measures within subjects, fixed effects SE method Prasad-Rao-Jeske-Kackar-Harville, covariance structure ‘variance components’ and Kenward-Roger method for calculating the degrees of freedom. To locate post hoc differences between days and time points, least squares means were calculated and t-type confidence limits were constructed for each of the least squares means.
CHAPTER 3 METABOLISM

3.1 Introduction

Epidemiological research has shown that SW may be at higher risk of developing CVD (Boggild & Knutsson 1999; De Bacquer et al. 2009; Ellingsen et al. 2007; Esquirol et al. 2009; Karlsson et al. 2001; Karlsson et al. 2003; Kawachi et al. 1995; Sookoian et al. 2007). This may, in part, relate to changes in hormone and metabolic responses (section 1.3.2). The mechanisms underlying these observations are still unclear, but it is thought that in SW the increased risk may, in part, be due to circadian misalignment (e.g. eating at night when metabolic activity is lower) (Knutsson & Boggild 2000) (section 1.4.3). Previous studies in SW (Knutsson et al. 2002; Lund et al. 2001; Simon et al. 2000) and studies on simulated shift work (Al-Naimi et al. 2004; Hampton et al. 1996; Ribeiro et al. 1998) or circadian misalignment (Scheer et al. 2003) have shown increased TAG, glucose and insulin responses and decreased NEFA levels when the food intake of subjects was at an inappropriate circadian time (section 1.7). In addition, sleep deprivation, for example in SW, may contribute to an increased risk for CVD (e.g. Gangwisch et al. 2006; Sabanayagam & Shankar 2010; Taheri et al. 2004) (section 1.2.2). In agreement with this, some laboratory studies have demonstrated decreased glucose tolerance and insulin sensitivity after partial sleep deprivation (PSD) (Buxton et al. 2010; Donga et al. 2010; Nedeltcheva et al. 2009; Spiegel et al. 1999; van Leeuwen et al. 2010) (section 1.6.2) and after total sleep deprivation (TSD) (Gonzalez-Ortiz et al. 2000; VanHelder et al. 1993) (section 1.6.1). In addition, a decrease in NEFAs was observed under hypoglycaemic clamp conditions (Schmid et al. 2007) after 1 night of TSD and a reduction in the TAG rhythm and average TAG levels was found after 3 to 5 days of total sleep deprivation (Vondra et al. 1986).

Furthermore, it is likely that there are inter-individual differences in the vulnerability to the effects of shift work and sleep deprivation (section 1.8), due to e.g. genetics, morningness-eveningness or shift work history. This suggests that NSW and experienced SW may respond differently when subjected to the same sleep restriction conditions and dietary intake.

The effects of TSD on the postprandial response to a standard breakfast have not been investigated before, although TSD is occurring during at least the first night shift in many
SW. In addition, differences between SW and NSW have not been assessed simultaneously in controlled laboratory conditions with food intake at a 'normal' circadian time.

3.1.1 Aims and hypotheses

This chapter investigates the effects of one night of TSD, a recovery nap and recovery sleep *per se* on basal and postprandial metabolic and insulin responses under controlled laboratory conditions. In addition, the responses of experienced SW (with a shift duration of 5 years or more) were compared with those of NSW.

It was hypothesised that:

- TSD will affect TAG, NEFA, glucose and insulin levels in both NSW and SW. Changes in these metabolite and insulin levels will indicate decreased insulin sensitivity after TSD.
- A nap and recovery sleep will have an impact on these postprandial changes by (partly) reversing the levels to those prior to TSD.
- There will be differences between SW and NSW. Indications of insulin insensitivity will be higher after TSD in SW than in NSW; changes in metabolites and insulin will not recover as quickly in SW as in NSW.
- There will be no differences in cholesterol and HDL throughout the protocol and there will be no differences between NSW and SW due to the matching during screening.

3.2 Methods

Methods specific to this chapter are described below. For general methods see chapter 2.

3.2.1 Subjects

Demographics of all 25 subjects (14 NSW, 11 SW) are shown in Table 3.1 and appendix F. One NSW (C2) had problems with blood sampling and too few samples could be collected in order to assess a reliable postprandial response. Another NSW (C13) had an extremely high insulin response and was excluded from all insulin analyses. The subject
was considered an 'outlier' as the areas under the curve (AUCs) after baseline sleep, TSD and recovery sleep were > 2 SD from the mean.

3.2.2 Blood samples

The sampling procedure is described in detail in section 2.5.3 and Figure 2.1. Plasma glucose was measured from blood collected in fluoride oxalate tubes, while all other metabolites and insulin were measured in plasma derived from blood collected in K-EDTA tubes. The plasma samples were stored at -20°C until assayed. Repeated freeze-thaw cycles were avoided in almost all cases. Although measurements were carried out for all 41 samples collected throughout the study, the results for the glucose, TAGs, NEFAs and insulin are only described after baseline sleep, TSD and recovery sleep as the postprandial response was assessed just on these days. Cholesterol and HDL were measured in all morning (0 time point) and evening samples (i.e. 9 samples per subject) as concentrations were not expected to change postprandially.

A preliminary analysis for the first 11 subjects was carried out for glucose, TAGs, NEFAs, insulin, HDL and cholesterol. The samples from the remaining 13 subjects were analysed in a second batch approximately 1 year later.

3.2.3 Measurements of the metabolites and insulin

3.2.3.1 Metabolites

For the preliminary analysis, the plasma was thawed, centrifuged at 1500 g for 5 min at 4°C and the tubes were placed in the I-lab 650 (Instrumentation Laboratory, Warrington, UK) for the measurement of the metabolites. For the remaining 13 subjects, the plasma was also thawed and centrifuged but the supernatant was then re-aliquoted into new plain tubes to optimise clarity of the sample and were placed in the I-lab for the measurements.

The plasma metabolite concentrations were determined with reagents for the detection of glucose (oxidase) (IL TestTM, catalogue number 0018250840, Instrumentation Laboratory), TAGs (IL TestTM, catalogue number 0018255640, Instrumentation Laboratory), NEFAs (catalogue number FA115, Randox Laboratories Ltd., Crumlin, UK), HDL-cholesterol (catalogue number CH2652, Randox Laboratories Ltd.) and cholesterol.
(IL TestTM, catalogue number 0018250540, Instrumentation Laboratory). All reactions were enzymatic, producing coloured end points, of which the absorbance was measured and directly proportional to the metabolite concentration (appendix K).

The l-lab was calibrated every day before the measurements were taken and quality control (QC) samples with a known concentration were measured and checked before measuring the samples of each subject to assess intra- and inter assay variability. Coefficients of variation (CVs) were 3 and 3.3, 3.8 and 4.2, 2.4 and 2.3, 7 and 8.8 and 4.3, 4.7 and 3.5 % for QC samples with 1.4 and 2.5 mmol/L TAG (n = 28), 2.4 and 5.1 mmol/L cholesterol (n = 20), 5.3 and 14.4 mmol/L glucose (n = 17), 1.1 and 1 mmol/L NEFA (n = 23) and 0.9, 1.4 and 2 mmol/L HDL (n = 21), respectively.

3.2.3.2 Insulin

Plasma insulin was measured with a human insulin specific radioimmunoassay (RIA) kit (catalogue number HI-14K, Millipore Ltd., Watford, UK). The plasma was thawed and centrifuged at 1500 g for 5 min at 4°C. Standards, QCs and samples were pipetted into LP3 tubes and the protocol supplied with the kit was followed (appendix L), with the exception of the following modifications. Four zero-binding tubes were used in order to improve precision. Two pairs of low and high QCs (before the first and after the last samples), or if samples of 2 subjects were assayed, 3 pairs of low and high QCs (before the first, in the middle of and after the last samples) were measured. Finally, the supernatant in step 10 was aspirated instead of decanted because the pellets were fragile. All samples from one subject were measured in one assay, usually those from one SW as well as one NSW and the day order was randomised to minimise any effects of possible assay drift.

After visual inspection, samples were measured again if the duplicates had a variation of approximately 10%, depending on the sample concentration. One value of the original duplicate closest to the repeated value was chosen as the final value. Samples were also diluted and repeated if insulin levels were higher than the linear part of the standard curve. The inter-assay CVs were 11.9 and 6.6% for 60.2 and 212.6 pmol/L insulin (n = 16).
3.2.4 Missing values and inter-/extrapolation

Repeated measures analyses cannot be carried out in SPSS, Statistica or GraphPad Prism on a data set with missing values. For TAGs, NEFAs, glucose and insulin, missing values had to be filled in using linear interpolation for missing values in the sampling period 15 to 180 min after breakfast and linear extrapolation for the samples taken 240 min after breakfast. Missing values for the samples taken either 15 or 0 min prior to breakfast were not interpolated but it was assumed that these 2 samples should have equal values. Missing values for the dinner and evening samples were not added. The percentage of values which needed to be inter-/extrapolated was as follows: glucose 3.6%, NEFAs 2.8%, TAGs 3.1% and insulin 3.8%. These added missing data are also shown in all graphs unless otherwise mentioned. Obviously, interpolation and extrapolation has limitations, especially when the missing value occurs exactly where the postprandial peak is expected and a linear assumption may not reflect the reality. However, this method has been chosen to avoid exclusion of too many subjects.

For cholesterol and HDL, interpolation is slightly different as samples were taken during a different time course (section 3.2.2). The values were added by taking the average of the remaining 3 time points 0 or 4 evening values (2.2% of the cholesterol and 1.8% of the HDL data, 1 NSW and 3 SW still had missing data).

3.2.5 Statistics

General statistics were described in section 2.6. Methods detailed below hold for all metabolites and insulin unless otherwise stated.

3.2.5.1 Basal levels

Basal levels (the mean of the -15 and 0 time points) of all metabolites and insulin on the baseline day of the study and subject demographics were compared between SW and NSW with an independent two-tailed Student's T-test. Basal levels of all metabolites were also compared after baseline sleep, TSD and recovery sleep. Analyses were carried out on the datasets of NSW and SW combined with a two-factor ANOVA (factors 'day' (3 levels: 'baseline sleep', 'TSD' and 'recovery sleep') and 'group' (2 levels: 'NSW' and 'SW')). Since statistically significant differences between the groups may not be
revealed in this combined analysis due to the small sample number, NSW and SW were also analysed separately using one-factor ANOVA (factor ‘day’).

3.2.5.2 Postprandial responses over 4 hours

Analyses were carried out on the datasets of NSW and SW combined with a three-factor ANOVA (factors ‘day’, ‘time’ (10 levels: time points 15 min prior to – 240 min after the standard breakfast) and ‘group’). NSW and SW were also analysed separately in two-factor ANOVA (factors ‘day’ and ‘time’).

As basal levels showed an effect of day (section 3.3.2), these basal levels were then corrected for. Thus, postprandial samples (time points 15-240 min) were calculated as a percentage of the basal levels prior to the breakfast. A three-factor ANOVA on the data sets of NSW and SW combined (factors ‘day’, ‘time’ and ‘group’) was then carried out on the normalised data. NSW and SW were also analysed separately in two-factor ANOVAs (factors ‘day’ and ‘time’).

3.2.5.3 Return to basal levels

An assessment was made as to whether TAG, NEFA, insulin and glucose return to basal levels 240 min after the standard breakfast. Firstly, a three-factor ANOVA on the data sets of NSW and SW combined (factors ‘day’, ‘time’ (2 levels: ‘basal state’ and ‘240 min’) and ‘group’) was carried out. NSW and SW were also analysed separately in two-factor ANOVA (factors ‘day’ and ‘time’).

Secondly, the basal levels were subtracted from the values for the 240 min time points and this difference was then calculated as a percentage of the fasting level. A two-factor ANOVA (factors ‘day’ and ‘group’) was carried out to assess whether there were any differences between the days and/or the groups.

3.2.5.4 Incremental area under the curve (IAUC)

The Incremental area under the curve (IAUC) was calculated for each individual on the 3 days (GraphPad Prism) by subtracting the area under the basal level from the total AUC (TAUC). The 3 days and the groups were compared with a two-factor ANOVA (factors
‘day’ and ‘group’). NSW and SW were also analysed separately in one-factor ANOVA (factor ‘day’). The same analyses were carried out for the TAUC.

3.2.5.5 Cholesterol and HDL-cholesterol

Cholesterol and HDL levels in the 9 samples were compared in a data set of NSW and SW combined by a two-factor ANOVA (factors ‘time points’ and ‘group’) of both the raw data and interpolated data. NSW and SW were also compared on the mornings and evenings of the adaptation and baseline days by an independent Student’s T-test.

3.3 Results

3.3.1 Comparison of shift workers and non-shift workers

The characteristics of all participants are given in Table 3.1. Individual data can be found in appendix F. Basal plasma glucose, TAG, NEFA and insulin levels measured on the baseline day of the laboratory study and other parameters determined on the screening day were not significantly different between SW and NSW, apart from the number of years the subjects had worked shifts for (P < 0.001) and the PSQI (P < 0.05). However, the significant difference between SW and NSW for the PSQI disappeared when using the values of the second assessment in SW, obtained during the week prior to or on the adaptation night of the laboratory study (see section 2.2). The PSQI in this group was now 5.1 ± 2.2 (mean ± SD), ranging from 2 – 10. In parallel with this, the BDI and ESS scores also came down in SW after the second assessment (4.7 ± 4.9, 0 -17 and 6.2 ± 3.3, 2 -11, respectively). The data did not show differences between NSW and SW in the PER3 polymorphism, but statistical analyses were not possible due the sample size. There was one SW and one NSW with the PER3<sup>5T</sup> genotype but there were more SW than NSW with the PER3<sup>6T</sup> genotype (5/11 vs 3/14) and, inversely related to this fewer subjects with the PER3<sup>4T</sup> genotype amongst SW than NSW (5/11 vs 10/14). Furthermore, no significant differences between SW and NSW were found in other screening parameters such as serum sodium, potassium, alanine transferase (ALT) and white blood cell counts (appendix F).

In addition, the ANOVAs showed that the ‘group’ effect (SW versus NSW) was not significant in any of the analyses described hereafter. However, separate analyses of the groups revealed some differences between NSW and SW.
Table 3.1. Characteristics of non-shift workers and shift workers on the first assessment.

<table>
<thead>
<tr>
<th></th>
<th>non-shift workers (n = 14)</th>
<th>shift workers (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SD</td>
<td>range</td>
</tr>
<tr>
<td>shift work (yrs)</td>
<td>0.03 ± 0.11</td>
<td>0 - 0.42</td>
</tr>
<tr>
<td>time since last shift (months)</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>age (yrs)</td>
<td>32.5 ± 6.2</td>
<td>25 - 42</td>
</tr>
<tr>
<td>weight (kg)</td>
<td>83.7 ± 13.5</td>
<td>67.8 - 109</td>
</tr>
<tr>
<td>height (m)</td>
<td>1.77 ± 0.06</td>
<td>1.66 - 1.85</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.6 ± 3.4</td>
<td>21.5 - 34.0</td>
</tr>
<tr>
<td>waist circumference (cm)</td>
<td>91.2 ± 9.4</td>
<td>76 - 107</td>
</tr>
<tr>
<td>hip circumference (cm)</td>
<td>100 ± 7.4</td>
<td>88 - 114</td>
</tr>
<tr>
<td>WHR</td>
<td>0.91 ± 0.04</td>
<td>0.84 - 0.96</td>
</tr>
<tr>
<td>cholesterol (mmol/L)</td>
<td>4.7 ± 0.5</td>
<td>3.9 - 5.5</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.3 ± 0.2</td>
<td>0.8 - 1.6</td>
</tr>
<tr>
<td>PER3^a (#)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>PER3^b (#)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HÖ</td>
<td>60.5 ± 6.0</td>
<td>51 - 72</td>
</tr>
<tr>
<td>PSQI</td>
<td>3.9 ± 1.9</td>
<td>1 - 7</td>
</tr>
<tr>
<td>BDI</td>
<td>3.3 ± 3.0</td>
<td>0 - 9</td>
</tr>
<tr>
<td>ESS</td>
<td>4.9 ± 3.2</td>
<td>1 - 11</td>
</tr>
<tr>
<td>currently smoke (units/day)</td>
<td>0 ± 0</td>
<td>0 - 0</td>
</tr>
<tr>
<td>past smoked (units/day)</td>
<td>3.6 ± 5.8</td>
<td>0 - 15.5</td>
</tr>
<tr>
<td>smoking duration (yrs)</td>
<td>2.6 ± 4.4</td>
<td>0 - 14</td>
</tr>
<tr>
<td>time since last smoked (months)</td>
<td>17.9 ± 24.1</td>
<td>0.5 - 60</td>
</tr>
<tr>
<td>scheduled bed time (decimal h)</td>
<td>23.3 ± 0.4</td>
<td>22.5 - 24</td>
</tr>
<tr>
<td>scheduled wake up time (decimal h)</td>
<td>7.2 ± 0.5</td>
<td>6 - 8</td>
</tr>
<tr>
<td>scheduled time in bed (decimal h)</td>
<td>7.9 ± 0.2</td>
<td>7.5 - 8</td>
</tr>
</tbody>
</table>

Values are means ± SD and ranges, measured on the screening day, the week prior to the study (scheduled bed and wake up time and bed time) or the baseline day of the laboratory protocol (for glucose, TAGs, NEFAs and insulin).

* P < 0.05 and *** P < 0.001 compared to NSW by independent two-tailed T-test.

3.3.2 Basal levels

Basal levels of plasma TAGs, NEFAs, glucose and insulin after baseline sleep, sleep deprivation and recovery sleep are shown in Figure 3.1 and Table 3.2. A significant effect of day was observed for the basal levels of all 4 parameters in the data sets with NSW and SW combined. There was a significant effect of day on basal TAG levels (F2,44 = 13.7, P < 0.001) with significantly lower TAG levels after TSD (1.2 ± 0.1 mmol/L (mean ± SEM)) than after baseline (1.3 ± 0.1 mmol/L) and recovery (1.5 ± 0.1 mmol/L) sleep (P < 0.05 and P < 0.001, respectively).

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Figure 3.1. Basal plasma metabolite and insulin levels.
Basal plasma (A) TAGs, (B) NEFAs, (C) insulin and (D) glucose (mean ± SEM) following baseline sleep (●), sleep deprivation (□) and recovery sleep (▲) in NSW, SW and all subjects.
Graphs display means (horizontal lines) and raw data points. * P < 0.05, ** P < 0.01 and *** P < 0.001. n = 13 for NSW (n = 12 for insulin) and n = 11 for SW. Basal = mean of -15 and 0 time points.
### Table 3.2. Basal plasma levels of TAGs, NEFAs, insulin and glucose

<table>
<thead>
<tr>
<th></th>
<th>baseline</th>
<th>NSW TSD</th>
<th>recovery</th>
<th>baseline</th>
<th>TSD</th>
<th>recovery</th>
<th>baseline</th>
<th>TSD</th>
<th>recovery</th>
<th>All subjects</th>
<th>TSD</th>
<th>recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAGs (mmol/L)</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 0.2**</td>
<td>1.3 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.1†</td>
<td>1.3 ± 0.1</td>
<td>1.2 ** ± 0.1</td>
<td>1.5 ± 0.1***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEFAs (mmol/L)</td>
<td>0.34 ± 0.04</td>
<td>0.28 ± 0.03</td>
<td>0.25 ± 0.02**</td>
<td>0.28 ± 0.02</td>
<td>0.32 ± 0.03</td>
<td>0.26 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>0.30 ± 0.02</td>
<td>0.25 ± 0.01*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>insulin (pmol/L)</td>
<td>67.4 ± 3.6</td>
<td>70.0 ± 5.4</td>
<td>66.0 ± 8.1**†</td>
<td>63.0 ± 5.1</td>
<td>64.7 ± 4.5</td>
<td>64.3 ± 5.1</td>
<td>65.3 ± 3.0</td>
<td>67.4 ± 3.5</td>
<td>75.6 ± 5.3</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>glucose (mmol/L)</td>
<td>5.1 ± 0.1</td>
<td>5.0 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>5.2 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>5.2 ± 0.1</td>
<td>5.2 ± 0.1</td>
<td>5.1 ± 0.0</td>
<td>5.2 ± 0.1</td>
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</table>

* P < 0.05 and ** P < 0.01, compared to after baseline sleep
† P < 0.05, †† P < 0.01 and ††† P < 0.001, compared to after TSD
When analysing the subject groups separately, both NSW and SW showed significant effects of day ($F_{2,24} = 11.1, P < 0.001; F_{2,20} = 4.4, P < 0.05$, respectively) with a larger increase in basal TAGs after recovery sleep compared to TSD in NSW ($P < 0.001$) than in SW ($P < 0.05$). There was a similar effect of day for basal glucose levels ($F_{2,44} = 3.3, P < 0.05$), with lower basal glucose levels after TSD ($5.1 \pm 0.0$ mmol/L) than after baseline sleep ($5.2 \pm 0.1$ mmol/L) and recovery sleep ($5.2 \pm 0.1$ mmol/L). Tukey post hoc test, however, was non-significant.

In the combined data set there was a significant effect of day on basal NEPA levels ($F_{2,44} = 4.2, P < 0.05$), with significantly lower NEPA levels after recovery ($0.25 \pm 0.01$ mmol/L) than after baseline sleep ($0.31 \pm 0.02$ mmol/L) ($P < 0.05$). When the groups were analysed separately, this appeared to be mainly due to an effect of day in NSW ($F_{2,24} = 6.4, P < 0.01$) with lower NEPA levels after recovery ($0.25 \pm 0.02$ mmol/L) than after baseline sleep ($0.34 \pm 0.04$ mmol/L) ($P < 0.01$). A significant day*group interaction was observed for basal insulin levels ($F_{2,42} = 4.2, P < 0.05$), revealing higher basal insulin levels after recovery ($86.0 \pm 8.1$ pmol/L) than after baseline sleep ($67.4 \pm 3.6$ pmol/L) ($P < 0.01, P < 0.05$ when the groups were analysed separately) and TSD ($70.0 \pm 5.4$ pmol/L) ($P < 0.05$) in NSW.

### 3.3.3 Postprandial responses

#### 3.3.3.1 Raw data

The time course for TAGs, NEFAs, insulin and glucose was assessed after baseline sleep, TSD and recovery sleep. The graphs of the mean data for NSW, SW and all subjects combined are shown in Figure 3.2. There was a significant effect of day on TAG levels in the combined subject group ($F_{2,44} = 10.9, P < 0.001$). TAGs were significantly increased after recovery sleep compared to TSD ($P < 0.001$) and baseline sleep ($P < 0.05$). When NSW and SW were analysed separately, both groups showed a significant effect of day, but the effect was stronger in NSW ($F_{2,24} = 8.2, P < 0.01$) than in SW ($F_{2,20} = 3.7, P < 0.05$).

Insulin showed a day*group interaction ($F_{2,42} = 4.2, P < 0.05$) with higher levels after recovery sleep compared to both TSD and baseline sleep in NSW ($P < 0.001$).
A TAGs

- NSW
- SW
- All subjects

B NEFAs

C Insulin

D Glucose

Figure 3.2. Plasma metabolite and insulin levels.
Plasma (A) TAGs, (B) NEFAs, (C) insulin and (D) glucose (mean ± SEM) prior to (-15 and 0 min) and after (15 to 240 min) a standard breakfast, following baseline sleep (●) sleep deprivation (○) and recovery sleep (▲) in NSW, SW and all subjects combined (mean ± SEM). n = 13 for NSW (12 for insulin) and n = 11 for SW.
When NSW and SW were analysed separately, both groups showed a significant effect of day, but the effect was stronger in NSW ($F_{2,22} = 10.9, P < 0.001$) than in SW ($F_{2,20} = 4.3, P < 0.05$). Post hoc tests showed that insulin levels were significantly higher after recovery sleep than after TSD and baseline sleep in NSW ($P < 0.01$) and that they were higher than after TSD in SW ($P < 0.05$). A significant effect of day was also observed for NEFAs in the combined subject group ($F_{2,44} = 5.2, P < 0.01$), with lower NEFA levels after recovery sleep compared to baseline sleep ($P < 0.01$). When the groups were analysed separately, the NEFA levels after recovery sleep were significantly lower compared to baseline sleep in NSW only ($P < 0.01$). For glucose, no significant effect of day was observed, but there was a significant interaction between day and time ($F_{8,383.2} = 2.2, P < 0.05$), indicating that the time course varied over the 3 days. However, the post hoc tests revealed that the only difference between the same time points on different days was an elevated glucose level 30 min after the standard breakfast following recovery sleep compared to after TSD ($P < 0.05$). Separate analyses of the groups did not show any significant effects of day or day*time interactions.

3.3.3.2 Normalised data

Since there was a statistically significant effect of day on basal TAG, NEFA, insulin and glucose levels (section 3.3.2), the postprandial response was analysed as a percentage of the basal level (i.e. mean of -15 and 0 time points). Using normalised data, no significant effects were found, apart from a day*time interaction for glucose ($F_{16,352} = 2.0, P<0.05$). In addition, in contrast to the raw data, there were no significant differences between the same time points on different days.

3.3.4 Return to basal levels

TAG, NEFA, insulin and glucose levels in the basal state and 240 min after the standard meal are shown in Figure 3.3. To assess the postprandial return to basal levels, the levels prior to the breakfast were compared to the levels 4 h after the breakfast (240 min time point). Statistical analyses showed a significant day*time interaction ($F_{2,44} = 3.9, P < 0.05$) for glucose in the combined subject group, revealing that glucose levels after the standard breakfast did not return to basal levels by 240 min after TSD ($P < 0.01$) (Figure 3.3 D a). Insulin in the combined subject group also showed a day*time interaction ($F_{2,42} = 4.2, P < 0.05$) with higher insulin levels 240 min after breakfast following TSD compared to baseline sleep ($P < 0.01$) (Figure 3.3 C b). TAG and NEFAs showed
significant effects of time in each of the groups, indicating that levels 240 min after the meal did not return to basal levels on any of the 3 days (Figure 3.3 A-C c), apart from those of NEFAs in NSW.

Figure 3.3. Plasma metabolite and insulin levels in the basal state and 240 min after the standard breakfast.
Plasma (A) TAG, (B) NEFA, (C) insulin and (D) glucose levels in the basal state and 240 min after the standard meal, following baseline sleep (●), sleep deprivation (□) and recovery sleep (△) in NSW (NSW), SW and all subjects combined. The letters represent the following comparisons: a, 240 min after the standard meal following TSD vs basal state following TSD; b, 240 min after the standard meal vs basal state on all 3 days; c, 240 min after the standard meal following TSD vs 240 min after the standard meal following baseline sleep; sleep. * P < 0.05 ** P < 0.01 and *** P < 0.001. n = 13 for NSW (12 for insulin) and n = 11 for SW.
Another way of assessing whether postprandial levels return to basal levels is by calculating the difference between basal levels and the plasma levels 240 min after breakfast as a percentage of the basal concentration. The glucose data showed a significant effect of day ($F_{2,44} = 3.9, P < 0.05$) and the relative difference between basal levels and the levels 240 min after breakfast was significantly higher after TSD compared to after recovery sleep ($P < 0.05$) and non-significantly higher after baseline sleep ($P = 0.0641$). There was no significant effect of day on this relative difference between basal TAG, NEFA and insulin levels and the levels 240 min after the standard breakfast.

### 3.3.5 Incremental area under the curve (IAUC)

Since there was a significant effect of day on basal levels (section 3.3.2), IAUCs were assessed. Results are shown in Figure 3.4 and Table 3.3. Analysis of the IAUCs showed a significant effect of day on the insulin response in the combined subject group ($F_{1,4,0.4} = 9.7, P < 0.01$). The insulin IAUC was increased after recovery sleep (83148 ± 8524 pmol/L.min) compared to both TSD (62644 ± 5422 pmol/L.min) and baseline sleep (59680 ± 5205 pmol/L.min) ($P < 0.01$). No significant effects were observed for the TAG and glucose responses. When NSW and SW were analysed separately, the effect of day on the insulin IAUC remained significant in NSW ($F_{1,4,15} = 7.8, P < 0.01$), with a larger insulin IAUC after recovery sleep (96299 ± 14174 pmol/L.min) compared to both baseline sleep (60923 ± 8622 pmol/L.min) ($P < 0.01$) and TSD (64089 ± 8323 pmol/L.min) ($P < 0.05$).

Although calculation of the TAUCs (Table 3.4) does not take differences in basal levels into account, the analyses gave similar results but, in addition, revealed a significantly larger TAG TAUC after recovery sleep compared to baseline sleep ($P < 0.05$) and TSD ($P < 0.001$) and a smaller NEFA TAUC after recovery sleep compared to baseline sleep ($P < 0.05$). The insulin TAUC showed a significant day*group interaction ($F_{1,4,30.2} = 4.0, P < 0.05$) with a higher TAUC after recovery sleep compared to baseline sleep and TSD ($P < 0.001$) in NSW. In addition, the effect of day on TAG, NEFA and insulin TAUCs appeared to be significant in NSW but not in SW, when the groups were analysed separately.
Analyses were also carried out for specified time periods within the TAUCs and IAUCs; from time points -15 to 90 min and 90 to 240 min. These analyses did not reveal any large discrepancies in differences between groups or days when compared to the observations resulting from the -15 min to 240 min TAUCs and IAUCs.

**Figure 3.4. Incremental areas under the curve for metabolites and insulin.**

Incremental areas under the curve (IAUCs) for (A) TAGs, (B) insulin and (C) glucose (mean ± SEM) after baseline sleep (●), sleep deprivation (□) and recovery sleep (◆) in NSW, SW and all subjects combined.

**P < 0.01. n = 13 for NSW (n = 12 for insulin) and n = 11 for SW.**
### Table 3.3. IAUCs of TAGs, insulin and glucose

<table>
<thead>
<tr>
<th></th>
<th>NSW baseline</th>
<th>NSW TSD</th>
<th>NSW recovery</th>
<th>SW baseline</th>
<th>SW TSD</th>
<th>SW recovery</th>
<th>All subjects baseline</th>
<th>All subjects TSD</th>
<th>All subjects recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAGs (mmol/L)</td>
<td>149.3 ± 18.3</td>
<td>160.4 ± 19.4</td>
<td>163.4 ± 14.6</td>
<td>154.1 ± 17.2</td>
<td>130.2 ± 14.3</td>
<td>147.7 ± 14.0</td>
<td>140.2 ± 12.5</td>
<td>146.6 ± 12.5</td>
<td>156.2 ± 15.1</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>60923 ± 8622.5</td>
<td>64089 ± 8333.0</td>
<td>96299 ± 14174.4</td>
<td>58323 ± 5952.0</td>
<td>61068 ± 7185.2</td>
<td>66801 ± 7324.5</td>
<td>59680 ± 5205.3</td>
<td>62644 ± 5422.0</td>
<td>83148 ± 8523.9</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>237.9 ± 28.9</td>
<td>311.9 ± 39.3</td>
<td>321.5 ± 30.6</td>
<td>241.7 ± 24.9</td>
<td>356.5 ± 35.4</td>
<td>360.0 ± 31.3</td>
<td>272.1 ± 19.8</td>
<td>309.4 ± 26.2</td>
<td>297.0 ± 19.7</td>
</tr>
</tbody>
</table>

### Table 3.4. TAUCs of TAGs, NEFAs, insulin and glucose

<table>
<thead>
<tr>
<th></th>
<th>NSW baseline</th>
<th>NSW TSD</th>
<th>NSW recovery</th>
<th>SW baseline</th>
<th>SW TSD</th>
<th>SW recovery</th>
<th>All subjects baseline</th>
<th>All subjects TSD</th>
<th>All subjects recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAGs (mmol/L)</td>
<td>502.3 ± 36.4</td>
<td>467.5 ± 39.6</td>
<td>566.0 ± 47.1</td>
<td>444.4 ± 51.9</td>
<td>420.9 ± 40.7</td>
<td>472.3 ± 40.9</td>
<td>475.8 ± 30.8</td>
<td>446.1 ± 28.2</td>
<td>523.0 ± 32.6</td>
</tr>
<tr>
<td>NEFAs (mmol/L)</td>
<td>53.3 ± 3.2</td>
<td>48.4 ± 3.6</td>
<td>44.5 ± 2.4</td>
<td>48.5 ± 3.6</td>
<td>44.7 ± 3.8</td>
<td>43.2 ± 2.1</td>
<td>49.6 ± 2.4</td>
<td>46.7 ± 2.6</td>
<td>43.9 ± 1.8</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>12989 ± 1506.5</td>
<td>13999 ± 1457.6</td>
<td>19688 ± 2606.6</td>
<td>12390 ± 1165.3</td>
<td>12917 ± 1360.5</td>
<td>14165 ± 1305.9</td>
<td>12701 ± 944.1</td>
<td>13273 ± 960.8</td>
<td>17055 ± 1576.9</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>1586.2 ± 35.6</td>
<td>1579.8 ± 49.7</td>
<td>1622.1 ± 51.7</td>
<td>1559.5 ± 32.5</td>
<td>1601.4 ± 33.3</td>
<td>1679.3 ± 63.3</td>
<td>1561.1 ± 29.3</td>
<td>1589.7 ± 30.4</td>
<td>1602.5 ± 28.8</td>
</tr>
</tbody>
</table>

* P < 0.05 and ** P < 0.01, compared to after baseline sleep
† P < 0.05 and †† P < 0.01, compared to after TSD
3.3.6 Correlations with shift durations

The SW (n = 11) had worked shifts for various lengths. Especially because there were 3 SW who had worked shifts for more than 15 years (aged 42 ± 3.6 years (mean ± SD)), it was of interest to assess whether these SW were different from the other 8 SW (aged 33.4 ± 6.9 years). Plots of TAGs, NEFAs, insulin and glucose in relation to shift duration are shown in Figure 3.5. Statistical analyses could not be carried out because of the small subject number and unequal distribution of shift duration across the subjects but there was no clear visual relation between either the IAUCs or basal TAG, NEFA or insulin levels and the duration of shift work.

3.3.7 Cholesterol and HDL-cholesterol

In agreement with the screening results described in Table 3.1, two-factor ANOVA did not show any overall differences between NSW and SW (Figure 3.6). In addition, the independent Student's T-tests did not reveal any differences between the groups in the mornings and evenings of the adaptation and baseline days. Furthermore, two-factor ANOVA did not show any significant effect of time point, indicating there was no time of day variation and no effect of sleep deprivation or recovery sleep on cholesterol and HDL levels.
Figure 3.5. Relationship between shift duration and individual incremental areas under the curve (IAUCs) as well as basal metabolite and insulin levels.

IAUCs (left) or basal levels (right) and shift work duration for TAGs, NEFAs (no IAUC), insulin and glucose following baseline sleep (●), total sleep deprivation (TSD) (□) and recovery sleep (▲). n = 13 (12 for insulin) for NSW and n = 11 for SW; N/A, not applicable.
Figure 3.6. Plasma cholesterol and HDL.
Plasma (A) cholesterol and (B) HDL on the first evening before adaptation sleep (○) and in the mornings (black) and evenings (grey) following adaptation sleep (○), baseline sleep (●), sleep deprivation (□) and recovery sleep (▲) in NSW, SW and all subjects combined. Graphs display means (horizontal lines) and raw data points. n = 14 for NSW and 11 for SW apart from some time points where values were missing. Interpolated values are not included.
3.4 Discussion

This is the first study to report the effect of total sleep deprivation and recovery sleep \textit{per se} on postprandial metabolic and insulin responses to a standard breakfast. The responses of experienced SW with long term exposure to shift work for 5 years or more were compared to NSW in well controlled laboratory conditions.

Morning basal TAG levels were significantly lower after TSD compared to after baseline and recovery sleep. This finding is in agreement with observations using a protocol consisting of isocaloric meals every 3 hours, which showed that 3 to 5 days of TSD deprivation resulted in a significant decrease in the amplitude of the TAG rhythm and significantly lower TAG levels in the morning (Vondra et al. 1986). Ilan et al. (1992) also reported that 76-80 h TSD resulted in a decrease of TAG levels, although food intake was \textit{ad libitum} and body posture was not controlled. The lower TAG levels in the morning after total sleep deprivation may, in part, be due to higher energy expenditure whilst staying awake during the night, even though in the current study the subjects were instructed to remain semi-recumbent throughout. In agreement with this hypothesis, animal studies have reported lower/unaltered TAG levels after sleep deprivation, accompanied by weight loss despite higher food/calorie intake, indicating a higher turnover of nutrients during sleep deprivation (Andersen et al. 2004; Everson & Wehr 1993; Martins et al. 2010). Alternatively, these observations could be due to impaired nutrient absorption. In agreement with the current finding, previous studies did not observe any differences in fasting glucose and insulin levels between baseline sleep and total sleep deprivation (Schmid et al. 2007) or partial sleep restriction (Buxton et al. 2010; Donga et al. 2010; Nedeltcheva et al. 2009). Other studies on partial sleep deprivation did not report the fasting glucose and insulin levels (Spiegel et al. 1999; Tasali et al. 2008).

Although energy utilisation may increase during sleep deprivation, the way the body deals with subsequent energy intake following a meal might be different. In the data of all subjects combined, glucose levels 4 h following the standard breakfast did not return to basal levels after TSD. In addition, insulin levels 4 h after the breakfast were significantly higher after TSD than after baseline sleep. The slower glucose clearance despite similar or increased insulin levels indicates insulin insensitivity (DeFronzo 1988; Reaven 2002). This finding is in accordance with the hypothesis and previous sleep deprivation studies (Buxton et al. 2010; Donga et al. 2010; Nedeltcheva et al. 2009; Spiegel et al. 1999;
Tasali et al. 2008; VanHelder et al. 1993). As glucose and insulin production and metabolism are tightly controlled by the autonomous nervous system (Iversen et al. 2000), it has been suggested (section 1.4.3) also by other authors (Spiegel et al. 1999), that the insulin insensitivity after sleep deprivation may be due to an altered balance between the parasympathetic and sympathetic nervous system. More specifically, it could be that the hypothalamus and in particular, the wake promoting factor orexin (Saper et al. 2005), plays a role in this process. Neurons from the hypothalamus innervate fat tissue, the liver and the pancreas (Kreier et al. 2006) and orexin has been shown to stimulate sympathetic neurons projecting to these tissues (van den Top et al. 2003), which would lead to, for example, increased glucose mobilisation and altered insulin sensitivity (Shiuchi et al. 2009; Yi et al. 2009). Postponing sleep and thus increasing orexin levels (section 1.4.3) could subsequently result in an overstimulation of the sympathetic nervous system and higher glucose mobilisation.

Significant effects of TSD were observed on basal TAG levels and 4 h postprandial glucose and insulin levels. After a 4 h recovery nap followed by an 8 h overnight recovery sleep the basal TAG levels were significantly increased compared to after the sleep deprivation night in both groups. In NSW the average basal TAG level after recovery sleep was elevated above the levels reported to be associated with smaller and denser low-density lipoprotein production (TAG levels > 1.5 mmol/L) which may increase the risk for CVD (Griffin et al. 1994). However, it has to be kept in mind that the basal samples in the current study were taken after a fasting period of 10.5 h and are thus not strictly considered fasting samples.

Basal NEFA levels were significantly lower after recovery sleep compared to baseline sleep in NSW and in all subjects combined. Basal insulin levels were significantly higher after recovery sleep than after baseline sleep and TSD in NSW, suggestive of insulin insensitivity.

The overall TAG and insulin responses were higher and NEFA levels were lower after recovery sleep. Such a decrease in NEFA levels has only been reported in one sleep deprivation study, but then after TSD (Schmid et al. 2007). The increased IAUC for insulin suggests that this increase after recovery sleep is likely to be independent of the change in basal levels. Similar glucose levels despite a significantly larger insulin response indicate insulin insensitivity (Figure 1.1). Moreover, this hyperinsulaemic state would result in larger hepatic conversion of NEFAs to TAGs and suppresses lipid mobilisation by HSL (section 1.5.1.1.2), leading to hypertriglyceridemia (DeFronzo 1988;
Reaven 2002), which may explain the lower postprandial NEFA levels after recovery sleep compared to baseline sleep. These results after recovery sleep are unexpected and not in agreement with the hypotheses as most of the parameters in other studies show the largest alterations following sleep deprivation and parameters either remain changed after recovery sleep or start to return to basal levels (e.g. Mullington et al. 2003; Spiegel et al. 1999; van Leeuwen et al. 2009) (section 1.6). However, in parallel with our findings another study showed that the TAG AUC was increased on the second day of night shift work compared to day shifts but was even larger after 2 days following the return to daytime work (Lund et al. 2001), indicating that responses can be largest after the recovery process. There is no satisfactory explanation, however, as to why some parameters would change after recovery sleep. This may either be a direct effect of recovery sleep or a delayed effect of sleep deprivation.

Future studies including a longer recovery period or longer periods of (partial) sleep deprivation may help to clarify this issue. In addition, most of the previous studies have used OGTTs and IVGTTs to assess insulin sensitivity (although the euglycaemic clamp is regarded as the gold standard (Wallace & Matthews 2002)). These tests, however, are technically harder to conduct than postprandial sampling, but they may be more accurate and sensitive than assessing postprandial responses after breakfast and therefore yield different results. Alternatively, another method to get an index of postprandial insulin sensitivity (although not validated) would be to use a model proposed by Caumo and colleagues (Caumo et al. 2000). Only AUCs for glucose and insulin need to be entered into the model and it is therefore a very practical method. However, due to time constraints and the fact that we only recorded oral carbohydrate rather than glucose intake (which is required by the model), these calculations were not carried out for the current study.

As expected cholesterol and HDL did not change throughout the experiment suggesting that these metabolites are indeed not affected by sleep deprivation or recovery sleep. In addition, there were no significant differences between NSW and SW, but there was one shift worker with higher HDL levels, which agreed with the screening results provided by the Royal Surrey Hospital (appendix F).

Although there were no significant differences between NSW and SW in TAGs, NEFAs, insulin and glucose when they were assessed in the same ANOVA, separate analyses of NSW and SW revealed some differences. In almost all analyses, the effects of sleep deprivation and recovery sleep were more pronounced in NSW than in SW. It could be
speculated that SW in this study appeared to be habituated to sleep deprivation, in
closest to the hypothesis. Although their postprandial responses appeared to be less
sensitive to variation, this does not necessarily provide an indication to their
cardiovascular risk. A 10-year follow-up study showed that initial improved glycaemic
control is related to decreased cardiovascular risk and that despite early loss in
differences in glycated haemoglobin (an important risk factor for microvascular disease)
the reduced microvascular risk persists during follow-up (Holman et al. 2008). These
observations could imply that cardiovascular damage may have an early onset and may
persist despite reversal of risk factors such as glycaemic control. The observed
differences between SW and NSW might also be explained by other factors linked to the
ability to cope with shift work and sleep deprivation and not to shift work directly. Some
examples of these factors are morning and eveningness as assessed by the HÖ
questionnaire, the PER3 polymorphism and genetic variation in the adenosinergic
system (section 1.8). Eveningness has been proposed to be associated with a better
tolerance for sleep deprivation and more flexibility in sleep timing, hence a better ability
to cope with shift work (Akerstedt & Torsvall 1981; Mongrain et al. 2006a; Mongrain et al.
2004; Taillard et al. 1999; Volk et al. 1994). Although SW and NSW in this study did not
have significantly different HÖ scores, SW had a slightly wider range of scores and a
lower mean HÖ score (Table 3.1). Since a lower HÖ score is associated with a stronger
preference for eveningness, this would support the previous statements, as the SW
seemed less responsive within the protocol. However the association with
morningness/eveningness is more a neurobehavioural one and it is not known whether
eveningness is also associated with a better physiological tolerance to sleep deprivation.
The lack of major differences in the postprandial responses between SW and NSW may
also be explained by the fact that the meal was given at the normal clock time. Previous
studies reporting postprandial differences have given meals at an abnormal clock time
(Hampton et al. 1996; Knutsson et al. 2002; Lund et al. 2001; Ribeiro et al. 1998; Scheer
et al. 2009) suggesting food intake at an inappropriate clock time may be a major
contributor to the adverse effects of shift work.

One of the limitations of this study was the fact that the postprandial response was only
measured for up to 4 h after the standard breakfast. Unfortunately, this time period could
not be lengthened because lunch had to be included in the protocol before the scheduled
nap. The peak of the TAG response was not observed, as this usually occurs up to 4 h
postprandially (section 1.5.1.1.2) and it can take approximately 9 h for TAGs to return to
basal levels after a meal (Lund et al. 2001; Ribeiro et al. 1998; Sopowski et al. 2001).
The decrease in TAGs observed after TSD may thus not be a decrease but a delay in
the TAG response. Unfortunately the protocol is unable to distinguish between these two phenomena. Future research should include longer sampling periods to assess how long it takes for TAGs, NEFAs, insulin and glucose to return to basal levels. Alternatively, subjects could be monitored after both breakfast and lunch as the postprandial hormone and metabolic responses may be additive if the time between the two meals is short.

In conclusion, this is the first study to assess basal and postprandial insulin and metabolic responses after TSD and recovery sleep and to compare NSW and SW in controlled laboratory conditions. Significantly lower basal TAG levels after TSD indicate higher energy expenditure during sleep deprivation, despite any increased physical activity. Postprandial TAG and insulin responses were larger after recovery sleep, suggestive of insulin insensitivity. These results might be explained by an altered balance between the parasympathetic and sympathetic nervous system. The more pronounced effects of sleep deprivation and recovery sleep observed in the NSW require further study.
CHAPTER 4 FLOW-MEDIATED DILATATION AND HEART RATE VARIABILITY

4.1 Introduction

Endothelial dysfunction, decreased flow-mediated dilatation (FMD) and changes in heart rate variability (HRV) have been shown to be risk factors for CVD (Bots et al. 2005; Buccelletti et al. 2009; Koskinen et al. 2009; Manfrini et al. 2008; Moens et al. 2005; Perciaccante et al. 2006; Rabbone et al. 2009; Shechter et al. 2009; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996; Thayer et al. 2010) (section 1.1). Although it is thought that the risk for CVD in SW depends on shift work duration, the extent of circadian misalignment and sleep deprivation (Knutsson & Boggild 2000) (sections 1.2.2 and 1.3.2), hardly any epidemiological studies have been conducted indicating an association between shift work or sleep deprivation on one hand and lower FMD or changes in HRV on the other hand. One field study has shown lower FMD in experienced SW (Amir et al. 2004). By contrast, HRV has been assessed more frequently in field studies of SW (Togo & Takahashi 2009) (section 1.7). These studies predominantly showed a shift in the balance between sympathetic and parasympathetic innervation. Only a few studies have reported changes in HRV (mainly higher sympathetic activity) and decreased vasodilation during and after (partial) sleep deprivation (Ewing et al. 1991; Sauvet et al. 2009; Spiegel et al. 1999; Zhong et al. 2005) (section 1.6).

These studies have indicated that sleep deprivation and shift work may contribute to a lower endothelial function and altered HRV. However, especially the shift work studies were not well-controlled for various confounding factors such as circadian phase, assessment of actual obtained sleep (or time in bed), physical activity and food intake. In addition, they often had a poor description of the shift work population and lacked an appropriate control group. Therefore, it is unclear whether the observed effects of shift work are due to these confounding factors or sleep deprivation per se. Moreover, to date SW have not been compared directly to NSW in the same study, endothelial function has not been assessed by the ultrasound method identical to the one used in our research group and HRV parameters in the time domain have not been reported widely.
4.1.1 Aims and hypotheses

The aim of the current study was thus to investigate the effect of one night of total sleep deprivation (TSD), a recovery nap and recovery sleep on FMD and HRV in controlled laboratory conditions and to assess the responses of experienced SW compared to NSW in the same study.

It was hypothesised that:

- TSD will induce changes in FMD and HRV. FMD and variance in HRV will be lower but sympathetic activity will be higher after TSD.
- A nap and recovery sleep will (partly) reverse these changes.
- SW and NSW will respond differently to TSD and recovery sleep. FMD and variance will be lower, whereas sympathetic activity will be higher after TSD in SW than in NSW and changes in FMD and HRV will not recover as quickly in SW as in NSW.

4.2 Methods

Methods specific to this chapter are described below. For general methods see chapter 2.

4.2.1 Subjects

All 25 subjects underwent ultrasound recordings and HRV assessments. Their individual screening results can be found in appendix F; the averages for the NSW and SW groups are given in Table 3.1.

4.2.2 Flow-mediated dilatation

4.2.2.1 Ultrasound recordings

High-resolution recordings with an ultrasound machine (Acuson Aspen, Siemens, Bracknell, Berkshire, UK) were used to assess endothelial function by flow-mediated dilation (FMD) (Celermajer et al. 1992) at 0.75 and 10.75 h after habitual wake up time
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(Figure 2.1). The technique and conditions used for the ultrasound recordings were established by Donald and colleagues at Great Ormond Street Hospital, London, UK (e.g. Charakida et al. 2005; Donald et al. 2006) and used and reported by our research group (Hampton et al. 2010; Walters et al. 2006) in accordance with guidelines from the International Brachial Reactivity Task Force (Corretti et al. 2002). Heart rate was recorded by the ultrasound machine with EGG electrodes (3 M (red dot) ECG monitoring electrodes, Medisave, Weymouth, UK) on 3 different locations; the upper and lower side of the sternum and near the lowest left rib. The subject was put in a supine position with the right arm in a 90° angle with respect to the body. The arm rested on a stool levelled with the bed. A pressure cuff was placed around the forearm immediately below the medial epicondyle, but left deflated until needed. The right brachial artery was imaged, 5 to 10 cm above the antecubital fossa, with a high resolution ultrasound probe held in a stereotactic clamp attached to the stool (Table 4.1).

Figure 4.1. Ultrasound set up.
Ultrasound images from the brachial artery in the right arm are recorded during 220 frames for 11 min, with an ultrasound probe held in a stereotactic clamp.

Location of the artery rather than a vein was confirmed by Doppler images. Optimal contact was achieved by applying ECG electrode gel (Scanmed medical instruments, Gloucestershire, UK). Depth and gain settings were chosen such that the lumen-intima contrast was optimal and kept the same within each recording. When a clear image of the artery had been obtained, the image was magnified. Hereafter, an 11 min recording was started (Brachial Imager™, Iowa City, Iowa, USA), capturing ECG-triggered images.
approximately every 3 sec. First of all, the brachial artery was imaged at rest for 1 min. Then the pressure cuff on the forearm was inflated to 200 mmHg, thereby occluding the brachial artery. After 5 min of occlusion the cuff was deflated and recording was continued for another 5 min when returning to rest. The images were recorded both onto a computer (Brachial Imager™) connected to the ultrasound machine and a video-tape inserted in the ultrasound machine (as a back up). Recording in Brachial Imager™ also allowed a second operator to make changes in contrast and brightness during the recording if necessary. Position of the probe (with respect to the antecubital fossa) and the arm were noted down and the position of the probe was marked after the first recording with a permanent marker to ensure that each recording was taken from the same part of the brachial artery.

4.2.2.2 Analysis

A region of interest (ROI) was chosen in which the brachial artery wall was tracked with edge detection software (Brachial Analyzer™, Iowa City, Iowa, USA) as clearly as possible, especially during rest and deflation. Within this ROI, the average arterial diameter was calculated for each of the 220 frames (Brachial Analyzer™) (Figure 4.2). A fit of the tracked edges with the arterial wall of over 80% was aimed for for each frame. In some cases Brachial Analyzer™ did not achieve this fit or did not fit the layer lining the vessel lumen (consistently) although the fit would be over 80 % (Figure 4.3 A). This can be due to either individual anatomy or the quality of the recording and any falsely tracked edges were rejected.

FMD was calculated as the maximum percentage change in diameter after cuff deflation compared to the baseline diameter. This percentage was calculated by the average of the 3 highest consecutive values for the diameter during and after cuff deflation as a percentage change of the average of 20 frames of resting diameters. The analyses were performed by myself with Dr Shelagh Hampton as a second operator. If the diameters at rest and at the end of deflation were not the same (Figure 4.3 B), the diameter after deflation was used for calculation of the FMD (34.8% of the re-assessed recordings), as this can be more reliable for example when occlusion causes movement resulting in under- or overestimation of the difference between the peak and resting diameter. The CVs averaged for all subjects, for the morning and afternoon resting diameters were 2.9 and 3.3% respectively, which was similar to an earlier reproducibility assessment by
Walters *et al.* (2006). Some data (7.5%) were not present because of missing data or poor quality recordings.

Figure 4.2. An example of the screen in Brachial Analyzer™.
The left window shows the ultrasound recording with the brachial artery, the region of interest (ROI), the tracked wall of the brachial artery, the ECG and part of the Doppler. The right window shows the diameter for every frame, composing a graph which displays the diameter at rest, during occlusion and deflation. The table contains the diameter (dia.), standard deviation (STD.) and percentage of the fit (Conf. (%)) for every frame and other information e.g. how many frames were valid.

Figure 4.3. Two examples of ultrasound recordings that needed editing.
In the left diagram (A) Brachial Analyzer™ does not track the layer lining the vessel lumen and therefore probably not the tunica intima. In the right diagram (B) the resting diameter before occlusion and at the end of deflation are not the same. Both situations can result in under- or overestimation of the FMD.
4.2.3 Heart rate variability

The ECG was derived from 2 positions, right mid-clavicular and around 6 cm under the left arm pit using ECG pads (electrode ECG wet gel adult short term 38 mm diameter foam backed offset stud Ambu Blue Sensor P-OO-S Pads, NHS Logistics Authority, Alfreton, UK) and ECG electrodes (Compumedics, Abbotsford, Australia). Data were acquired, stored, converted and imported into Somnologica Studio (version 5.0.1 build 1662, Embla, supplied by Stowood Scientific Instruments Ltd., Oxford, UK) for further analysis as described in section 2.5.4. HRV was assessed at set periods. On each of the four study days 5 x 5-min analysis intervals were chosen 0.25, 4.25, 11.5, 12.5 and 13.5 h (Figure 2.1) after habitual wake up time when the subjects were required to be semi-recumbent for the Karolinska Drowsiness Test (KDT) (section 5.2.3). During the sleep deprivation night, data were combined into 13 x 30-min analysis periods starting 17 h after habitual wake up time. The detection of QRS complexes was automatically done using Somnologica algorithm settings (5 min analysis bins, data ± 5 SD from the mean of the preceding 5 s of data were considered as potential R-tops, a minimum 5 consecutive R-tops were needed for a valid sequence, an output sampling rate of 1 Hz was used, minimum and maximum heart rate was set at 25 and 350 beats per minute (bpm), respectively in compliance with the Task Force recommendations (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996). Correct detection was manually confirmed and falsely detected or undetected QRS complexes (artefacts, mainly caused by noise or a lost signal) were removed (< 1% of the analysis period for the day time assessments and < 3% for the sleep deprivation night). Examples of recordings with and without artefacts are shown in Figure 4.4.

An HRV report based on the artefact-free data was automatically generated containing the following parameters: NN interval (interval between adjacent QRS complexes), RMSSD, standard deviation of differences between NN intervals (SDSD), SDNN/mean of the standard deviations of all NN intervals for all 5 min segments in the recording (SDNN index), percentage of NN intervals longer than 50 ms (pNN50), very low frequency power (VLF: 0.003 – 0.04 Hz), low frequency power (LF: 0.04 – 0.15 Hz), high frequency power (HF: 0.15 – 0.4 Hz), total power (TP), LF norm (LF/(LF+HF)), HF norm (HF/(LF+HF)) and LF/HF. All 13 analysis bins for the sleep deprivation night were complete apart from one NSW whose NN-interval data were excluded as an outlier (> 2.3 SD from the mean).
Figure 4.4. Examples of ECGs and heart rate, NN interval and heart rate variability traces in Somnologica.

The figures represent parts of 30 s epochs of the ECG (upper panel), heart rate (bpm) (2nd panel), NN interval (RR interval (s), 3rd panel) and heart rate variability (evenly sampled NN interval differences (bpm/s)) (bottom panel). Figure A is an example of a recording without artefacts and Figure B shows how Somnologica automatically removed the ECG artefact, caused by a lost signal, in the other 3 traces.
Figure 4.4. Examples of ECGs and heart rate, NN interval and heart rate variability traces in Somnologica.

The figures represent parts of 30 s epochs of the ECG (upper panel), heart rate (bpm) (2nd panel), NN interval (s) (RR interval, 3rd panel) and heart rate variability (evenly sampled NN interval difference (bpm/s)) (bottom panel). Failure to detect a heart beat (C) or detection of a heart beat while there was no heart beat present (D) results in false negatives and false positives in Somnologica's heart rate, NN interval and heart rate variability traces (E).
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Figure 4.4. Examples of ECGs and heart rate, NN interval and heart rate variability traces in Somnologica.

The figures represent parts of 30 s epochs of the ECG (upper panel), heart rate (beats per minute (bpm)) (2nd panel), NN interval (s) (RR interval, 3rd panel) and heart rate variability (evenly sampled NN interval difference (bpm/s)) (bottom panel). The detection of heart beats (QRS complexes) is checked. False negatives (C) or positives (D) are manually marked as artefacts in the ECG (E) and Somnologica removes these parts from the heart rate, NN interval and heart rate variability trace (F).
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For the 5 min analysis bins some data were missing and some bins were considered outliers according to the same criteria (total missing values comprised < 7.5% of the data set).

4.2.4 Statistics

The HRV assessments during the sleep deprivation night were analysed using a two-factor ANOVA (factors ‘time’ (13 levels) and ‘group’ (SW and NSW)). A mixed-model regression analysis was conducted in SAS 9.1 (section 2.6.4) to determine main effects of ‘group’, ‘day’ and ‘time’ and interactions between those effects on resting brachial diameter, FMD and HRV. Subsequently, body weight was entered into the analysis of resting brachial diameter as a random effect in order to assess whether there was an association between body weight and brachial diameter.

4.3 Results

4.3.1 FMD

There was no significant effect of day or time on the resting brachial diameter and no day*time interaction. However, there was a significant effect of group ($F_{1,169} = 12.8$, $P < 0.001$) with a significantly higher resting diameter in SW compared to NSW. Body weight as a random effect was significantly associated with resting diameter ($P < 0.001$) and the observed effect of group on resting diameter became non-significant when body weight was used as a random effect.

The %FMD in both SW and NSW in the morning and afternoon is shown in Figure 4.5. There were no significant effects of day, time, group or interactions on %FMD. However, a trend for a group effect was observed ($F_{1,169} = 3.3$, $P = 0.07$) with a lower %FMD in SW compared to NSW.

4.3.2 HRV

4.3.2.1 HRV during TSD

Examples of time and frequency domain parameters, SDNN index and LF/HF, during sleep deprivation are shown in Figure 4.6.
Figure 4.5. Flow-mediated dilatation.
%FMD (mean ± SEM) in (A) the morning (0.75 h after habitual wake time) and (B) the afternoon (10.75 h after habitual wake time), following adaptation sleep, baseline sleep, sleep deprivation and recovery sleep in NSW (•) (n = 14) and SW (○) (n = 11).

Figure 4.6. SDNN and LF/HF during sleep deprivation.
HRV during (A) SDNN and (B) LF/HF (mean ± SEM) in NSW (•) (n = 14) and SW (○) (n = 11) during sleep deprivation (from 17 to 23 h after waking). * P < 0.05, ** P < 0.01 and *** P < 0.001 compared to 17 h after waking.
Average values for the other HRV parameters across the TSD night are shown in Table 4.1. There was a significant effect of time on SDNN ($F_{4,6,106} = 8.9$, $P < 0.001$), VLF ($F_{7,1,164} = 11.1$, $P < 0.001$), LF ($F_{5,6,129} = 2.7$, $P < 0.05$) and TP ($F_{6,6,151} = 11.5$, $P < 0.001$) with the values increasing through the night. There were no significant effects of group, time or interaction on any of the other parameters.

Table 4.1. HRV parameters of non-shift workers and shift workers during sleep deprivation.

<table>
<thead>
<tr>
<th>Time domain</th>
<th>Non-shift workers</th>
<th>Shift workers</th>
</tr>
</thead>
<tbody>
<tr>
<td>NN interval (ms)</td>
<td>917 ± 26</td>
<td>937 ± 24</td>
</tr>
<tr>
<td>pNN50 (%)</td>
<td>22 ± 4</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>SDNN index (ms)</td>
<td>88 ± 6</td>
<td>87 ± 7</td>
</tr>
<tr>
<td>SDSD (ms)</td>
<td>33 ± 4</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>RMSSD (ms)</td>
<td>47 ± 5</td>
<td>42 ± 4</td>
</tr>
</tbody>
</table>

Frequency domain

<table>
<thead>
<tr>
<th></th>
<th>Non-shift workers</th>
<th>Shift workers</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP (ms$^2$)</td>
<td>20664 ± 1455</td>
<td>21225 ± 1896</td>
</tr>
<tr>
<td>VLF (ms$^2$)</td>
<td>13216 ± 1307</td>
<td>13563 ± 1501</td>
</tr>
<tr>
<td>LF (ms$^2$)</td>
<td>6014 ± 477</td>
<td>6386 ± 644</td>
</tr>
<tr>
<td>HF (ms$^2$)</td>
<td>1335 ± 112</td>
<td>1179 ± 132</td>
</tr>
<tr>
<td>LF/HF</td>
<td>5.3 ± 0.6</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>LF norm (n.u.)</td>
<td>0.8 ± 0.0</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>HF norm (n.u.)</td>
<td>0.19 ± 0.02</td>
<td>0.15 ± 0.02</td>
</tr>
</tbody>
</table>

Data are means ± SEM during the sleep deprivation night (17 – 23 h after waking). $n = 11$ for SW and $n = 14$ for NSW (apart from the NN interval for which $n = 13$). No significant effects of group or group*time interactions were shown by two-factor ANOVA.

Abbreviations used: HF, high frequency power (0.15 – 0.4 Hz); HF norm, normalized high frequency power (HF/(LF+HF)) in normalized units (n.u.); LF, low frequency power (0.04 – 0.15 Hz); LF norm, normalized low frequency power (LF/(LF+HF)) in normalized units (n.u.); LF/HF, low frequency power divided by high frequency power; NN-interval, normal-to-normal interval (intervals between QRS complexes); pNN50, percentage of intervals longer than 50 ms; RMSSD, square root of the mean of the sum of the squares of differences between NN intervals; SDNN index, mean of the standard deviations (SD) of all NN intervals for all 5 min segments in the recording; SDSD, SD of differences between NN intervals; TP, total power; VLF, very low frequency power (0.003 – 0.04 Hz).
4.3.2.2 HRV across the study days

The SDNN and LF/HF after adaptation and baseline sleep, during and after sleep deprivation and after recovery sleep are shown in Figure 4.7. Although for completeness the data during TSD are shown again in Figure 4.7, the statistical analysis mentioned hereafter only includes the 5 min day time points on each of the 4 study days. There was a significant effect of group ($F_{1,430} = 15.1, P < 0.001$) and a significant day*time interaction ($F_{12,430} = 2, P < 0.05$) on SDNN. SDNN was significantly lower in SW than in NSW ($P < 0.001$). The most consistent observation emerging from the day*time interaction was that SDNN following a night of TSD (0.25 h after habitual wake up time) was significantly higher than at all other time points ($P < 0.001$ for all points apart from 0.25 h after waking up following baseline sleep for which $P < 0.01$) (Figure 4.7 A).

There was also a significant effect of group ($F_{1,417} = 4.2, P < 0.05$), day ($F_{3,417} = 5.4, P < 0.01$) and time ($F_{4,417} = 12.7, P < 0.001$) on LF/HF. LF/HF was significantly higher in SW than in NSW ($P < 0.05$). LF/HF was significantly lower after adaptation sleep than after baseline sleep and TSD ($P < 0.01$) and recovery sleep ($P < 0.001$). In addition, LF/HF was significantly higher 13.5 h after waking than at 4.25 h after waking and significantly higher at 13.5, 12.5 and 4.25 h than at 11.5 and 0.25 h after waking (Figure 4.7 B).

Most of the other HRV parameters also showed significant effects of group, day and time (Table 4.2). NN was higher and pNN50 was significantly lower in SW than in NSW ($P < 0.05$). Both NN and pNN50 changed significantly across study days with higher NN and pNN50 after adaptation sleep than after baseline sleep, TSD and recovery sleep and higher NN and pNN50 after baseline sleep and TSD than after recovery sleep (Figure 4.8 A and B). RMSSD and SDSD were significantly lower in SW than in NSW ($P < 0.001$ and $P < 0.01$, respectively). In addition, RMSSD and SDSD were significantly higher after adaptation sleep, baseline sleep and TSD than after recovery sleep (Figure 4.8 C and D). LFnorm had the same significant effects as LF/HF and estimates for the effects were in the same direction, whereas the estimates for HF norm had the opposite sign. Both TP and VLF showed no group effect but a day*time interaction ($P < 0.01$). A significant effect of time was observed for LF ($P < 0.01$) with higher LF 0.25 h after waking than at the other time points.
A adaptation baseline sleep deprivation recovery

SDNN (ms)

Time after waking (h)

Figure 4.7. Daytime SDNN and LF/HF.

(A) SDNN and (B) LF/HF (mean ± SEM) in NSW (●) (n = 14) and SW (○) (n = 11) 0.25, 4.25, 11.5, 12.5 and 13.5 h after habitual wake time following adaptation sleep, baseline sleep, sleep deprivation and recovery sleep.
### Table 4.2. P-values for main effects and interactions for all HRV parameters at 5 time points across the 4 study days.

<table>
<thead>
<tr>
<th>Time domain</th>
<th>NN</th>
<th>pNN50</th>
<th>SDNN</th>
<th>SDSD</th>
<th>RMSSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.0002</td>
<td>0.0021</td>
<td>0.0003</td>
</tr>
<tr>
<td>Time</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Group</td>
<td>0.0465</td>
<td>0.0333</td>
<td>0.0001</td>
<td>0.0013</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Day*time</td>
<td>0.5997</td>
<td>0.0666</td>
<td>0.0267</td>
<td>0.1328</td>
<td>0.2869</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Frequency domain</th>
<th>TP</th>
<th>VLF</th>
<th>LF</th>
<th>HF</th>
<th>LF/HF</th>
<th>LF norm</th>
<th>HF norm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>0.0012</td>
<td>0.0034</td>
<td>0.0747</td>
<td>&lt;.0001</td>
<td>0.0012</td>
<td>0.0007</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Time</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.0086</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Group</td>
<td>0.0546</td>
<td>0.0896</td>
<td>0.3487</td>
<td>0.0008</td>
<td>0.0405</td>
<td>0.0268</td>
<td>0.0130</td>
</tr>
<tr>
<td>Day*time</td>
<td>0.0070</td>
<td>0.0037</td>
<td>0.3307</td>
<td>0.5211</td>
<td>0.9377</td>
<td>0.7508</td>
<td>0.4848</td>
</tr>
</tbody>
</table>

Values in bold indicate statistical significance. n = 11 for SW and n = 14 for NSW. No significant day*group, time*group and day*time*group interactions were shown by two-factor ANOVA.

Abbreviations used: HF, high frequency power (0.15 – 0.4 Hz); HF norm, normalized high frequency power (HF/(LF+HF)) in normalized units (n.u.); LF, low frequency power (0.04 – 0.15 Hz); LF norm, normalized low frequency power (LF/(LF+HF)) in normalized units (n.u.); LF/HF, low frequency power divided by high frequency power; NN-interval, normal-to-normal interval (intervals between QRS complexes); pNN50, percentage of intervals longer than 50 ms; RMSSD, square root of the mean of the sum of the squares of differences between NN intervals; SDNN, standard deviation (SD) of all NN intervals; SDSD, SD of differences between NN intervals; TP, total power; VLF, very low frequency power (0.003 – 0.04 Hz).
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4.3.3 Correlations with shift durations

As in section 3.3.6 the association between shift duration and the assessed parameters was explored. There was no relation between shift duration and FMD in either the morning or the afternoon after TSD or recovery sleep (Figure 4.9 A). Furthermore, there was no relation between shift duration and relative power in the frequency domain such as LF/HF (Figure 4.9 B) during TSD. By contrast the SW with the longest shift work duration (> 15 years) had the lowest variance, for example SDNN ($r^2 = 0.7$, $P < 0.01$) (Figure 4.9 C) and the lowest pNN50 during TSD.

Figure 4.8. Day time NN interval, pNN50, SDSD and RMSSD.

(A) NN interval, (B) pNN50, (C) SDSD and (D) RMSSD after adaptation sleep, baseline sleep, sleep deprivation and recovery sleep in NSW (■) ($n = 14$) and SW (▲) ($n = 11$). Values are mean (of the day time averages of all subjects) ± SEM. See text for P-values.
Figure 4.9. Relation between FMD, SDNN and LF/HF and shift duration.
(A) FMD in the afternoon after sleep deprivation, (B) LF/HF during sleep deprivation and (C) SDNN during sleep deprivation versus shift duration.
4.4 Discussion

The present study investigated the effects of sleep deprivation and recovery sleep on cardiovascular function in controlled laboratory conditions. Non-invasive methods to assess cardiovascular function were used, namely FMD and HRV (Bots et al. 2005; Buccelletti et al. 2009; Koskinen et al. 2009; Manfrini et al. 2008; Moens et al. 2005; Perciaccante et al. 2006; Rabbone et al. 2009; Shechter et al. 2009; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996; Thayer et al. 2010). Experienced SW were compared directly to NSW matched for age, BMI and cholesterol levels in an identical laboratory setting with strict control of body posture, physical activity, meals, circadian phase and ambient lighting.

The results showed a trend towards a lower FMD in SW compared to NSW. This finding is in agreement with results from a study by Amir and colleagues (2004) who showed that baseline FMD was lower in physicians who had worked shifts for more than 3 years (8.6 ± 3.4% FMD) compared to those with a shorter shift work history (14.3 ± 4.1% FMD). This result may in part be due to sleep deprivation as daytime FMD in the physicians after a night shift was also significantly smaller (6.7 ± 4.8% FMD) compared to daytime FMD without a previous night shift (10.5 ± 4.5% FMD). In addition, vasodilatation has been shown to be lower after TSD when assessed by endothelium-dependent vasodilatation (after acetylcholine administration) at 12:00 and by endothelium-independent dilatation (after sodium nitroprusside solution administration) at both 12:00 and 18:00 (Sauvet et al. 2009). In contrast to the hypothesis, the current study, however, was not able to observe a significant effect of TSD on FMD. This discrepancy could be due to methodological differences between the studies (e.g. control of circadian phase and physical activity or a different method used to assess endothelial function).

In addition, the analysis of the resting brachial diameter revealed a larger diameter in SW than in NSW. This is in agreement with previous studies that showed a larger resting diameter in individuals with a lower FMD (Oflaz et al. 2006; Shechter et al. 2009; Witte et al. 2005). As these patient groups also had a higher BMI and weight seemed to be most related to resting diameter in the current study, weight was entered into the model for resting diameter analysis as a random effect. Weight and resting diameter appeared to be positively associated and entering weight into the model rendered the effect of group
non-significant, tying in with previous observations. These results indicate that resting diameter may be related to weight rather than shift work itself.

Significant differences were also observed between SW and NSW in HRV parameters. SDNN, RMSSD and SDSD were significantly lower and LF/HF was significantly higher in SW compared to NSW. This finding is in agreement with the idea that lower SDNN, RMSSD and SDSD reflect a lower variance and higher LF/HF indicates higher sympathetic activity, both factors being associated with a higher risk for CVD (Buccelletti et al. 2009; Perciaccante et al. 2006; Rabbone et al. 2009) in SW. Most previous studies (section 1.7) have shown that sympathetic activity (e.g. normalized LF or LF/HF) was higher on night duty than when off duty and asleep at night (Ewing et al. 1991; Furlan et al. 2000; Ito et al. 2001; Mitani et al. 2006; Rauchenzauner et al. 2009; Su et al. 2008) and was higher on days including a night shift compared to days including a morning shift (Van Amelsvoort et al. 2001a). Sympathetic activity has also been shown to be higher in SW compared to NSW when both groups work day shifts (Ishii et al. 2005; Ishii et al. 2004) or are asleep at home (Chung et al. 2009). One study in SW found lower SDNN and RMSDD during a night shift compared to sleep at night (Su et al. 2008). This is strengthened by the observation that the 3 SW with the longest shift work duration (> 15 years) had the lowest variance, e.g. SDNN, during sleep deprivation. By contrast, some studies have not been able to show this association between higher LF/HF and lower variability and shift work (Freitas et al. 1997; Murata et al. 2005; van Amelsvoort et al. 2001b). The current study confirmed that these predominant observations of a higher sympathetic activity and lower variability in field studies of SW are also detectable in SW studied in controlled laboratory settings. The consistent but non-significant trend of a higher LF/HF ratio in SW during TSD suggests that SW have a higher sympathetic activity than NSW during this period similar to when they are on day shifts (Ishii et al. 2005) or asleep (Chung et al. 2009). Thus, both the FMD and HRV data suggest that SW may exert higher levels of sympathetic activity on the cardiovascular system. A lack of a significant day*group interaction indicates that, in contrast to the hypothesis, this is an overall difference which is not only induced by acute sleep deprivation.

As in previous studies, it is not possible to extract a single cause for the observed differences between the SW and NSW. The long term effects of shift work may be a plausible explanation as all SW in the current study had worked shifts for 5 years or more and there are indications that alterations in endothelial function are already discernable after 3 years (Amir et al. 2004). The studies that have reported effects of shift work on HRV differ in the duration of shift work (range 0.5 – 37 years (Ishii et al. 2005; Mitani et
al. 2006; Munakata et al. 2001), average 3 – 17 years (Amir et al. 2004; Chung et al. 2009; Furlan et al. 2000; Murata et al. 2005; Su et al. 2008)) section 1.3.2. In addition, repeated sleep deprivation and/or circadian misalignment (Knutsson & Boggild 2000) of rhythms in HRV and endothelial function with the imposed lifestyle (e.g. sleep/wake cycle and physical activity) in unadapted SW could contribute to the effect of shift work duration on HRV.

In the present study, FMD and the daytime SDNN values in SW and NSW were lower than the values reported in healthy volunteers, namely a FMD above 7-10% (Moens et al. 2005; Shechter et al. 2009) and a SDNN above 70 ms (Buccelletti et al. 2009). This difference might be a result of the different study methodologies (e.g. controlled posture, diet and light in the current study may play a role) but is unlikely to be due to the techniques used as we have reported higher average FMD values using similar equipment and software (Hampton et al. 2010; Walters et al. 2006). In addition, time of day effects were observed for the HRV parameters. These findings suggest that for correct interpretation of FMD and HRV data, use of strict thresholds to indicate cardiovascular risk is not possible and that comparison with an appropriate control group in controlled conditions would be more valid.

The HRV parameters not only showed significant differences between SW and NSW but also across study days. The significantly higher SDNN observed following a night of TSD is in agreement with a study from Viola et al. (2002). The reason for this higher variance (also seen in RMSSD and SDSD) after sleep deprivation, is not known. A few studies have reported significantly higher sympathetic activity (Sauvet et al. 2009; Spiegel et al. 1999; Zhong et al. 2005) and a lower NN50 (Ewing et al. 1991) during and after TSD and PSD however, other studies have found no difference in HRV parameters in these conditions (Gould et al. 2009; Muenter et al. 2000). The reason for lower LF/HF after adaptation sleep compared to the other 3 study days is also not clear. Other unexpected observations were the lower RMSSD and SDSD observed after recovery sleep indicating a lower variance and a decrease in NN-interval and pNN50 throughout the study.

In addition to changes across days, changes within a day and circadian rhythms have been reported in LF (peak at 6:15 h) and HF (peak at 5:16 h) (Vandewalle et al. 2007), but not in other HRV parameters such as LF/HF in CR studies (Vandewalle et al. 2007; Viola et al. 2002). Although not a CR study, the current study also observed an increase in LF and no effect of time on LF/HF throughout the sleep deprivation night as well as a significant effect of time on HF during the day. Compared to the previous findings
(Vandewalle et al. 2007; Viola et al. 2002), however, HF did not show a significant increase during the sleep deprivation night and LF/HF showed a significant effect of time during the day.

SDNN also showed significant variation over the day and a significant increase during TSD. These observations are supported by previous studies which have shown a significant circadian rhythm in SDNN with a peak between 4:45 and 7:00 h (Bonnemeier et al. 2003; Vandewalle et al. 2007; Viola et al. 2002). It is unclear how an increase of SDNN throughout the night (with higher SDNN being associated with better cardiovascular health) is related to reports of a sudden increase in CVD events and stroke around habitual wake up time (e.g. Mulcahy et al. 1996; Muller 1999; Peckova et al. 1998; Turin et al. 2009; van der Palen et al. 1995). However, the contribution of endogenous circadian rhythms towards this phenomenon has rarely been separated from external factors (such as activity, food intake, report rate and presence of medical care) using appropriate study conditions such as a ‘CR’ protocol (Walters et al. 2003). On the other hand, the increased risk of experiencing life-threatening cardiovascular events in the early morning may be unrelated to the observed SDNN peak.

As mentioned in section 6.3.1 Somnologica software was not very flexible for HRV analysis and did not allow for data manipulation other than artefact removal. Therefore it would be useful to further explore HRV analysis with free software available online, which has extra options such as for detrending (of a low frequency baseline trend) and NN interval interpolation (Niskanen et al. 2004). The only requirement for the data to be imported is a series of NN intervals in a text file (without time stamps). This can be achieved by using Somnologica but is not straightforward. The software can either manually export the whole trace of 1 recording (e.g. 12 h) or event data if a period of interest is marked as an event. However, both methods create large, inaccessible files with 0 values where no QRS complex was detected and NN-intervals when there was a QRS complex. Alternatively, in the analysis settings the option ‘export NN intervals to patient folder’ can be chosen, which creates a text file with the NN interval per second for each recording. Also in this case, the time stamps are somewhat unclear and artefacts are omitted but not indicated in the file. This latter method is therefore applicable to generate a series of NN intervals but not ideal for very precise data and artefact manipulation. However, with appropriate expertise and training both programs could be used alongside each other to record the ECG, generate NN intervals and analyse HRV.
In conclusion, significantly lower variance, higher sympathetic activity and a trend for a lower FMD was observed in the SW compared to the NSW. These findings suggest that HRV and FMD have potential as non-invasive assessments of cardiovascular function and may be useful to assess CVD risk in this vulnerable group.
CHAPTER 5 CIRCADIAN PHASE, SLEEP, ALERTNESS AND MOOD

5.1 Introduction

Previous chapters have dealt with the main aims of this thesis; namely to assess the effects of sleep deprivation and shift work on metabolic and cardiovascular function. Whereas alterations in these parameters may be more evident after chronic exposure to sleep deprivation and shift work, mood and especially alertness are likely to show mainly acute responses to sleep deprivation.

There is ample research showing that sleep deprivation affects alertness (Lim & Dinges 2008, 2010; Rogers et al. 2003) (section 1.6). Although it is clear that sleep deprivation results in decreased alertness, the impact on mood seems to be less consistent. In most healthy people, sleep deprivation has been shown to increase negative mood (Pilcher & Huffcutt 1996) (section 1.6), while sleep deprivation has been reported to be a very effective treatment for depressive patients (Giedke & Schwarzler 2002; Hemmeter et al. 2010; Ringel & Szuba 2001).

Other studies have shown that shift work increases measures of sleepiness, due to the need to work at the wrong circadian phase and the concurrence of sleep deprivation (Akerstedt 1998, 2003; Akerstedt & Wright 2009; Harma et al. 1998) (section 1.7). Effects of shift work on wellbeing have been shown (Costa 2003) mainly when different shifts were compared (Cavallo et al. 2002; Folkard et al. 1993) or in simulated shift schedules (Smith et al. 2009), but there are not many reports that describe the differences in mood between SW and NSW. Some of these studies show that the frequency of unhappiness was higher in night SW (Kaliterna et al. 2004). A recent meta-analysis indicated that SW have a higher odds ratio for depression than day workers (Driesen et al. 2010) but these authors and others (Costa et al. 2006) suggest that this association is likely to be related to high job demand and low control over shift schedules.

Few previous studies, however, have assessed the effects of sleep deprivation and recovery sleep on alertness and mood in SW when they were tested in a laboratory setting. In addition, SW and NSW have not been compared directly when both were subjected to the same sleep deprivation and recovery conditions in a laboratory protocol.
5.1.1 Aims and hypotheses

The aim of the study was to assess the effects of one night of TSD, a recovery nap and recovery sleep on objective and subjective alertness and subjective mood and to compare responses from SW to those of NSW in an identical laboratory setting. Moreover, the correlation between subjective and objective alertness was determined to explore whether this correlation changed as a result of sleep deprivation and recovery sleep and whether it was the same in SW and NSW.

It was hypothesised that:

- Alertness and mood will be affected by TSD, a recovery nap and recovery sleep. Subjective sleepiness, reaction times and lapse frequency will increase, reciprocal reaction time will decrease and mood will be less positive after TSD. A nap and recovery sleep will (partly) reverse these changes.
- SW and NSW will respond differently to TSD and recovery periods. Subjective sleepiness will be less severe and mood will be more positive during and after TSD in SW than in NSW. Reaction times and lapse frequency will not be different between the groups. Subjective sleepiness after recovery sleep will have returned to baseline levels to a larger extent in SW than in NSW.
- The correlation between subjective sleepiness and PVT performance will be different between days and between groups. TSD will gradually lead to a loss of correlation between these 2 parameters. In addition, the correlation will be stronger in NSW than in SW.
- Circadian phase and phase angle of entrainment will not be different between NSW and SW.

5.2 Methods

5.2.1 Assessment of circadian phase

Saliva was collected into scintillation vials (PerkinElmer, Beaconsfield, UK) every 30 min for 4 h before the participants' self-selected bed time and 9 saliva samples were collected immediately following waking until 4.75 h after waking (approximately 1 sample every 30 min) (Figure 2.1). Salivary melatonin concentrations were measured on the evening before baseline sleep according to an established protocol (English et al. 1993)
with rabbit anti-melatonin antiserum (code R/R/19540-16876, Stockgrand Ltd., University of Surrey, Guildford, UK), overnight incubation at 4°C with $^{125}$I-iodomelatonin (PerkinElmer, Beaconsfield, UK) and separation by cellulose-linked donkey anti-rabbit IgG (SacCell, IDS Ltd., Boldon, Tyne & Wear, UK). In addition, 1 mL Brij/saline wash solution (0.9% w/v NaCl, 0.06% Brij 35, Sigma Ltd., Poole, UK) was added to all samples prior to the centrifugation step. In every assay samples from SW and NSW were assayed together. The average limit of detection for the saliva melatonin assay was 1.5 ± 1.2 pg/ml (mean ± SD) (n = 5). The inter-assay CVs were 15.9, 13.0 and 7.7 % for quality control samples with 7.1 (n = 6), 19.5 (n = 6) and 44.1 pg/ml (n = 5) melatonin, respectively. Dim light melatonin onset (DLMO) as a marker of circadian phase was determined for each subject as the time that the melatonin level exceeded the calculated threshold of > 2 SD from the mean basal concentration (mean of the 3 or 2 (in 3 subjects due to missing data) consecutive lowest concentrations) and did not return below this basal level (Burgess et al. 2003). The DLMO could not be assessed in one shift worker (subject S8) as elevated melatonin concentrations were already present at the time the first saliva sample was collected. For this subject and 2 other subjects, melatonin concentrations were also determined in the morning after the baseline sleep in order to confirm a melatonin rhythm. In addition, individual phase angles were computed by calculating the difference between DLMO and bed time.

5.2.2 Assessment of sleep

Polysomnographic recordings were taken continuously throughout the study (section 2.5.4). Signals for the EEG were derived from gold plated electrodes (Astromed Inc., Slough, UK) (occipital O1 and O2, central C3 and C4 and frontal F3 and F4 electrodes referenced to mastoid M1 and M2 electrodes) using tape measure and wax pencil to indicate the correct positions, according to the American Academy of Sleep Medicine (AASM) guidelines (Iber et al. 2007) (Figure 5.1 A & B). Left and right EOG and EMG (2 electrodes placed 2 cm below the inferior edge of the mandible) were also derived using the gold electrodes as well as subject ground and system reference for which the electrodes were placed on the forehead (Figure 5.1 C).

All electrodes were applied with EC2 paste (Astromed Inc., Slough, UK) with gauze or tape after cleaning of the skin with NuPrep gel (Astromed Inc., Slough, UK). Data were acquired, converted into European Data Format (EDF) and imported into Somnologica Studio (version 5.0.1 build 1662, Embla, supplied by Stowood Scientific Instruments Ltd., Oxford, UK) as described in section 2.5.4.
Figure 5.1. EEG, EOG and EMG electrode positions.
The international 10-20 electrode placement system from (A) the left side (adapted from Malmivuo & Plonsey, (1995)) and (B) the top (adapted from Iber et al. (2007)) and (C) frontal electrodes.
The nasion, inion and preauricular points are used as landmarks from which the head is measured and sites for electrode placement, each 10 or 20% apart from each other, are determined. M = mastoid (behind the ear (A)), F = frontal, C = central, O = occipital, ROG and LOG are right and left oculogram, EMG = electromyogram, GND = subject ground and Ref = system reference.

To determine the extent of sleep/wakefulness during TSD, recordings were scored for wake, non-rapid eye movement sleep (NREM) stage 1, NREM 2, NREM 3 and rapid eye movement sleep (REM) from habitual bed time on the baseline day until the nap after TSD (comprising a period of 14.4 ± 0.3 h, mean ± SD) according to the AASM criteria.
(apart from 3.5 ± 2.8 % data loss due to changing of batteries or technical issues). Difficult epochs due to noise or signal loss were scored as wake as the sleep statistics report could not be generated if epochs were left unscored. The sleep statistics report contained several parameters such as total sleep time (TST), sleep onset latency (SOL) (time until the first occurrence of 60 s of consecutive sleep events) and number of awakenings (at least 30 s of wakefulness). TST, time spent in NREM 1, NREM 2 and NREM 3 and REM was also calculated as a percentage of the total scoring time). In order to assess the duration of single sleep periods, TST was divided by the number of awakenings + 1 (for the final awakening as this awakening is not counted by Somnologica) for each subject. Although this is not a conventional method, it was chosen to give an impression about sleep continuity.

5.2.3 Alertness and mood

5.2.3.1 Subjective alertness and mood

Subjective alertness and mood were assessed at 0.25, 4.25, 5.25, 7, 8, 9, 11.5, 12.5 and 13.5 h following adaptation sleep, baseline sleep, TSD and recovery sleep (with exception of time points 7, 8, and 9 during the nap after TSD) (Figure 2.1). During the TSD night alertness and mood assessments were carried out every hour starting 16.5 h after waking up. For the first 6 subjects this was done relative to their individual wake up time but for later subjects, assessments for the two subjects in the study session were synchronised for feasibility. As wake up time for the two subjects in a session was always 30 min apart, this implied a 30 min margin in the timing relative to wake up time, e.g. subjects with wake up times at 7:00 and 7:30 h would both have assessments during TSD at 23:30, 00:30, etc., resulting in one subject starting 16 h and the other subject starting 16.5 h after waking. Whether the assessment was started at 16 or 16.5 h was alternated between NSW and SW. In addition, for the first 6 subjects, the alertness and mood assessments started 17.5 h after waking up as the assessment at 16.5 h had not yet been added in.

Subjective mood and alertness were assessed by Karolinska Sleepiness Scale (KSS) (Akerstedt & Gillberg 1990), with descriptions for each odd number ranging from 1 ‘very alert’ to 9 ‘very sleepy’ (appendix M) and 9-digit rating scales with only the descriptions for the extreme values (1 ‘very alert’ - 9 ‘very sleepy’, 1 ‘very cheerful’ – 9 ‘very miserable’, 1 ‘very calm’ – 9 ‘very tense’ and 1 ‘very depressed’ – 9 ‘very elated’). In order to explore whether adjustments of the raw data would make a difference to the
visual representation of the data, KSS scores of each subject were z-scored in two ways: 
(value - mean of the values for the subject after baseline sleep)/standard deviation of the 
values for the subject after baseline sleep ('z-scored to baseline day') and (value – mean 
of all values for the subject)/standard deviation of all values for the subject ('z-scored to 
overall mean'). KSS scores were also calculated as a fraction of the mean baseline day 
values ('normalised to mean baseline day').

5.2.3.2 Objective alertness

The subjective alertness and mood assessments were directly followed by a modified 
Karolinska Drowsiness Test (KDT) (Akerstedt & Gillberg 1990; Cajochen et al. 1999; 
Lafrance & Dumont 2000) for which the data will not be described in this thesis. In 
summary, the subjects were semi-recumbent and the distance between the volunteers’ 
head and the fixation point was approximately 120 cm with the black 4 cm fixation dot on 
a white A4 paper placed at the end of the bed. The volunteer was requested to “maintain 
a stable, relaxed position (try not to move your head and arms) and fixate on the black 
dot in front of you for 5 minutes with your eyes open.” (After 5 minutes) “Now close your 
eyes and remain in this position for 1 more minute”.

After the KDT, objective alertness was assessed at 0.35, 4.35, 5.35, 7.1, 8.1, 9.1, 11.6, 
12.6 and 13.6 h following adaptation sleep, baseline sleep, TSD and recovery sleep (with 
the exception of time points 7.1, 8.1, and 9.1 h during the nap after TSD) by visual PVT 
(Dinges & Powell 1985) (Ambulatory Monitoring Inc., Ardsley, NY, USA). The first 6 
subjects, 4 NSW and 2 SW, were excluded as by mistake they performed both an audio 
and visual PVT simultaneously. The PVT box was placed on the volunteer’s lap with their 
forearms/wrists/hands resting on their thighs and the device tilted slightly so that the 
stimulus window was visible. The distance between eyes and display was approximately 
35-55 cm and the volunteer was instructed to use the same hand and finger throughout 
all tests performed. Further instructions were given according to the PVT manual 
(Ambulatory Monitoring Inc.). All subjects performed a ‘practice trial’ to familiarise 
themselves with the equipment and test. The PVT is assumed to have only a small 
learning effect, i.e. during the first 1-3 trials (Kribbs & Dinges 1994). The total trial time 
(10 min) and interstimulus interval (minimum 2 and maximum 10 s) were set according to 
the default settings. Data were downloaded using PVTcommW (Version 2.10.1.1, 
Ambulatory Monitoring Inc.) and analysed using REACT (Version 1.1.05, Ambulatory 
Monitoring Inc.). For each 10-min epoch the following performance indices were
calculated for comparison with previous studies (e.g. Cajochen et al. 1999; Dinges et al. 1997; Vgontzas et al. 2004): mean reaction time per 10-min epoch (mean RT), reciprocal reaction time (1/RT or RRT), median RT, increases in the response duration in the lapse domain (i.e. slowest 10% RTs per 10-min epoch, SRT), the shift in optimum RT (i.e. fastest 10% RTs per 10-min epoch, FRT) and the number of lapses (i.e. reaction times (RTs) ≥ 500 ms). According to the default settings, lapses were included in these parameters. The only previous study which mentions that lapses were excluded from the reaction time calculations is a recent study by Horne & Burley (2010).

5.2.4 Statistical analysis

5.2.4.1 Circadian phase

Melatonin DLMOs and phase angles in SW and NSW were compared by two-tailed independent Student's T-test.

5.2.4.2 Sleep

Sleep parameters were compared between SW and NSW with unpaired Student's t-tests (or Mann-Whitney tests if the data were not normally distributed). A Pearson correlation test was carried out to determine whether the number of awakening was related to the TST.

5.2.4.3 Alertness and mood

The alertness and mood assessments during the sleep deprivation night were analysed using a two-factor ANOVA (factors 'time' (13 levels) and 'group' (SW and NSW)) (SPSS 16, SPSS Inc.). If applicable, P-values were Greenhouse-Geisser adjusted when Mauchly's test for sphericity was significant.

A mixed-model regression analysis was conducted in SAS 9.1 (TS level 1M3, SAS Institute Inc.) to cope with missing data in the daytime alertness and mood assessments (section 2.6.4).

The data were also analysed omitting all the afternoon measurements (i.e. those around 7, 8 and 9 h after wake up time) as the imbalance between the presence of these
measurements after adaptation sleep, baseline sleep and recovery sleep but not after TSD caused problems for the post hoc tests for the main effects and day*group interactions in some cases.

The correlation between objective alertness (median RT) and subjective alertness (KSS) was tested with a Pearson correlation at all different time points in NSW and SW separately, in a similar way to the association between subjective wellbeing and subjective sleepiness in two different protocols (Birchler-Pedross *et al.* 2009). As there was variance in TST, the association between TST and the assessed parameters was also investigated with a Pearson correlation.

### 5.3 Results

#### 5.3.1 Circadian phase

Figure 5.2 shows the individual melatonin profiles for SW (n = 11) and NSW (n = 14) on the evening before baseline sleep and the morning after baseline sleep for 3 subjects. A brief case study for subject S8 can be found in appendix N, showing the melatonin profile measured before baseline sleep in the laboratory despite a regular sleep wake cycle the week prior to the study (confirmed by actigraphy, a subjective sleep diary and a time stamped voicemail). Average melatonin profiles and those for subjects with the longest and shortest phase angle are shown in Figure 5.3 A & B. The DLMO was not significantly different in SW compared to NSW (21.1 ± 0.5 h (mean ± SD) and 21.2 ± 0.7 h, respectively) (Figure 5.3 C). In addition, the phase angle between DLMO and bed time was also similar in SW and NSW (2.2 ± 0.5 h and 2.1 ± 0.6 h, respectively) (Figure 5.3 D).
Figure 5.2 A. Individual saliva melatonin profiles in non-shift workers.
Saliva melatonin profiles in NSW on the evening before baseline sleep (from 4 - 0.5 h before bed time).
Figure 5.2 B. Individual saliva melatonin profiles in shift workers.
Saliva melatonin profiles in SW on the evening before baseline sleep (from 4 – 0.5 h before bedtime) and in the morning following baseline sleep (from 0 – 4.75 h after waking) in 3 SW. Note, y-axis scales differ between graphs.
Figure 5.3. Saliva melatonin profiles, DLMOs and phase angles on the evening before baseline sleep.

Upper diagrams show saliva melatonin profiles in (A) NSW showing the average (± SEM) in all NSW (△) (n = 14), the subject with the longest phase angle (●) and the subject with the shortest phase angle (■), and in (B) SW showing the average (± SEM) in all SW (△) (n = 10), subject with the longest phase angle (●) and subject with the shortest phase angle (■).

Bottom diagrams show (C) individual DLMOs and mean ± SEM and (D) individual phase angles and mean ± SEM in NSW (●) (n = 14) and SW (○) (n = 10).
5.3.2 Sleep

The assessment of the amount and architecture of sleep during the sleep deprivation period is shown in Figure 5.4 and Table 5.1. No significant differences were observed between SW and NSW in any of the parameters. However, there were large interindividual differences in the subjects' ability to stay awake (TST was 35 ± 8 min, 7 – 83 (mean ± SEM, range)) in SW and 29 ± 7 min, 0 - 74 in NSW) and how long it took before they fell asleep (SOL was 263 ± 51 min, 50 – 547 in SW and 370 ± 41 min, 113 – 547 in NSW) (Figure 5.4 A and B). The number of awakenings was significantly positively correlated with the TST ($r^2 = 0.79$, $P < 0.001$) (Figure 5.4 D), showing that subjects who had a longer TST did not necessarily sleep longer compared to those with a shorter TST, but fell asleep more often. To further assess continuity of the sleep episodes, the TST was divided by the number of awakenings. It appeared that NSW had on average 1.6 min of sleep and SW 2.2 min before they awoke (Table 5.1). Finally, the data showed that there was no difference between the groups in the time spent in each of the sleep stages (Table 5.1). Only one SW had a REM episode.

5.3.3 Alertness and mood

5.3.3.1 Subjective alertness and mood

Raw, z-scored and normalised KSS data are shown in Figure 5.5. As the different data forms did not make a big difference in the visualisation, all subjective data are presented as raw data. Subjective alertness and mood parameters are shown in Figure 5.6 and for the sleep deprivation night in Figure 5.7. Both KSS and the 9-digit scale score for alertness showed significant effects of day ($F_{3,751} = 49.4$, $P < 0.001$ and $F_{3,751} = 34.8$, $P < 0.001$, respectively), time ($F_{8,751} = 3.9$, $P < 0.001$ and $F_{8,751} = 5.1$, $P < 0.001$, respectively) and day*time interactions ($F_{21,751} = 4.1$, $P < 0.001$ and $F_{21,751} = 5.6$, $P < 0.001$, respectively). Subjects were less alert according to the KSS and 9-digit scale score in the morning following TSD (0.25, 4.25 and 5.25 h after habitual wake up time) than at all other time points. When the same time points on different days were compared, additional significant findings were increased alertness on the KSS in the late afternoon and evening following recovery sleep compared to following baseline sleep (8, 12.5, 13.5 h after habitual wake up time) and increased alertness in the evening after recovery sleep compared to after TSD (at 12.5 and 13.5 h).
Figure 5.4. Sleep parameters during the TSD night.

(A) Total sleep time, (B) sleep onset latency and (C) total number of awakenings during sleep deprivation in NSW (●) and SW (○) (individual and mean ± SEM) and (D) the correlation between the number of awakenings and total sleep time (●) ($r^2 = 0.79$, $P < 0.001$) from habitual bed time on the sleep deprivation night until the nap. $n = 14$ for NSW and $n = 11$ for SW.
Table 5.1. Sleep parameters during the sleep deprivation night in non-shift workers and shift workers.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-shift workers</th>
<th>Shift workers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sleep time (min)</td>
<td>29 ± 7</td>
<td>35 ± 8</td>
</tr>
<tr>
<td>SOL (min)</td>
<td>370 ± 41</td>
<td>263 ± 51</td>
</tr>
<tr>
<td>Number of awakenings</td>
<td>18 ± 4</td>
<td>19 ± 5</td>
</tr>
<tr>
<td>Average sleep duration before awakening (min)</td>
<td>1.6 ± 0.3</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>Total sleep time (%)</td>
<td>3.7 ± 0.8</td>
<td>4.5 ± 0.9</td>
</tr>
<tr>
<td>NREM 1 (%)</td>
<td>2.6 ± 0.4</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td>NREM 2 (%)</td>
<td>1.3 ± 0.4</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>NREM 3 (%)</td>
<td>0.1 ± 0.0</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>REM (%)</td>
<td>0.0</td>
<td>0.04 ± 0.04</td>
</tr>
</tbody>
</table>

Data are means ± SEM during the sleep deprivation night (from habitual bed time until the scheduled nap on average 14.4 h later). % is relative to total scored time. 

n = 14 for NSW and n = 11 for SW.
Figure 5.5. Raw, z-scored and normalised day time KSS scores.

(A) raw KSS scores, (B) KSS z-scored to all days, (C) KSS z-scored to the baseline day and (D) KSS scores normalised to the baseline day (mean ± SEM) in NSW (•) and SW (○) 0.25, 4.25, 5.25, 7, 8, 9, 11.5, 12.5 and 13.5 h after habitual wake up time following adaptation sleep, baseline sleep, sleep deprivation and recovery sleep. n = 14 for NSW and n = 11 for SW.
Figure 5.6. Subjective day time alertness and mood.

(A) KSS and 9-digit scale scores for (B) alertness, (C) cheerfulness (D) calmness and (E) depression (mean ± SEM) in NSW (●) and SW (○) 0.25, 4.25, 5.25, 7, 8, 9, 11.5, 12.5 and 13.5 h after habitual wake up time following adaptation sleep, baseline sleep, sleep deprivation and recovery sleep. n = 14 for NSW and n = 11 for SW. Note, y-axis scales differ between graphs.
Figure 5.7. Subjective alertness and mood during TSD.

(A) KSS and 9-digit scale scores for (B) alertness, (C) cheerfulness, (D) calmness and (E) depression (mean ± SEM) in NSW (●) and SW (○) during TSD (from 16.5 to 23.5 h after waking). n = 14 for NSW and n = 11 for SW. Note, y-axis scales differ between graphs.
The 9-digit scale also showed increased alertness at the last time point (13.5 h) after recovery sleep compared to after adaptation sleep and after baseline sleep and TSD. Moreover, both the KSS and 9-digit scale showed a significant effect of group ($F_{1,751} = 15.7, P < 0.001$ and $F_{1,751} = 7, P < 0.01$, respectively) revealing that SW felt more alert than NSW.

There was a significant effect of day ($F_{3,751} = 11.0, P < 0.001$) and a day*time interaction ($F_{21,751} = 1.7, P < 0.05$) for cheerfulness. When the same time points were compared between days, subjects felt significantly less cheerful in the morning after TSD compared to in the morning after adaptation sleep (at 0.25, 4.25 and 5.25 h), baseline sleep (at 4.25 and 5.25 h) and recovery sleep (at 0.25, 4.25 and 5.25 h). Subjects also felt significantly more cheerful at the last time point (13.5 h) after recovery sleep compared to all the other days. There were also significant differences between the groups. The SW were significantly more cheerful than NSW ($F_{1,751} = 22.9, P < 0.001$). SW also felt significantly calmer ($F_{1,751} = 32.0, P < 0.001$) and more elated ($F_{1,751} = 46.8, P < 0.001$) than the NSW. Finally, there was a significant effect of day ($F_{3,751} = 2.9, P < 0.05$) on depression ratings. When the afternoon measurements were left out the effect of day became non-significant ($F_{3,544} = 2.3, P = 0.0735$) but subjects were significantly more elated after recovery sleep than after TSD ($P < 0.05$). There was also a trend for more depression after TSD compared to after adaptation sleep ($P = 0.0511$).

During the sleep deprivation night there was a significant effect of time on the KSS score ($F_{7,176} = 6.3, P < 0.001$) and the 9-digit score for alertness ($F_{7,176} = 7.4, P < 0.001$), caused by an increase in sleepiness throughout the night. All parameters were significantly different between SW and NSW, with SW feeling less sleepy according to the KSS ($F_{1,176} = 5.5, P < 0.05$) and 9-digit scale ($F_{1,176} = 5.8, P < 0.05$) and more cheerful ($F_{1,176} = 12.5, P < 0.001$), elated ($F_{1,176} = 10.2, P < 0.01$) and calmer ($F_{1,176} = 9.0, P < 0.01$).

### 5.3.3.2 Objective alertness

PVT parameters were measured at regular intervals throughout the protocol (Figure 2.1). All assessed PVT parameters on all time points can be found in Figure 5.8 with a selection for the TSD night in Figure 5.9.
Figure 5.8. Objective day time alertness.
(A) mean RT, (B) median RT, (C) mean RRT, (D) lapses, (E) mean SRT and (F) mean FRT (mean ± SEM) in NSW (•) and SW (○) 0.35, 4.35, 5.35, 7.1, 8.1, 9.1, 11.6, 12.6 and 13.6 h after habitual wake up time following adaptation sleep, baseline sleep, sleep deprivation and recovery sleep. n = 10 for NSW and n = 9 for SW.
Figure 5.9. Objective alertness during TSD. 
(A) mean RT, (B) median RT, (C) mean RRT, (D) lapses, (E) mean SRT and (F) mean FRT (mean ± SEM) in NSW (●) and SW (○) during TSD (from 16.6 to 23.6 h after waking). n = 10 for NSW and n = 9 for SW.
There appeared to be a significant effect of day ($F_{3,556} = 32.3$, $P < 0.001$), time ($F_{8,556} = 2.7$, $P < 0.01$) and day*time interaction ($F_{21,556} = 3.9$, $P < 0.001$) for mean RT as well as for mean SRT ($F_{3,556} = 28.5$, $P < 0.001$; $F_{8,556} = 2.8$, $P < 0.01$ and $F_{21,556} = 4.3$, $P < 0.001$, respectively) and for lapses ($F_{3,556} = 24.9$, $P < 0.001$; $F_{8,556} = 3.3$, $P < 0.01$ and $F_{21,556} = 2.2$, $P < 0.01$, respectively). There was a significant effect of day ($F_{3,556} = 40.7$, $P < 0.001$) and a day*time interaction ($F_{21,556} = 2$, $P < 0.01$) on mean RRT. Similar to subjective alertness, post hoc tests revealed a decrease in alertness shown by a significant increase in mean RT and SRT in the morning (0.35, 4.35 and 5.35 h) after TSD compared to all other time points. In addition, mean RT and SRT were significantly slower at 0.35 h than at 4.35 h and at 4.35 h than at 5.35 h after TSD. In parallel with these findings, mean RRT was significantly lower (i.e. reaction time was slower) and lapses were more frequent in the morning (0.35, 4.35 and 5.35 h) following TSD than almost all other time points. However, some additional differences were seen such as a significantly lower mean RRT at 11.6 h after TSD and recovery sleep compared to baseline sleep whereas other previously found differences in other PVT parameters were not present. The number of lapses at 5.35 h after TSD did not differ from that at 8.1 h after baseline sleep and 5.35, 8.1 and 9.1 h after recovery sleep. Furthermore, median RT and FRT did not only show a significant effect of day ($F_{3,556} = 25.3$, $P < 0.001$ and $F_{3,556} = 9.4$ and $P < 0.001$, respectively) but also a significant day*group interaction for median RT ($F_{3,556} = 2.9$, $P < 0.05$) and a group effect for mean FRT ($F_{1,556} = 6.2$, $P < 0.05$). When the afternoon assessments (at 7.1, 8.1 and 9.1 h) were left out, median RT showed significant effects of day ($F_{3,403} = 23.2$, $P < 0.001$) and day*time interaction ($F_{15,403} = 1.7$, $P < 0.05$). As for mean RT and SRT, median RT was longer in the morning following sleep deprivation than all other time points, apart from the reaction times at 4.35 h and 5.35 h after TSD which were not different from those at 5.35 h after recovery sleep. Moreover, median RT showed an effect of group ($F_{1,403} = 6.6$, $P < 0.05$) and a day*group interaction ($F_{3,403} = 3.1$, $P < 0.05$). It appeared that both NSW and SW had a higher median RT after TSD than after adaptation and baseline sleep. However, NSW had a significantly longer median RT than SW after TSD and recovery sleep. In parallel with this, NSW had significantly longer RTs after TSD compared to after recovery sleep and longer RTs after recovery sleep than after adaptation and baseline sleep. For FRT a trend for an effect of group remained after omitting the afternoon measurements ($F_{1,403} = 3.8$, $P = 0.0517$) with a tendency for slower FRT in SW. The significant effect of day ($F_{3,403} = 9.8$, $P < 0.001$) was still present with longer FRT after TSD compared to all other days.
During TSD none of the PVT parameters showed significant group effects nor time*group interactions. Significant increases throughout the TSD night were observed for mean RT ($F_{7,134} = 3.5, P < 0.01$), median RT ($F_{7,134} = 2.6, P < 0.05$), mean SRT ($F_{7,134} = 3.2, P < 0.01$) and number of lapses ($F_{7,134} = 3.4, P < 0.01$), but a decrease in mean RRT ($F_{7,134} = , P < 0.001$) (Figure 5.9). Post hoc tests showed that in the NSW mean RT and SRT were significantly slower around 1.5 h before habitual wake up time than 2.5 and 0.5 h before, while the complete data set indicates that in SW this performance 'dip' appears to occur an hour later and was much smaller.

5.3.4 Correlation between subjective alertness and PVT performance

Figure 5.10 illustrates that there was no clear change in the association between subjective alertness (KSS) and objective alertness (median RT) across time points or between SW and NSW.

5.3.5 Association between sleep during TSD and assessed parameters

As the protocol aimed to induce TSD, it was of importance to assess whether the extent of incomplete sleep deprivation was related to the measured parameters. There was no significant association between TST during TSD and KSS or median RT just before the nap (Figure 5.11). Similarly, there was no significant correlation between TST during TSD and either the IAUCs of TAGs, insulin and glucose or the AUCs of TAGs, NEFAs, insulin and glucose after TSD and after recovery sleep. There was also no correlation between TST and FMD in the morning and afternoon after TSD and after recovery sleep. Nor was there a correlation between TST and SDNN or LF/HF at any time point following TSD, or average RMSSD, SDSD, NN interval or pNN50 after recovery sleep (data not shown).
Figure 5.10. Correlation between subjective and objective alertness.
Correlation between KSS and median RT (A) throughout the laboratory study and (B) during sleep deprivation (with the time points for median RT) in NSW (●) and SW (○). n = 10 for NSW and n = 9 for SW. During sleep deprivation the statistically significant correlations are indicated by an asterisk (P < 0.05).
Figure 5.11. Correlation between sleep during TSD and subjective and objective alertness. Correlation between total sleep time during sleep deprivation and (A) KSS at 5.25 h and (B) median RT at 5.35 h after habitual wake up time following sleep deprivation in NSW and SW. n = 25 for KSS and n = 19 for median RT.
5.4 Discussion

Sleep deprivation has been shown to decrease alertness and affect mood (section 1.6). These findings may also be observed in SW, either due to the acute effects of insufficient sleep or the circadian phase at which alertness and mood are measured during a shift. A major aim of the current study was to investigate the chronic rather than acute effects of shift work on alertness and mood responses to sleep deprivation and recovery sleep by subjecting experienced SW to identical laboratory settings as NSW. In addition, a preliminary analysis was conducted to explore whether the correlation between objective and subjective mood and alertness differed across the sleep deprivation protocol as well as between SW and NSW. Additional aims were to determine circadian phase and phase angle of entrainment and to assess wakefulness during the sleep deprivation night in all subjects.

Circadian phase was assessed by measuring the DLMO, considered a reliable marker for circadian phase (Arendt 2003; Burgess et al. 2003; Klerman et al. 2002). The results show that using the week at home study period (prior to the laboratory session), control of the participants’ circadian phase was achieved in this study since there was no significant difference in the DLMO of SW compared to NSW. In addition, the data confirmed that the SW were normally entrained to the light/dark cycle since the time interval (phase angle) between DLMO and bed time was not only very similar in SW and NSW (2.2 ± 0.5 h and 2.1 ± 0.6 h, respectively) but also comparable to previous reports in healthy young volunteers (phase angle of 2 ± 1.4 h and similar DLMO at 21.7 ± 1.5 h) (Burgess et al. 2003).

Despite intensive monitoring by staff, subjects still fell asleep during the TSD night. This also occurred in other studies that aimed to achieve TSD (many other studies did not report polysomnographic sleep or did not analyse sleep outside the planned sleep episodes). In previous studies, ranges of sleep lengths have been shown 0 – 16.5 min (Urrila et al. 2007), 0 - 49 min NREM 1 (Cajochen et al. 1999), with an average length of sleep episodes and sleep architecture (Urrila et al. 2007) similar to those found in the current study. Faraut et al. (2010) only observed 2 sleep episodes of 7 and 8 min of NREM 2 outside the permitted hours and other studies demonstrated an average sleep duration less than the restricted hours that were allowed (Donga et al. 2010; Nedeltcheva et al. 2009; Spiegel et al. 1999). However, TST in some of the current subjects was long. This discrepancy may be due to differences in the study designs. Most importantly, in the current study subjects were required to lie very still for at least 20
min during the ultrasound recordings for FMD, which is especially sleep-inducing. In addition, in some previous studies, subjects were allowed to eat and move around in light brighter than the dim light used in the current study (Faraut et al. 2010; Nedeltcheva et al. 2009; Spiegel et al. 1999; Urrila et al. 2007) which would have helped the subjects to stay awake.

Not surprisingly, both the KSS and the 9-digit scale score showed a significant increase in subjective sleepiness across the TSD night. In accordance with this, an increase in mean RT, median RT, mean SRT and lapse frequency but a decrease in mean RRT indicates increasing objective sleepiness during prolonged wakefulness. This increase in reaction time, lapse frequency and sleepiness may be part of the rising phase of the circadian rhythm observed in subjective and objective alertness which has been shown to display a maximum deterioration around the body temperature minimum (around 2 h before habitual wake up time) (Cajochen et al. 1999; Cajochen et al. 2001; Dijk et al. 1992; Graw et al. 2004; Kline et al. 2010; Lee et al. 2009; Wyatt et al. 1999) (section 1.5.4.1). As other authors have mentioned (Cajochen et al. 1999), the current data also indicated some improvement of performance around the last hour before habitual wake up time in NSW and after wake up time in SW, although it has to be kept in mind that the resolution may not be high enough to distinguish in the timing between NSW and SW as tests during TSD were taken within a 30 min margin with respect to wake up time (section 5.2.3). In contrast to the other PVT parameters, FRT did not change significantly during TSD. However, a circadian rhythm in FRT with an increase, albeit possibly less steep, throughout the night is likely to exist (Cajochen et al. 1999; Graw et al. 2004; Kline et al. 2010; Wright et al. 2002). In addition, there were no significant changes in the mood parameters during the TSD night although previous studies indicated a circadian rhythm in mood with a trough around the temperature minimum (Boivin et al. 1997; Kline et al. 2010).

When the day time measurements (0.25/0.35 h – 13.5/13.6 h after habitual wake up time) were analysed, both the KSS and 9-digit scale demonstrated that subjects felt less alert in the morning after sleep deprivation (before the nap) than at any other time point. Similarly, mean RT and mean SRT were higher in the morning after TSD than at any other time point. In addition, mean RRT was lower and median RRT and the number of lapses were higher the morning after TSD than most but not all other time points. Mean RRT was also lower at 11.6 h after TSD than after baseline sleep. These observations are not surprising and are in agreement with previous studies showing that alertness is decreased after TSD (Gould et al. 2009; Jay et al. 2007; Kahn-Greene et al. 2007;
Zhong et al. 2005), PSD (Axelsson et al. 2008; Buxton et al. 2010; Cohen et al. 2010; Dinges et al. 1997; Faraut et al. 2010; Haavisto et al. 2010; Mollicone et al. 2010; Sallinen et al. 2008; Spiegel et al. 1999; Vgontzas et al. 2004) and in sleep deprived subjects compared to subjects with normal sleep (Franzen et al. 2008) (section 1.6). FRT was slower after TSD compared to all other days but the effect was not as strong as for the other parameters, suggesting that FRT is less sensitive to sleep deprivation. This result is supported by findings from a study that did not find differences in FRT between a high sleep pressure protocol (CR) and a low sleep pressure protocol (nap protocol) (Graw et al. 2004). The time course of changes in PVT parameters across the baseline day and the sleep deprivation night was similar to that observed in other studies with a fairly stable performance up to 16 h after habitual wake up time and deterioration beyond this point (Cajochen et al. 1999; Dijk et al. 1992; Urrila et al. 2007).

The current study showed a trend but no significant effect for an increase in depression ratings after TSD compared to after adaptation sleep. By contrast, subjects were less cheerful in the morning after TSD than on the other mornings. Most studies have also shown deteriorations in some aspects of mood but not in others and inconsistent results within a sub-type of mood (e.g. depression) after TSD (Kahn-Greene et al. 2007), PSD (Dinges et al. 1997; Haack & Mullington 2005) and in sleep deprived compared to non-sleep deprived subjects (Franzen et al. 2008).

The first recovery period after the TSD night was the 4-h nap. The data indicate that the nap has been able to partly reverse the increased sleepiness in the morning after TSD as there were no significant differences in the evening subjective and objective measures after the nap compared to the evenings of the adaptation and baseline day. This is in agreement with findings that a nap is helpful (Dinges et al. 1987; Gillberg et al. 1996) but the current data do not provide evidence that a nap mostly improves performance above subjective sleepiness (Dinges et al. 1987; Gillberg et al. 1996). Moreover, a lower mean RRT at 11.6 h following TSD, compared to following baseline sleep and the absence of a day*time interaction for FRT (but an overall increase after TSD) are the only indications that objective vigilance was not fully recuperated after the nap. Alternatively, a significant increase in sleepiness in the morning after TSD but not after the nap could also be due to circadian modulation of the effects of sleep deprivation. Previous studies have shown that the worst effects of sleep deprivation were displayed between the body temp minimum and shortly after habitual wake up time (Cohen et al. 2010; Lee et al. 2009; Mollicone et al. 2010).
The recuperative effect of the nap, however, may be temporary and therefore it is of interest to assess the effect of recovery sleep. The effectiveness of recovery sleep appeared to depend on the parameter measured. For example, subjective sleepiness, mean RT and SRT showed full recuperation after recovery sleep. In agreement with this, other studies have also shown that 9 h recovery sleep following 40 h TSD was sufficient for subjective sleepiness and RT to return to baseline levels (Jay et al. 2007) and that subjective sleepiness recovered after 1 PSD night (2 h sleep) plus a 30-min nap and 8 h recovery sleep (Faraut et al. 2010). However, reversal to baseline levels was possibly less complete in lapse frequency and median RT (the 5.35 h time point following recovery sleep did not differ from the same time point after TSD) and mean RRT (still lower at 11.6 h after recovery sleep compared to after baseline sleep). In addition, one of the next paragraphs will discuss that NSW not only had a longer median RT after TSD but also after recovery sleep than after baseline and adaptation sleep. In agreement with this, other studies have shown that subjective sleepiness may recover faster than objective sleepiness, especially lapses (Axelsson et al. 2008; Haavisto et al. 2010; Lamond et al. 2007). Despite this observation, overall recovery seems faster in the current study than in previous studies on repeated PSD where it took at least 2 recovery nights for PVT parameters (and in some cases subjective sleepiness) to return to baseline levels (Axelsson et al. 2008; Dinges et al. 1997; Sallinen et al. 2008). However, recovery from repeated PSD may take longer than a single night of TSD and, in addition, there was no nap opportunity in those studies.

Furthermore, the results from the KSS revealed increased alertness in the evening following recovery sleep compared to following baseline sleep and TSD and the 9-digit scale showed higher alertness levels at the last time point (13.5 h) after recovery sleep compared to after adaptation and baseline sleep and TSD. In addition, subjects felt more elated after recovery sleep compared to after TSD. Furthermore, subjects were more cheerful during the very last assessment compared to the same time point on other days. The alertness and mood data are suggestive of the so-called ‘end-of-study-effect’. This phenomenon is characterised by an increase in positive mood towards the end of an experiment due to the prospect of completing the study (despite the fact that in the current study the subjects were not told exactly when the last measurement will take place). This may reflect feelings of relief as staying in a laboratory has been indicated to have a negative influence on mood (Paterson et al. 2010).

The current protocol did not only show effects of sleep deprivation and recovery sleep on alertness and mood, but also of shift work. In accordance with the hypotheses,
throughout the sleep deprivation night SW felt less sleepy according to the KSS and 9-digit scale. By contrast, PVT parameters showed no significant differences between the groups. During the day time measurements SW felt more alert than NSW according to the KSS and 9 digit scale. Also objectively, NSW may have been less alert as they had a significantly longer median RT than SW after TSD and recovery sleep and higher median RT after recovery sleep than after adaptation sleep and baseline sleep. The latter also indicates that NSW do not recover as well from sleep deprivation as SW, although the hypothesis was that this would be observed in subjective rather than in objective alertness. Remarkably, the only indication of the SW performing worse than NSW was the observation that FRT tended to be slower in SW than in NSW. During TSD, SW did not only feel more alert but also more cheerful, elated and calmer than NSW. This consistent pattern was seen during the day time measurements as well when SW felt significantly more cheerful, elated and calmer than NSW, which confirmed the expectations but this has not been reported before.

There were no significant differences between groups in circadian phase and sleep parameters during TSD so differences in alertness and mood between the groups are probably not due to this. There are no previous reports studying SW and NSW under identical settings for comparison with the current findings. A plausible explanation for the difference between SW and NSW may be selection into and out of shift work rather than the long term consequences of shift work itself. Primary selection into shift work of those who rate themselves as more tolerant to shift work and sleep deprivation can influence the outcome of studies of differences between SW and NSW (Boggild 2009). Along the same lines, it may be those who are least affected by shift work and sleep deprivation, that continue their jobs in shift work (Knutsson 2004). Therefore, the SW in the current study may be those who feel that they can tolerate sleep deprivation fairly well (also confirmed by personal communications with the SW). These phenomena and the fact that the current study did not investigate the acute effects of being in a shift schedule but compared SW and NSW when subjected to sleep deprivation may explain why the increased sleepiness and negative mood as observed in SW (Akerstedt & Wright 2009; Driesen et al. 2010; Kaliterna et al. 2004) (section 1.7) is not seen in the current study. Moreover, as suggested in section 1.7.1.4, it may be the flexibility aspect of shift work that affects irritability, anxiety and depression (Ahlberg et al. 2004; Costa et al. 2006) rather than sleep deprivation itself. However, van Dongen (2006) noted there was still a fair amount of variation in a self-selected group of F-117 pilots in flight simulator performance. These differences between groups and individuals could also be related to
genotype (Retey et al. 2005; Van Dongen & Belenky 2009; Viola et al. 2007) 1.8 but this is not likely to play a role in the current study (section 6.4.1).

The differences between NSW and SW also indicated that during TSD a more alert feeling in SW was not accompanied by a better PVT performance. In agreement with this, the KSS scores and median RT were not correlated in SW during TSD around 22.6 h after waking up while there was a significant correlation in NSW. By contrast, the only indication that subjective alertness correlates with objective alertness in SW could be found in the day time data that showed that SW felt more alert and also performed better, at least according to the median RT, after TSD and recovery sleep. The preliminary analysis on the correlation between subjective (KSS) and objective alertness (median RT) showed no clear difference across days and between groups. In contrast to expectations, the data do not suggest that sleep deprivation results in the loss of the capability to judge one's own alertness as the correlation was not better before sleep deprivation. Neither do the correlation data confirm the hypothesis that NSW would generally be able to accurately judge how sleepy they are whereas SW would overestimate their performance (feel more alert but are objectively not more alert) (only at 22.6 h after waking) as the TSD analyses indicated.

The data rather indicate there is no pattern in the correlation of an individual's subjective and objective alertness. This may suggest that subjective and objective alertness are correlated within a group of subjects (e.g. SW) but not within a subject. This is in agreement with previous studies which have shown no good correlation between subjective and objective sleepiness (section 1.5.4.2), apart from a situation where the KSS was administered during the PVT and the within PVT correlation was determined (Horne & Burley 2010). For example, Van Dongen et al. (2004) found significant differences between subjects in the number of lapses whereas their subjective sleepiness (on the SSS) was similar. Similarly, when subjects were divided into resistant or vulnerable to sleep deprivation according to their increase in SRT during the selection night, it appeared that during a subsequent sleep deprivation night, resistant subjects were able to maintain a fairly stable reaction time whereas vulnerable subjects showed an increase in reaction times, while both groups felt equally sleepy (KSS) (Galliaud et al. 2008). Additional results also demonstrated independence between neurobehavioural performance on one hand and subjective sleepiness on the other hand (Franzen et al. 2008; Frey et al. 2004). Another study showed that during consecutive simulated night shifts subjects had a moderate ability to rate their actual performance before or after PVT and that these pre-test self-ratings were correlated to subjective alertness but the study
does not reveal whether subjective alertness also directly correlates with PVT performance (Dorrian et al. 2003). Finally, when a dose-response relationship between hours of sleep in the previous night and either sleepiness (SSS) or PVT performance was investigated, it also appeared that though both improved in a saturating exponential manner with more sleep, subjective sleepiness improved more slowly (Jewett et al. 1999). It is not clear as to why there would be a poor correlation between subjective sleepiness and PVT performance. One possible reason would be that different brain areas or downstream pathways are involved in each of the behaviours. A second explanation could be that the correlation is dependent on the specific parameters compared and the study conditions present. The correlation between subjective and objective alertness could be improved, for example in PSD by sitting still with eyes closed for 1 min prior to the assessment in order to minimise transient activation (Yang et al. 2004).

In agreement with previous studies, the current study demonstrated that during and after sleep deprivation subjective alertness and PVT performance deteriorated. Mood did not change during the sleep deprivation night but cheerfulness was lower in the morning following the TSD night. Both subjective sleepiness and PVT performance returned to baseline levels after a nap plus recovery sleep. When SW were compared to NSW in identical settings (e.g. circadian phase, physical activity, food intake and light exposure), it appeared that SW felt better and more alert than NSW, possibly due to selection in and out of shift work, leaving mostly those individuals amongst the experienced SW who felt that they could tolerate shift work and sleep deprivation.
CHAPTER 6 GENERAL DISCUSSION

This study investigated the effect of sleep deprivation, recovery sleep and shift work on metabolic and cardiovascular function. In addition, the consequences for mood and alertness were assessed. Epidemiological studies have indicated that short sleep and shift work are associated with alterations in metabolic and cardiovascular function. Smaller scale field and laboratory studies have supported this relationship. However, there is a paucity of information about the effects of TSD on the postprandial metabolic and insulin responses to a standard breakfast as well as endothelial function and heart rate variability (HRV) (especially the time domain parameters). Furthermore, previous studies were often not well-controlled for environmental conditions. Moreover, in many cases the shift work population was poorly characterised and not compared to the appropriate control group.

Thus, these metabolic and cardiovascular parameters were assessed following TSD in a laboratory setting with strict control of body posture, physical activity, meals, circadian phase and ambient lighting. In addition, experienced SW were compared directly to NSW matched for age, BMI and cholesterol levels in these identical settings.

6.1 Discussion of the results

Previous chapters have shown changes in the assessed parameters on different days throughout the protocol. Here the changes in these parameters will be discussed per day and SW will be compared to NSW.

6.1.1 Adaptation sleep

First of all, a lower LF/HF, longer NN intervals and higher pNN50 were observed after adaptation sleep than on the other days. A plausible explanation would be that this is a consequence of adapting to the laboratory environment. This explanation would imply that stress levels and therefore sympathetic activity (Black & Garbutt 2002) would be slightly higher than on all other days. However, a lower LF/HF indicates that sympathetic activity is lower after adaption sleep than on all other days. Opposite reasoning would be that the first day is the least stressful day as other studies have shown that negative feelings increase by staying in a laboratory (Paterson et al. 2010).
6.1.2 One night of TSD

After one night of TSD basal TAG levels were significantly decreased. This suggests that wakefulness during TSD (without an increase in physical activity) may induce an increase in the use of nutrients (section 3.4) possibly by an increased metabolic rate (Koban & Swinson 2005; Young et al. 1998). Insulin levels 4 h after the standard breakfast following TSD were higher than the levels after baseline sleep. However, glucose levels 4 h postprandially did not return to basal levels after TSD. This reduced suppression of glucose levels by raised insulin concentrations indicates insulin insensitivity (Figure 1.1), which has also been observed after sleep deprivation by others (Buxton et al. 2010; Donga et al. 2010; Nedeltcheva et al. 2009; Spiegel et al. 1999; Tasali et al. 2008; VanHelder et al. 1993) (section 1.6). In agreement with results from Viola et al. (2002) the current study also demonstrated a peak in variance (most clearly in SDNN but also visible in RMSSD and SDSD) around habitual wake up time. However, there is no explanation for the increased variance after sleep deprivation. FMD was not significantly affected by TSD. Nevertheless, the metabolism and HRV data reflect effects of sleep deprivation on the autonomic nervous system, most likely a shift in the balance between sympathetic and parasympathetic modulation. Other authors found a shift towards sympathetic activity after TSD (Sauvet et al. 2009; Zhong et al. 2005) and PSD (Spiegel et al. 1999).

An interesting candidate to link the effects of sleep deprivation on the brain with those occurring in the rest of the body could be the wake promoting factor orexin (section 1.4.3). Orexin neurons have been shown to project to the ventral medulla thereby affecting heart rate (Berthoud et al. 2005) and to stimulate sympathetic neurons innervating tissues involved in metabolism (van den Top et al. 2003). Sleep deprivation has also been shown to increase orexin levels in the cerebrospinal fluid of both squirrel monkeys and rats and hypothalamic prepro-orexin mRNA levels in rats were higher (Deboer et al. 2004; Martins et al. 2010; Zeitzer et al. 2007). Increased exposure to orexin during sustained wakefulness may therefore result in a shift between sympathetic and parasympathetic modulation, affecting metabolism, HRV and possibly FMD. In addition, there are other sleep-promoting factors (Borbely & Tobler 1989) that could play a role in ‘peripheral effects’ of sleep deprivation. If these factors are able to promote sleep, they are likely to show differences after sleep deprivation when sleep needs to be induced. It would be very interesting to explore the possibilities to find a ‘biomarker of sleep deprivation’ that could be related to metabolic and cardiovascular function and that can be sampled from humans for example in blood, saliva or urine. Examples that could
be proposed as such ‘biomarkers’ are prostaglandin $D_2$ (PGD$_2$) (Borbely & Tobler 1989) and lipocalin-type prostaglandin D synthase (L-PGDS) (that produces PGD$_2$), which are thought to be involved in the regulation of sleep in addition to their function in inflammation and regulation of vascular tone (Sasaguri & Miwa 2004). L-PGDS has been shown to be decreased in plasma at night during TSD (Jordan et al. 2004) and in CSF of subjects with excessive daytime sleepiness (Bassetti et al. 2006). Insulin and acetylcholine have also been suggested as sleep promoting factors (Borbely & Tobler 1989).

In the present study, not only metabolic and cardiovascular function were affected by sleep deprivation. Subjective and objective sleepiness significantly increased throughout TSD, probably reflecting the rising phase of the circadian rhythm in sleepiness (Cajochen et al. 1999; Cajochen et al. 2001; Dijk et al. 1992; Graw et al. 2004; Kline et al. 2010; Lee et al. 2009; Wyatt et al. 1999) (section 1.5.4.1). In agreement with previous studies on sleep deprivation, both subjective and objective sleepiness were higher in the morning after TSD compared to after baseline sleep (Axelsson et al. 2008; Buxton et al. 2010; Cohen et al. 2010; Dinges et al. 1997; Faraut et al. 2010; Gould et al. 2009; Haavisto et al. 2010; Jay et al. 2007; Kahn-Greene et al. 2007; Mollicone et al. 2010; Sallinen et al. 2008; Spiegel et al. 1999; Vgontzas et al. 2004; Zhong et al. 2005) (section 1.6). Cheerfulness was lower in the morning after TSD as has been shown by some of the results in previous studies (Dinges et al. 1997; Franzen et al. 2008; Haack & Mullington 2005; Kahn-Greene et al. 2007). Decreased PVT performance and alertness have been related to changes in brain activity (Drummond et al. 2005) and the basal forebrain was one of the areas affected by sleep deprivation (Thomas et al. 2000). Another wake promoting factor, adenosine, accumulates in the basal forebrain after sleep deprivation (Porkka-Heiskanen et al. 2003) (section 1.4.3) and increased adenosine levels have been shown to impair PVT performance in the rat (Christie et al. 2008). These data suggest that the basal forebrain and elevated adenosine levels may be important in mediating the impairing effects of sleep deprivation on PVT performance and alertness.

6.1.3 Recovery sleep

In the present study, metabolic function, HRV, alertness and mood were affected by sleep deprivation but some important changes were also observed after recovery sleep. Basal and postprandial NEFA levels were lower and basal and postprandial insulin and
TAG levels were higher after recovery sleep. Higher TAG levels in the presence of elevated insulin levels indicate insulin insensitivity as insulin insensitivity can cause an enhanced release of TAGs by the liver and impaired plasma clearance of TAGs by LPL (section 1.5.1.1). This process has been reported to result in increased TAG levels in the circulation (DeFronzo 1988; Griffin 2009; Reaven 2002). Mobilisation of lipids due to hormone sensitive lipase activity, however, may still be suppressed by the higher insulin levels as the NEFA response was smaller after recovery sleep (section 1.5.1.1). Similar patterns in TAG, NEFA and insulin responses have been observed after phase shifting (Hampton et al. 1996; Lund et al. 2001; Ribeiro et al. 1998) (section 1.7). In parallel with our findings another study showed that the TAG AUC was increased on the second day of night shift work compared to day shifts but was even greater after 2 days after the return to daytime work (Lund et al. 2001), indicating that responses can be largest after the recovery process.

After recovery sleep, a lower variance (SDSD, RMSSD) as well as a decreased pNN50 and duration of NN intervals were also observed. Sauvet et al. (2009) found significant increases in intercellular adhesion molecule-1 (ICAM-1), IL-6 and norepinephrine, after recovery sleep but not after TSD. The observation that both HRV parameters and metabolites and insulin change after recovery sleep indicates that both pathways may be related. As suggested earlier for the effects of sleep deprivation, the effects of recovery sleep could also be mediated by a common substrate for both metabolism and HRV, such as the autonomic nervous system. Alternatively, the changes in the parameters could be ‘serial’ rather than ‘parallel’ with alterations in one parameter inducing changes in a second parameter. Some authors for instance have suggested that insulin insensitivity and the compensatory hyperinsulinemic response may induce sympathetic activity and increase heart rate (Facchini et al. 1996). However, most studies show return to baseline levels after recovery sleep (section 1.6) and the reasons why these changes in metabolism and HRV occur after recovery sleep but not after TSD remain to be elucidated.

In contrast to the metabolic parameters and HRV, subjective alertness and PVT performance did not show their largest responses after recovery sleep but returned to pre-sleep deprivation levels. Only in the evening after recovery sleep, did subjects feel more alert and cheerful better than on the other evenings, probably due to the prospect of going home (the so-called ‘end-of-study-effect’).
6.1.4 Comparison of shift workers and non-shift workers

The metabolic data did not show any overall statistically significant differences between SW and NSW. However, when the groups were analysed separately, the responses of NSW were more pronounced than those of SW, with higher basal insulin levels, lower basal NEFA levels and an increased postprandial insulin but decreased NEFA response after recovery sleep in NSW but not in SW. By contrast, HRV showed significant overall group differences with a lower SDNN, SDSD, RMSSD and pNN50, increased NN-interval and higher LF/HF in SW compared to NSW. SW also showed a trend for a lower FMD. The observations in HRV and FMD agree well with previous findings (section 1.7). Furthermore, SW felt significantly more alert, happier, more cheerful and calmer throughout the protocol.

The differences between the groups in subjective alertness and mood could be explained by selection into shift work (Boggild 2009) and coping with shift work (Knutsson 2004) of those individuals who feel that they are least affected by sleep deprivation. However, this statement may not hold for the physiological data in the current study as a trend for decreased FMD and lower variance and higher sympathetic activity might be related to increased cardiovascular risk in the SW. This suggests a mismatch between parameters assessed and/or acute and chronic effects of sleep deprivation. Although SW appear to cope with acute sleep deprivation, chronic shift work may adversely affect their cardiovascular health.

It is difficult to reconcile an explanation for these cardiovascular changes with one for the stronger insulin and NEFA response to recovery sleep in NSW compared to SW. A possible explanation may be based on the 10-year follow-up study mentioned in section 3.4 by Holman et al. (2008) which indicated that microvascular damage is likely to have an early onset and may persist despite reversal of risk factors such as glycaemic control. The authors suggest the mechanism underlying this could be that ‘long-term hyperglycaemia is associated with a slow onset of microvascular disease, which may be mediated by the gradual accumulation of advanced glycation end products that are subsequently slowly degraded with intensive glycaemic control. This mechanism may also be implicated in the development of CVD.’ In agreement with this, other researchers state that endothelial dysfunction and increased sympathetic activity are thought to be caused by repeated exposure to postprandial hyperglycaemia, hyperinsulinaemia and hyperlipidaemia (Konukoglu et al. 2003; Nesto 2004; O’Keefe & Bell 2007; Wascher et al. 2005). Alternatively, others suggest that there is a mutual interaction between insulin
resistance and endothelial dysfunction (Hadi & Suwaidi 2007) or even that changes in endothelial function may show before changes in glucose control and insulin sensitivity. An animal study, for example, showed that changes in endothelial function occurred earlier in a high fat diet model than changes in fasting insulin, TAG and glucose levels and peripheral insulin resistance (Kim et al. 2008), suggesting that the endothelium is more sensitive to this high fat load and that these early changes in endothelial function make the tissue more sensitive to be damaged by later metabolic stress. These observations indicate that damage to cardiovascular system (e.g. HRV and FMD) may have already occurred earlier in the SW and that this damage persists even though glucose and TAG responses do not show major differences compared to NSW. However, it is not clear how this could explain why SW show less divergent insulin and NEFA responses to recovery sleep compared to NSW.

The observed differences between SW and NSW were found despite both groups being matched in their screening data. In order to be able to match SW and NSW, some parameters such as cholesterol and BMI were allowed to exceed the official cut off values as for example cholesterol and BMI have been shown to be higher than 'normal' in SW (section 1.3.2). Some other parameters were also allowed larger margins, such as the HÖ, BDI, PSQI and ESS. It appeared that many of the questions in these questionnaires are influenced by shift work and do not necessarily indicate extreme diurnal preference or sleep disorders. Answers to questions such as ‘During the past month, when have you usually got up in the morning?’ in the PSQI or question 16 on sleep in the BDI (appendix E) may be influenced by the time of day and the particular shift during which the questionnaire is given and the instructions provided. This was observed with the BDI, PSQI and ESS scores that dropped significantly when the questionnaires were completed in the week of regular sleep before the study with the instruction to keep only day shift or days off in mind. In addition, many SW find it difficult to answer questions like: ‘Considering only your own “feeling best” rhythm, at what time would you get up if you were entirely free to plan your day?’ or ‘If there is a specific time you have to get up in the morning, to what extent are you dependent on being woken up by an alarm clock?’ and their answers may be different depending on whether they keep morning or evening shifts in mind when replying to the question. To address these issues an adapted version of the Munich ChronoType Questionnaire (Roenneberg et al. 2007; Roenneberg et al. 2003) for SW has been developed (Juda 2010), but for many other questionnaires a differential outcome for SW has not been considered.
In parallel with this, SW and NSW were also not different in other screening parameters such as serum sodium and potassium (affecting de- and repolarisation of myocardial cells (Fox 1996), and therefore the shape of the ECG), ALT (a marker of hepatotoxicity and altered gluconeogenisis (Ozer 2008)) and white blood cell counts, although previous studies have suggested that SW at risk for the metabolic syndrome have elevated ALT levels (Lin et al. 2009) and that an increased leukocyte count is independently associated with shift work duration (Sookoian et al. 2007). Another subject demographic was the PER3 VNTR polymorphism which has been associated with vulnerability to sleep deprivation (Viola et al. 2007). Those having the PER3<sup>6/6</sup> genotype were shown to live under higher sleep pressure compared to matched individuals with the PER3<sup>6/4</sup> genotype (section 1.8). This has also been related to a physiological measure, namely lower HRV (and possibly worse cardiovascular function) in PER3<sup>6/5</sup> individuals (Viola et al. 2008). In most populations around 10% of individuals are homozygous for PER3<sup>6/6</sup>, whereas approximately 50% are homozygous for PER3<sup>6/4</sup> (Ciarleglio et al. 2008; Nadkarni et al. 2005). Due to the small sample size of the current study and no prior matching of the participants for genotype, statistical analyses could not be carried out on the current data set. Therefore, the data were thus only visually explored and the 2 subjects with the PER3<sup>6/6</sup> genotype (1 SW and 1 NSW) did not show any overt differences compared to the other subjects, except for the observation that C13 had the highest TAG I AUCs of all subjects, was excluded from all the insulin analysis and had the highest glucose IAUCs of the NSW. However, this could also be related to ethnicity as this subject was Asian. Asians have been shown to have a more insulin resistant-like profile, probably despite a similar BMI (Wulan et al. 2010). The 5/5 genotype did not have clear effects on FMD, SDNN and LF/HF, but especially during TSD these two subjects (C13 and 35) were at the lower end of the NN spectrum. In addition, their median RT during TSD was in the upper range of the spectrum. Amongst the NSW subject C13 had the longest total sleep time during TSD.

The assessment of circadian phase using DLMO showed that there were no differences in the DLMOs between SW and NSW. Moreover, both groups had a similar phase angle between DLMO and habitual bed time. This finding implies that all the parameters described in previous chapters were assessed at a similar circadian phase for all subjects and thus any differences observed between the groups are unlikely to be due to differences in circadian timing. PSG-derived sleep was assessed during the TSD night. There were no differences between SW and NSW in TST, SOL, number of awakenings and time spent in each of the sleep stages as a percentage of total scored time. This
also supports the statement that any differences seen between the groups cannot be explained by differences in sleep during the TSD night.

6.2 Impact

As screening parameters (e.g. cholesterol and BMI) were not significantly different between the groups, the current findings suggest that non-invasive FMD and HRV assessments may be more sensitive to detect alterations in cardiovascular function than other parameters. This agrees with research showing that attenuated FMD predicts cardiovascular events in groups of subjects with comparable other risk factors for CAD (Shechter et al. 2009). Therefore, FMD and HRV might be predictive of altered cardiovascular function, for example in SW in our study, which were otherwise similar to NSW in many other parameters (e.g. screening parameters, glucose and TAGs). However, care should be taken with the conditions in which FMD and HRV are assessed and the control groups against which they are compared.

It is likely that the differences between SW and NSW in metabolic and hormonal responses and cardiovascular function assessed in epidemiological studies and smaller scale studies with more flexible conditions (such as circadian phase, physical activity and food intake) were not solely due to acute differences in these conditions at the time of assessment as differences between SW and NSW persisted in the current study when environmental conditions were strictly controlled.

Despite indications that cardiovascular physiology may be negatively affected by shift work, subjective alertness and mood data suggest that SW felt better under the sleep deprivation conditions. There thus appears to be a mismatch between the SW response to the acute effects of SD and the long term consequences of shift work for cardiovascular health. This emphasises that shift schedules should be designed to allow for optimal physiological recovery as subjectively SW may underestimate how much recovery time they need. The alertness and mood data suggest a nap is an effective countermeasure for the neurobehavioural effects of sleep deprivation and should be considered more often to be incorporated in shift schedules and other environments where sleep deprivation occurs.

It is not clear from the current data, however, how many recovery nights are needed for metabolic and cardiovascular parameters to return to baseline levels following one night
of TSD. For SW working a number of night shifts, recovery is impossible to predict at this stage. Although a nap proved very effective for recuperation of mood and alertness, one night of recovery sleep following the nap may not be sufficient for recovery of metabolic and cardiovascular function. Although the changes are probably reversible initially, repeated shortened recovery periods in environments where sleep deprivation occurs frequently, such as in (rotating) shift work, may result in the build up of disturbances in metabolism, HRV and FMD, which become chronic and may contribute to increased cardiovascular risk.

6.3 Limitations

The main limitation of this study was the sample size. The initial power calculations were based on the FMD measurements as these were thought to have the highest variation of all the parameters. The calculations (based on the study by Amir et al. (2004)) indicated that a sample size of $n = 14$ would be needed to reveal significant differences within the same subject (e.g. between days) and ($n = 8$ to observe differences between NSW and SW) with a power of 0.8 (see application for ethical approval, appendix A). As this number was achieved for NSW but not for SW, this may imply that differences within the groups, e.g. before and after sleep deprivation, do not reach statistical significance. However, it was very difficult to recruit SW who fitted the inclusion criteria (Table 2.1), especially the requirement of having worked shifts for 5 years or more and not suffering from any medical conditions (allergies especially were common amongst all applicants) and the request to be working day shifts or have days off for the week prior to the laboratory study.

6.3.1 Assessed parameters

As mentioned earlier, the assessment of FMD is a technique with more variability than many other methods. However, FMD is thought to be more robust than other techniques such as pulse wave analysis or pulse contour analysis to assess endothelial function (Donald et al. 2006). Although the coefficient of variation for within subject %FMD between study visits is not much higher at 7.1 -15% (Donald et al. 2006; Walters et al. 2006) than for example for a plasma insulin or saliva melatonin radioimmunoassay, reviews suggest that higher numbers of subjects are needed than those in the current study to be able to detect effects between interventions and groups (Corretti et al. 2002; Moens et al. 2005). Variability in FMD can arise from different sources, such as anatomy
of the subject, methods and study conditions and operators (as also described by others (Corretti et al. 2002; Stout 2009)). The current study used several standards to decrease variability and increase reproducibility such as brachial diameter calculation using 220 frames, ECG-triggered images, marking of the ultrasound probe location of the arm, same operator to set up and conduct the recording (as also suggested in a methodological review by the Brachial Reactivity Task Force (Corretti et al. 2002) and Stout (2009)), whereas previous studies did not always have all of these conditions thus making comparison of data on reproducibility or interventions more difficult. Most noticeably, in some previous studies the brachial diameter was measured in only 3 frames: at rest, ~1 min after deflation and again after 10-15 minutes of rest (or in 4 frames for comparison with endothelium-independent dilatation following administration of a NO donor such as nitroglycerin) (Amir et al. 2004; Fathi et al. 2004; Ofiaz et al. 2006; Sorensen et al. 1995). An interesting analysis to reduce variability further is to calculate FMD normalised to the amount of shear stress for the duration of time-to-peak as reactive hyperaemia shows interindividual differences (Padilla et al. 2008). Another suggestion to deal with interindividual differences would be to report absolute change in diameter as larger arteries have been shown to dilate relatively less than smaller arteries (Corretti et al. 2002), a trend which was also seen when SW and NSW were compared in the current study.

Another technical limitation was the fact that Somnologica software was not so flexible for HRV analysis. There were some bugs in the software’s artefact removal. Sometimes the artefact was not adequately removed in the NN trace and the length and place of the artefact identification had to be altered slightly to achieve satisfactory results. Furthermore, there were no options for other manipulations of the data which have been used in some other studies, e.g. interpolation of removed NN intervals (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996). Also inherent to the calculation of HRV parameters, it must be kept in mind that the interpretation of absolute VLF, LF and HF power and TP has to be cautious as these indices may be sensitive to (removal of) artefacts. Therefore, normalised indices (LF norm, HF norm and LF/HF) were given more weight when drawing conclusions about the data. Also related to the calculation of HRV parameters, it is not surprising that LF norm, HF norm and LF/HF yield very similar P-values and estimates (except for the sign of HF norm estimates) as Burr (2007) noted ‘these 3 indices are algebraically pairwise redundant at the level of definition’. However, he still regarded the use of these parameters as appropriate and informative but emphasised
that these interdependencies should not be forgotten. Likewise, the parameters in the
time domain may show similar results as they are derived from an identical ECG.

Finally, saliva sampling for melatonin measurements was only conducted in the evening
before bed time and in the morning after waking up. However, circadian phase
calculations using the basal levels and the melatonin peak (Revell et al. 2005; Sletten et
al. 2009) may be more reliable as the resolution is better. However, this would require
subjects to be woken up for saliva sampling or too frequent blood sampling (exceeding
limits of blood volume allowed to be taken within 3 months) and was therefore not
feasible in the current study.

6.3.2 Study design

One of the comments often made on sleep deprivation protocols, is that sleep
depprivation is likely to cause stress. Stress increases the release of hormones such as
cortisol and epinephrine (Black & Garbutt 2002). Both cortisol and (nor)epinephrine
influence metabolism; cortisol has catabolic effects by increasing lipid mobilisation but
these effects are long term as they involve gene expression and occur over a period of
days (Frayn & Akanji 2003). The direct action of hormones is faster, for instance the
mobilisation of fuels through stimulation of β-receptors by epinephrine. Stress also
affects other aspects of the autonomic nervous system and could therefore also
influence HRV and FMD. In accordance with this hypothesis, (evening) cortisol and
(nor)epinephrine have been shown to be increased after sleep deprivation (Buxton et al.
2010; Irwin et al. 1999; Leproult et al. 1997; Nedeltcheva et al. 2009; Schussler et al.
2006; Spiegel et al. 1999). However, other studies have shown that 1 night of TSD
resulted in a decrease in morning cortisol and had no overall effects on (nor)epinephrine
(Schmid et al. 2007; Schmid et al. 2009). Since the amount of stress caused by sleep
depprivation is probably also subject to interindividual differences, as is the response to
other stressors (Kudielka & Wust 2010) this may add an additional layer of variance onto
the differences that are already present. The amount of stress during TSD may be
reduced by allowing moderate food intake or physical activity. Although this could be
done in controlled manner, such a protocol would be most informative if the differences
between groups were the main focus. However, this would compromise comparisons
between baseline sleep, sleep deprivation and recovery sleep.
Related to this is the fact that the current study did not include a control group who did not undergo sleep deprivation. Although a within subject design is statistically more robust in order to reveal intervention effects, it can not rule out that some parameters may have decreased or increased due to the stay in the laboratory. This has been shown in the case of depression, where both sleep deprivation and staying in a laboratory increased depressive feelings (Paterson et al. 2010). However, it is unlikely that the laboratory stay is a main explanation for the findings as many changes were also observed after sleep deprivation but not after recovery sleep at the end of the protocol. Moreover, some studies including a control group have shown that merely staying in a laboratory did not affect parameters such as glucose and insulin (van Leeuwen et al. 2010).

Including a 4 h nap in the protocol created some difficulties when analysing the results. As mentioned in section 5.2.4.3 SAS post hoc differences for main effects of day and day*group interactions for the alertness and mood data could not be determined as there were 9 time points after adaptation, baseline and recovery sleep but only 6 time points after TSD due to the presence of a nap period. In order to assess these post hoc effects the 3 afternoon measurements after adaptation, baseline and recovery sleep had to be left out of some of the analyses, leading to the loss of possibly informative data from these 9 time points. In addition, the data observed after recovery sleep have to be considered a combined effect of the nap plus recovery sleep. For the same reason we decided not to analyse the HRV data during the 4-h afternoon periods as initially intended, since the days would be incomparable; on 3 days the observations would be the result of adaptation, baseline and recovery sleep, whereas after TSD the obtained data would show the effect of both sleep deprivation and sleep during the nap.

### 6.4 Future research

#### 6.4.1 Parameters to assess

Much of the future research could focus on extending the analyses. This also holds for the alertness and mood data, for which for example interindividual differences could be teased apart further. The first suggestion would be to further analyse the subjective and objective alertness data by a multilevel regression type analysis which could take into account multiple correlations, for example within a day, across days and within a group and identify whether these correlations are significantly different between time points,
days and groups. It would also be interesting to conduct an analysis similar to Santhi and colleagues (2007) who computed a cumulative RT percentile distribution. This method may give a more complete view of the whole RT spectrum (rather than taking the FRT or SRT) and is possibly able to reveal more subtle differences. In addition, the PVT generates other, not so commonly reported, parameters such as the number of false starts, standard deviation of the reaction times and the change in the RRT regression line from beginning to the end of the trial. These could be informative, especially in combination with other parameters. For example, faster reaction times may sometimes coincide with more false starts and the ratio between this could give an idea about the speed/accuracy trade off. In addition, variation across a trial or a condition could test the 'state instability' hypothesis and the relation between false starts and lapses may enable one to distinguish between compensatory effort and lack of motivation (Doran et al. 2001). Also within the PSG, other parameters could be assessed, such as the distribution of sleep episodes across the night. This is done in a hypnogram but Somnologica has no simple method of quantifying or extracting these data. In addition, the nap and recovery night could be scored for sleep stages to assess whether NSW and SW recover to a similar extent and in the same way. Ideally, the adaptation and baseline night should also be scored to ensure both groups had similar sleep quantities and architecture. Finally, the additional chapters of the SSI could be used to explore sleep, fatigue, health, wellbeing and the social and domestic situation and to detect whether and which coping strategies are present in the current group of SW.

Although the PER3 genotypes were determined in the current study, the study was not specifically designed to assess the effect of genotype on metabolic and cardiovascular function or to detect whether the PER3 genotype frequencies were different between SW and NSW. Directions for future research would be either to conduct a large epidemiological study or, in order to have more control over the environmental and experimental conditions, match subjects for genotype and then investigate the responses of interest as has been done in some studies (Viola et al. 2008). The same conditions hold for investigating the characteristics of different genotypes in the adenosinergic system that may be important in the regulation of sleep (section 1.8). The G/A genotype of ADA has been linked to various observations such as fewer awakenings, more SWS and deeper sleep than the G/G genotype (Retey et al. 2005). However the G/A genotype is only present in 8-12% of the population while 88-92% of people have the G/G genotype, so matching of subjects for ADA genotype would be necessary to assess whether they respond differently to sleep deprivation. Another interesting candidate would be the adenosine A2A receptor, although its C/C genotype only showed enhanced
power in the high theta/low alpha range compared with the T/T genotype (Retey et al. 2005). Polymorphisms in this receptor may also affect vulnerability to sleep deprivation as sensitivity for its antagonist, caffeine, influence the neurobehavioural response to sleep deprivation (Retey et al. 2006).

SW working shifts for 5 years or more participated in the present study. Three of the 11 SW had a shift work duration of longer than 15 years. Although there were no obvious differences between them and the 8 SW that had worked shift for 5-7 years (apart from a lower SDNN during the TSD night in the 3 SW with the shift work duration longer than 15 years (section 4.3.3)), future research should compare SW with long shift durations to those with a shorter shift work duration e.g. more than 15 years vs 3-5 years and match them for other screening parameters.

Another suggestion would be to match subjects for socioeconomic status as both this factor and health problems have been shown to be independently associated with the amount of self-reported sleep problems in the British population (Arber et al. 2009). Therefore, differences in socioeconomic status between subject groups may affect their attitude towards sleep and how they respond to sleep deprivation. Although mental and physical health aspects of the screening data were not significantly different between SW and NSW, they may have been dissimilar in their socioeconomic status (data not captured).

Some parameters that were initially planned to be measured in the current study but could not be assessed due to time and financial constraints are inflammatory markers and factors influencing endothelial function. This aim was based on research showing that even though the metabolic component is still linked to the development of CVD, inflammation is receiving more attention (Grundy et al. 2004; Libby 2006; Shaw et al. 2005). Proinflammatory cytokines (such as IL-6 and TNF-α) as well as changes in insulin concentrations are thought to stimulate the expression of vascular cell adhesion molecules (V-CAMs), which in turn, attract leukocytes. Upon migration of leukocytes into the vascular intima, macrophage proliferation is stimulated which results in matrix remodelling. This may eventually lead to collagen degradation and plaque rupture and thrombosis, characteristic for CVD (Libby 2006). The role of inflammation is thought to be partially reflected in moderately, but constantly elevated high sensitivity C-reactive protein (hsCRP) levels (Grundy et al. 2004; Libby & Ridker 2004).
These inflammatory markers and cell adhesion molecules have indeed been shown to be affected by sleep deprivation (Mullington et al. 2009). TSD for 40 - 88 h or PSD for 5 days increased hsCRP and the level was still elevated after recovery sleep (Frey et al. 2007; Meier-Ewert et al. 2004; van Leeuwen et al. 2009). Furthermore, 1 night to 4 nights of TSD or PSD affected I-CAM, L/E-selectin and various factors in the inflammatory and immune system such as interleukins and TNF-α pathways (Frey et al. 2007; Irwin et al. 2006; Irwin et al. 2008; Redwine et al. 2004; Redwine et al. 2000; Shearer et al. 2001; van Leeuwen et al. 2009; Vgontzas et al. 2007; Vgontzas et al. 2004). By contrast, there are hardly any studies reporting the effects of shift work on cell adhesion molecules or factors involved in inflammation. However, some studies have demonstrated alterations in SW in other related pathways contributing to changes in vascular function, for example alterations in fibrinolytic activity in rotating SW (Andreotti & Kluft 1991) or increased plasma homocysteine in shift working bus drivers compared to day workers. Another study investigated several parameters (normetanephrine / creatinine, von Willebrand factor, anti-thrombin III, T-plasminogen activator – plasminogen activator inhibitor 1 - complex, hematocrit) before, during and after a night shift in taxi drivers (Hattori & Azami 2001). Most of these factors showed differences between morning, midnight and next morning, depending on what time the shift was finished. According to the authors, these results indicated endothelial dysfunction. However, as in many shift work studies, the differences may be due to differences in physical activity and/or circadian phase, not necessarily the night shift or sleep deprivation itself.

Finally, it would be informative to study other factors involved in metabolism, such as the hormones leptin and ghrelin, signalling satiety and hunger, respectively. A decrease in leptin amplitude was observed after sleep deprivation, indicating reduced satiety (Mullington et al. 2003; Spiegel et al. 2004). In support of these findings, other studies have observed increased hunger ratings and ghrelin levels after both PSD and TSD (Schmid et al. 2008) and increased ghrelin levels in the early evening after sleep deprivation (before recovery sleep) compared to before sleep deprivation (Schussler et al. 2006). These findings support the increased energy expenditure during/after sleep deprivation that was suggested based on the observed decrease in basal TAG levels. By contrast, others have shown that leptin increased and satiety and hunger did not change after PSD (Schmid et al. 2009; Schussler et al. 2006; van Leeuwen et al. 2010). Differences between studies, however, may be related to the extent of ad libitum food intake.
6.4.2 Study design

The most important next step to take with respect to the study design would be to extend the recovery period. As discussed in previous sections, a longer period of recovery sleep would be needed to investigate further the time course of these observed changes in metabolism and HRV following recovery sleep. To elucidate why these changes take place after recovery sleep but not after sleep deprivation also requires further study. Other studies have also suggested that one night of recovery sleep may not be sufficient because some changes only became overt after recovery sleep (Sauvet et al. 2009). Alternatively some studies on alertness have indicated that one recovery night of 8 h may not be enough to reverse the effects of sleep deprivation and either longer hours (a longer recovery night or the inclusion of a nap) (Faraut et al. 2010) or more days (section 1.6.1.2) are needed.

Another modification to the study design could be to study an even more defined group of SW. The current study included SW with a range of shift durations, schedules and job types. In addition to restricting the range of shift work durations (see previous section), only one type of shift schedule could be specified, for example the selection of SW rotating in a clockwise direction. However, the current study criteria were left a little wider in order to be able to recruit a sufficient number of subjects. In addition, the job type was left unspecified as this may result in comparing the effect of certain job against day work, whereas the aim of the study was to assess the effects of shift work in general.

6.5 Conclusion

The data from the current study have demonstrated that sleep deprivation resulted in significantly decreased basal TAG levels and postprandial glucose clearance as well as a peak in HRV variance around habitual wake up time. Lower variance in HRV and indications of a hyperinsulinaemic response and insulin insensitivity were observed after recovery sleep. Alertness and mood significantly deteriorated after TSD but showed effective recuperation after recovery sleep. These statistically significant changes were observed despite the small sample number. Future studies should increase the sample number and include longer periods of sleep deprivation and recovery sleep to assess the time course of these changes and to investigate why some changes only take place after recovery sleep but not after TSD. Furthermore, shift workers showed decreased HRV variance and increased sympathetic activity, a trend for lower FMD and a smaller
decrease in NEFA and increase in insulin levels after recovery sleep compared to non-shift workers. It would be of interest to elucidate which factors are responsible for the observed differences in metabolic and cardiovascular function between shift workers and non-shift workers by either studying a different shift work population or additional parameters that can reveal information about the underlying mechanisms. Interindividual differences, for example in the response to sleep deprivation and the mechanisms underlying these, are also important areas of future research.
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Poster presentations


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Registration grant from the European Biological Rhythms Society to attend their XI. Congress, Strasbourg, France, 22-28 August 2009.
Appendix B Email questionnaire

E-mail questionnaire for the study:

“The effects of total sleep deprivation on metabolic and cardiovascular function”

Name:
Phone #:
Email:
Address:
Post code:

Thank you for your interest in our study. Our selection process is pretty extensive. After receiving your completed questionnaire, we will review it and may give you a follow-up call to collect some additional information. We then may determine that you are not eligible for this study. If you are eligible, we will ask you to fill out some more questionnaires, to come to our unit so that we can show you around, explain the study in more detail, and ask you to provide a blood and urine sample for a standard screen. Only after the completion of this visit and once we have support from your GP we will be able to determine whether or not we can fit you into our study.

Attached is a brief overview of the study. If you are still interested, please complete and return via email the following questionnaire. If you would rather not complete these forms via email, we can ask you these questions over the phone. To do so, or if you have any further questions, please call us at 01483 689705. If you return the filled out questionnaire, you should hear from us within the next few days. Thanks again for your interest.

Please fill out the entire questionnaire as completely and precisely as possible; this will speed up the selection process.

The information you provide will be kept confidential and will not be shared with anyone other than the research staff!

1. Confirm the correct spelling of your name and address, as well as your correct phone number in the section above.

2. a Confirm your age.

   How old are you? (In order to be able to participate, you must be 25-45) ____
   What is your date of birth? ____/____/19____

2. b What is your
Appendices

Weight (kg) Height (cm)

3. How did you hear about our study?
   - Poster at: ________  - Newspaper  - Email  - Other: _________

4. Are you afraid of blood and/or needles?  □ Yes  □ No  □ I don’t know

5. Have you ever participated or are you currently participating in another research study or clinical trial?
   - Yes  □ No  If yes, please state what kind of study, where and when ___________________

6. Do or did you take any regular medication (This includes any pills, creams, injections or anything you need a doctor’s prescription for)?
   - Yes  □ No  If yes, please state name, dosage, time and frequency of administration and date of last use
   ................................................................................................................................................
   ................................................................................................................................................

7. Do or did you take any regular ‘over the counter medication’ (medication for which no prescription was needed such as Aspirine, Paracetamol, Ibuprofen, medication for soar throats and colds or homeopathic medication such as St Johns Wort) or food supplements (such as vitamins / minerals and fish oils)?
   - Yes  □ No  If yes, please state name, dosage, time and frequency of administration and date of last use
   ................................................................................................................................................
   ................................................................................................................................................

8. The following is a list of medical conditions. Please, circle ‘yes’ if you have or ever had any of them.

   Heart disease  Yes/No  Vascular disease  Yes/No
   Blood Pressure  Yes/No  Gastrointestinal disorders  Yes/No
   Endocrine disorders  Yes/No  Asthma  Yes/No
   Liver disease  Yes/No  Arthritis  Yes/No
   Diabetes  Yes/No  Cancer  Yes/No
   Anaemia  Yes/No  Thyroid disorders  Yes/No
   Any allergies e.g. for food or pollen (hay fever)  Yes/No
   Psychiatric conditions (i.e. depression/schizophrenia)  Yes/No
   Anything else?  Yes/No

9. Have you ever smoked?
Appendices

☐ Yes  ☐ No

If yes:  For how long have you been smoking/were you smoking?
What do/did you smoke (cigarettes, cigars, pipe)?
How many units do/did you smoke a day?

10. Do you drink / did you use to drink alcohol?

☐ Yes  ☐ No

If yes:  For how long have you been drinking/were you drinking?
How much alcohol do you drink / did you drink on average (units per week)?
(1 pint = 2 units; 1 measure of alcohol = 1 unit)

None  1-4 units  5-14  15 or more

11. Do you use / have you used recreational drugs?

☐ Yes  ☐ No  If yes, please state name, dosage, time and frequency of administration and date of last use

Recreational drugs are prohibited throughout the study. You will be asked to provide a urine sample for a drug screen at the start of the study, and possibly at other random times during the study. Would you consent on this?

☐ Yes  ☐ No

12. How many cups of tea/coffee or other caffeinated drinks (such as red bull or coke) do you drink a day?

None  1-3 cups  4 or more cups

13. Would you be able to refrain from smoking, alcohol, caffeine and/or taking the prescribed medication/over the counter medication/food supplements for some time before and during the study?

☐ Yes  ☐ No

14. Have you given more than 400 ml of blood in the past 3 months?

☐ Yes  ☐ No

15 a. What is your occupation?

15 b. What kind of shift patterns you have been working up to now?

☐ only day shifts  ☐ permanent nights

☐ only early and / or late shifts  ☐ other / mixed

☐ rotating shifts
Appendices

15c Regarding the shift pattern(s) you have ticked for question 15b: for how many months / years have you been working each of the patterns and when was it you have worked these patterns?

16. Have you crossed any time zones in the past month?
☑ Yes  ☐ No  If yes, where did you go and when did you return?____________________

17. We would like to know more about your habitual sleep schedule.

What time are you currently going to bed during the week / when you are on day shifts?
What time are you currently waking up during the week / when you are on day shifts?
Are you waking up because you have to (i.e. to get to class or work or any other reason)?  ☑ Yes  ☐ No

So, you usually get about (calculate Average Sleep Duration) _______ hours of sleep per night during the week / day shifts

What time are you currently going to bed during the weekend / days off?
What time are you currently waking up during the weekend / days off?

So, you usually get about (calculate Average Sleep Duration) _______ hours of sleep per night during the weekend / days off?

How often do you wake up during the night? _______________

When you awaken, how long are you awake for? ________ 

Thank you very much for completing this questionnaire!
The aim of the study is to investigate the effect of a night of total sleep deprivation (as is experienced during the first night of a night shift) on a range of metabolic, inflammatory and cardiovascular responses. Different groups of people (shift workers and non-shift workers) will be studied. The effect of a recovery nap and recovery sleep will also be investigated.

You will be asked to stay in the laboratory on one occasion for 4 nights and 4 days. After two nights of sleep, we will ask you to stay awake for one night, followed by a recovery nap and recovery sleep. During the study you will be in controlled conditions regarding dim light, body posture and food intake. Throughout the study we will need to take saliva samples and regular blood samples to investigate the above mentioned metabolic, inflammatory and cardiovascular responses. You are also asked to wear electrodes on your head, face and chest for sleep recordings. Furthermore, you will undergo ultrasound recordings of the artery in your upper arm (flow mediated dilatation (FMD)) to measure functioning of the blood vessels. These non-invasive methods are all painless and harmless. Mood, alertness and performance will also be measured.

As mentioned before, there are numerous pre-screening procedures. Before the study, you will be screened for drugs of abuse in urine, and a standard hematological and biochemical blood screen will also be done. We will write to your medical practitioner to ask whether he/she considers you suitable for the study and to request information about medications or medical disorders that may affect the study. If your medical practitioner considers that you should not, for whatever reason, participate in the study, we will be obliged to accept this advice.

Once we have considered you to be suitable to take part and you have decided to give your consent, you will be asked to collect a buccal swab (samples of cells lining the inside of the cheek). Samples of cheek cells will be used to provide a DNA sample for the sole measurement of specific genes involved in sleep and circadian rhythms. All research records including DNA samples will be assigned alphanumeric codes to ensure confidentiality and collected data will not be available to unauthorised persons.

Prior to the laboratory study, your sleep/wake cycle, diet, food supplements, medication and exercise will be strictly controlled. You will be required to keep a regular sleep-wake cycle for 1 week prior to the study, to refrain from caffeine, alcohol, certain foods and heavy exercise the day before and during the laboratory session and from certain medication and food supplements for a certain wash out period. To ensure you are doing this, we will ask you to wear activity and light monitors, ring the lab-voice mail and complete a sleep-wake diary the week prior to the study. You will also be asked to record your consumptions and exercise pattern the week prior to the study.

Payment is £600. If you do not complete the study, you will be paid on a pro-rata basis.
Appendix C Whole blood, serum and urine screening parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole blood haematology</strong></td>
<td></td>
</tr>
<tr>
<td>haemoglobin (Hb)</td>
<td></td>
</tr>
<tr>
<td>white blood cells (WBC)</td>
<td></td>
</tr>
<tr>
<td>platelets</td>
<td></td>
</tr>
<tr>
<td>packed cells volume (PCV)</td>
<td></td>
</tr>
<tr>
<td>mean cell/corpuscular volume (MCV)</td>
<td></td>
</tr>
<tr>
<td>mean cell/corpuscular haemoglobin (MCH)</td>
<td></td>
</tr>
<tr>
<td>red blood cells (RBC)</td>
<td></td>
</tr>
<tr>
<td>mean cell/corpuscular haemoglobin concentration (MCHC)</td>
<td></td>
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<tr>
<td>neutrophils</td>
<td></td>
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<tr>
<td>lymphocytes</td>
<td></td>
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<tr>
<td>monocytes</td>
<td></td>
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<tr>
<td>eosinophils</td>
<td></td>
</tr>
<tr>
<td>basophils</td>
<td></td>
</tr>
<tr>
<td><strong>Serum biochemistry</strong></td>
<td></td>
</tr>
<tr>
<td>sodium</td>
<td>135 - 145 mmol/L</td>
</tr>
<tr>
<td>potassium</td>
<td>3.5 - 5.1 mmol/L</td>
</tr>
<tr>
<td>urea</td>
<td>2.6 - 6.5 mmol/L</td>
</tr>
<tr>
<td>creatinine</td>
<td>60 - 120 µmol/l</td>
</tr>
<tr>
<td>total bilirubin</td>
<td>&lt; 23 µmol/l</td>
</tr>
<tr>
<td>alkaline phosphatase</td>
<td>25 - 120 IU/l</td>
</tr>
<tr>
<td>alanine amino transferase (ALT)</td>
<td>&lt; 40 IU/l</td>
</tr>
<tr>
<td>albumin</td>
<td>34 - 48 g/l</td>
</tr>
<tr>
<td>cholesterol</td>
<td>&lt; 5 mmol/l</td>
</tr>
<tr>
<td>HDL</td>
<td>&gt; 1 mmol/l</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td></td>
</tr>
<tr>
<td>opiates (morphine, codeine, dihydrocodeine, pholcodine)</td>
<td></td>
</tr>
<tr>
<td>amphetamines / ecstasy</td>
<td></td>
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<tr>
<td>methadone</td>
<td></td>
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<tr>
<td>barbiturates</td>
<td></td>
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<tr>
<td>benzodiazepines</td>
<td></td>
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<tr>
<td>cocaine</td>
<td></td>
</tr>
<tr>
<td>cannabis</td>
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</tr>
</tbody>
</table>
Appendix D Measurement of waist and hip circumference

Schematic presentation of the levels at which the waist, hip and thigh circumferences should be measured.
According to the recommendations issued by the World Health Organization. Waist: midway between the lower rib margin and iliac crest; hip: widest over the great trochanters; thigh: highest level at the gluteal fold. From Van der Kooy & Seidell (1993).
Appendix E Screening questionnaires

General health questionnaire

Subject Identification Code: Date:

Date of Birth: Age:

Dominant hand:

Height (cm): Weight (kg):

Waist circumference: Hip circumference:

Do you do/have you done shift work?

Yes/No (If yes: please, fill in the ‘standard shift work index’)

Do or did you take any regular medication (This includes any pills, creams, injections or anything you need a doctor’s prescription for)?

☐ Yes ☐ No If yes, please state name, dosage, time and frequency of administration and date of last use

........................................................................................................................................................................................................

........................................................................................................................................................................................................

Do or did you take any regular ‘over the counter medication’ (medication for which no prescription was needed such as Apirine, Paracetamol, Ibuprofen, medication for soar throats and colds or homeopathic medication such as St Johns Wort) or food supplements (such as vitamins / minerals and fish oils) ?

☐ Yes ☐ No If yes, please state name, dosage, time and frequency of administration and date of last use

........................................................................................................................................................................................................

........................................................................................................................................................................................................

207
Do you have or have you had any major health problems?

<table>
<thead>
<tr>
<th>Condition</th>
<th>Yes/No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psychiatric conditions (i.e. depression/schizophrenia)</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Migraine</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Heart disease</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Vascular disease</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Gastrointestinal disorders</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Endocrine disorders</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Asthma</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Retinal disease</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Kidney disease</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Autoimmune disease</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Liver disease</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Arthritis</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Yes/No</td>
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<tr>
<td>Cancer</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Hernia</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Lung problems</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Anaemia</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Thyroid disorders</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Food or other allergies</td>
<td>Yes/No</td>
</tr>
</tbody>
</table>
General sleep questionnaire

Identification Code: ......................... Date:.....................

1. On a normal day how many times do you go outside?
   < 1/day  1/day  > 1/day

2. Would you regard your mobility as:
   good  fair  poor

3. What time do you normally go to bed (h:min)?

4. How many hours per night do you sleep on average?

5. How long does it take you to fall asleep?
   < 10 min  10-30 min  > 30 min

6. How often do you wake up during the night?

7. How often do you get up during the night?

8. Why do you get up during the night?

9. What time do you normally wake up (h:min)?

10. Do you go through phases of getting to sleep earlier and earlier or later and later each night?

11. Do you have any difficulty getting up in the morning? Y/N

   If yes, what kind of difficulty?
12. Do you fall asleep during the day? Y/N
   If yes, how often and for how long?

13. Do you feel you have any problems sleeping? Y/N
   If yes - What kind of problems?

14. Does it occur regularly?

15. Do you do anything to try and overcome this problem?
   (i.e. sleeping pills, relaxation techniques, herbal remedies, routine)

16. Are these strategies effective?

17. How long have you had this sleep problem?
   < 6 months  6-12 months  >12 months

18. Do you ever skip a night’s sleep?

19. Is your sleep pattern different during work free periods (e.g. holidays, weekends)?
   If yes, how is it different?

20. Does your sleep pattern affect your social or occupational life? Y/N If yes, how?
   If yes, in what way?
Horne-Östberg questionnaire

This questionnaire will used to assess whether you are a “morning” type (i.e. a lark) or an “evening” type (i.e. an owl) person.

a) Please read each question very carefully before answering.
b) Answer all questions
c) Answer questions in numerical order
d) Each question should be answered independently of others. Do NOT go back and check your answers.
e) For some questions you are required to respond by placing a cross alongside your answer. In such cases, select ONE answer only.
f) Please answer each question as honestly as possible. Both your answers and results will be kept in strict confidence.

QUESTION 1

Considering only your own “feeling best” rhythm, at what time would you get up if you were entirely free to plan your day?

AM 5 6 7 8 9 10 11 12

QUESTION 2

Considering only your own “feeling best” rhythm, at what time would you go to bed if you were entirely free to plan your evening?

PM 8 9 10 11 12 AM 1 2 3
QUESTION 3

If there is a specific time you have to get up in the morning, to what extent are you dependent on being woken up by an alarm clock?

a. Not at all dependent  [  ]
b. Slightly dependent  [  ]
c. Fairly dependent  [  ]
d. Very dependent  [  ]

QUESTION 4

Assuming adequate environmental conditions, how easy do you find getting up in the morning?

a. Not at all easy  [  ]
b. Not very easy  [  ]
c. Fairly easy  [  ]
d. Very easy  [  ]

QUESTION 5

How alert do you feel during the first half hour after having woken in the morning?

a. Not at all alert  [  ]
b. Slightly alert  [  ]
c. Fairly alert  [  ]
d. Very alert  [  ]
QUESTION 6

How is your appetite during the first half hour after having woken in the morning?

a. Very poor
b. Fairly poor
c. Fairly good
d. Very good

QUESTION 7

During the first half hour after having woken in the morning, how tired do you feel?

a. Very tired
b. Fairly tired
c. Fairly refreshed
d. Very refreshed
QUESTION 8

When you have no commitments the next day, at what time do you go to bed compared to your usual bedtime?

a. Seldom or never later
b. Less than one hour later
c. 1-2 hours later
d. More than 2 hours later

QUESTION 9

You have decided to engage in some physical exercise. A friend suggests that you do this one hour twice a week and the best time for him is between 0700 and 0800h. Bearing in mind nothing else but your own inclinations, how do you think you would perform?

a. Would be on good form
b. Would be on reasonable form
c. Would find it difficult
d. Would find it very difficult

QUESTION 10

At what time in the evening do you feel tired and as a result in need of sleep?
QUESTION 11

You wish to be at your peak for a test which you know is going to be mentally exhausting and lasting for two hours. You are entirely free to plan your day and considering only your own "feeling best" rhythm, which ONE of the four testing times would you choose?

a. 0800 – 1000 [ ]

b. 1100 – 1300 [ ]

c. 1500 – 1700 [ ]

d. 1900 – 2100 [ ]

QUESTION 12

If you went to bed at 2300h at what level of tiredness would you be?

a. Not at all tired [ ]

b. A little tired [ ]

c. Fairly tired [ ]

d. Very tired [ ]

QUESTION 13

For some reason you have gone to bed several hours later than usual, but there is no need to get up at any particular time the next morning. Which ONE of the following events are you most likely to experience:

a. Wake up at the usual time and not go back to sleep [ ]

b. Wake up at the usual time and doze [ ]

c. Wake up at the usual time and go back to sleep [ ]

d. Wake up later than usual [ ]
QUESTION 14

One night you have to remain awake between 0400 and 0600h. You have no commitments the next day. Which ONE of the following suits you best:

a. Not to go to bed until 0600h

b. Nap before 0400h and sleep after 0600h

c. Sleep before 0400h and nap after 0600h

d. Sleep before 0400h and remain awake after 0600h

QUESTION 15

You have to do two hours of hard physical work. You are entirely free to plan your day and considering only your own "feeling best" rhythm which ONE of the following times would you choose?

a. 0800 - 1000

b. 1100 - 1300

c. 1500 - 1700

d. 1900 - 2100

QUESTION 16

You have decided to engage in some physical exercise. A friend suggests that you do this between 2200 and 2300h twice a week. Bearing in mind nothing else but your own "feeling best" rhythm how well do you think you would perform?

a. Would be on good form

b. Would be on reasonable form

c. Would find it difficult

d. Would find it very difficult
QUESTION 17

Suppose that you can choose your own work hours. Assume that you worked a FIVE hour day (including breaks) and that your job was interesting and paid by results. Which FIVE CONSECUTIVE HOURS would you select:

![Time Selection](https://example.com/time_selection.png)

QUESTION 18

At what time of day do you think that you reach your “feeling best” peak?

![Time Selection](https://example.com/time_selection.png)

QUESTION 19

One hears of “morning” and “evening” types. Which do you consider yourself to be?

a. Morning type [ ]
b. More morning than evening [ ]
c. More evening than morning [ ]
d. Evening type [ ]
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 the questions.

1) During the past month, when have you usually gone to bed at night?
   Usual bed time

2) During the past month, how long (in minutes) has it usually take you to fall asleep each night?
   Number of minutes

3) During the past month, when have you usually got up in the morning?
   Usual getting up time

4) During the past month, how many hours of actual sleep did you get at night? (This may be different from the number of hours spent in bed.)
   Hours of sleep per night

For each of the remaining questions, check the one best response. Please answer all questions.

5) During the past month, how often have you had trouble sleeping because you.....

a) Cannot get to sleep within 30 minutes
   Not during the past month_____ Less than once a week_____ Once or twice a week_____ Three or more times a week_____

b) Wake up in the middle of the night or early morning
   Not during the past month_____ Less than once a week_____ Once or twice a week_____ Three or more times a week_____

c) Have to get up to use the bathroom
   Not during the past month_____ Less than once a week_____ Once or twice a week_____ Three or more times a week_____

d) Cannot breathe comfortably
   Not during the past month_____ Less than once a week_____ Once or twice a week_____ Three or more times a week_____
Appendices

e) Cough or snore loudly
   Not during the past month ___
   Less than once a week ___
   Once or twice a week ___
   Three or more times a week ___

f) Feel too cold
   Not during the past month ___
   Less than once a week ___
   Once or twice a week ___
   Three or more times a week ___

g) Feel too hot
   Not during the past month ___
   Less than once a week ___
   Once or twice a week ___
   Three or more times a week ___

h) Had bad dreams
   Not during the past month ___
   Less than once a week ___
   Once or twice a week ___
   Three or more times a week ___
i) Have pain
   Not during the past month ___
   Less than once a week ___
   Once or twice a week ___
   Three or more times a week ___

j) Other reason(s), please describe

_____________________________________________________________________
_____________________________________________________________________

How often during the past month have you had trouble sleeping because of this?

Not during the past month ___
   Less than once a week ___
   Once or twice a week ___
   Three or more times a week ___

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

   Very good ___________
   Fairly good __________
   Fairly bad ___________
   Very bad _____________

7) During the past month, how often have you taken medicine (prescribed or “over the counter”) to help you sleep?

Not during the past month ___
   Less than once a week ___
   Once or twice a week ___
   Three or more times a week ___

8) During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?

Not during the past month ___
   Less than once a week ___
   Once or twice a week ___
   Three or more times a week ___
9) During the past month, how much of a problem has it been for you to show enthusiasm to get things done?

- No problem at all
- Only a very slight problem
- Somewhat of a problem
- A very big problem

10) Do you have a bed partner or roommate?

- No bed partner or roommate
- Partner/roommate in other room
- Partner in same room, but not same bed
- Partner in same bed

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

a) Loud snoring
   - Not during the past month
   - Less than once a week
   - Once or twice a week
   - Three or more times a week

b) Long pauses between breaths while asleep
   - Not during the past month
   - Less than once a week
   - Once or twice a week
   - Three or more times a week

c) Legs twitching or jerking while you sleep
   - Not during the past month
   - Less than once a week
   - Once or twice a week
   - Three or more times a week

d) Episodes of disorientation or confusion during sleep?
   - Not during the past month
   - Less than once a week
   - Once or twice a week
   - Three or more times a week

e) Other restlessness while you sleep; please describe

Not during the past month
Less than once a week
Once or twice a week
Three or more times a week
Beck Depression Inventory

**UNIVERSITY OF SURREY**

**Identification Code:** ................. **Date:** ...............  

Choose one statement from among the group of four statements in each question that best describes how you have been feeling during the **past few days**. Circle the number beside your choice.

<table>
<thead>
<tr>
<th>Question</th>
<th>Statement 0</th>
<th>Statement 1</th>
<th>Statement 2</th>
<th>Statement 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I do not feel sad.</td>
<td>I feel sad.</td>
<td>I am sad all the time and I can't snap out of it.</td>
<td>I am so sad or unhappy that I can't stand it.</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>I am not particularly discouraged about the future.</td>
<td>I feel discouraged about the future.</td>
<td>I feel I have nothing to look forward to.</td>
<td>I feel that the future is hopeless and that things cannot improve.</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>I do not feel like a failure.</td>
<td>I feel I have failed more than the average person.</td>
<td>As I look back on my life, all I can see is a lot of failure.</td>
<td>I feel I am a complete failure as a person.</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>I get as much satisfaction out of things as I used to.</td>
<td>I don't enjoy things the way I used to.</td>
<td>I don't get any real satisfaction out of anything anymore.</td>
<td>I am dissatisfied or bored with everything.</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>I don't feel particularly guilty.</td>
<td>I feel guilty a good part of the time.</td>
<td>I feel quite guilty most of the time.</td>
<td>I feel guilty all of the time.</td>
</tr>
<tr>
<td></td>
<td>0</td>
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<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
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<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>6</td>
<td>I don't feel I am being punished.</td>
<td>I feel I may be punished.</td>
<td>I expect to be punished.</td>
<td>I feel I am being punished.</td>
</tr>
<tr>
<td>7</td>
<td>I don't feel disappointed in myself.</td>
<td>I am disappointed in myself.</td>
<td>I am disgusted with myself.</td>
<td>I hate myself.</td>
</tr>
<tr>
<td>13</td>
<td>I make decisions about as well as I ever could.</td>
<td>I put off making decisions more than I used to.</td>
<td>I have greater difficulty in making decisions than before.</td>
<td>I can't make decisions at all anymore.</td>
</tr>
<tr>
<td>14</td>
<td>I don't feel that I look any worse than I used to.</td>
<td>I am worried that I am looking old or unattractive.</td>
<td>I feel that there are permanent changes in my appearance that make me look unattractive.</td>
<td>I believe that I look ugly.</td>
</tr>
<tr>
<td>15</td>
<td>I can work about as well as before.</td>
<td>It takes an extra effort to get started at doing something.</td>
<td>I have to push myself very hard to do anything.</td>
<td>I can't do any work at all.</td>
</tr>
<tr>
<td>19</td>
<td>I haven't lost much weight, if any, lately.</td>
<td>I have lost more than five pounds.</td>
<td>I have lost more than ten pounds.</td>
<td>I have lost more than fifteen pounds.</td>
</tr>
<tr>
<td>16</td>
<td>I can sleep as well as usual.</td>
<td>I don't sleep as well as I used to.</td>
<td>I wake up 1-2 hours earlier than usual and find it hard to get back to sleep.</td>
<td>I wake up several hours earlier than I used to and cannot get back to sleep.</td>
</tr>
<tr>
<td>20</td>
<td>I am no more worried about my health than usual.</td>
<td>I am worried about physical problems such as aches and pains, or upset stomach, or constipation.</td>
<td>I am very worried about physical problems, and it's hard to think of much else.</td>
<td>I am so worried about my physical problems that I cannot think about anything else.</td>
</tr>
<tr>
<td>17</td>
<td>I don't get more tired than usual.</td>
<td>I get tired more easily than I used to.</td>
<td>I get tired from doing almost anything.</td>
<td>I am too tired to do anything.</td>
</tr>
<tr>
<td>21</td>
<td>I have not noticed any recent change in my interest in sex.</td>
<td>I am less interested in sex than I used to be.</td>
<td>I am much less interested in sex now.</td>
<td>I have lost interested in sex completely.</td>
</tr>
<tr>
<td>18</td>
<td>My appetite is no worse than usual.</td>
<td>My appetite is not as good as it used to be.</td>
<td>My appetite is much worse now.</td>
<td>I have no appetite at all anymore.</td>
</tr>
</tbody>
</table>
### Epworth Sleepiness Scale

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

- **0** = would *never* doze or sleep.
- **1** = *slight* chance of dozing or sleeping
- **2** = *moderate* chance of dozing or sleeping
- **3** = *high* chance of dozing or sleeping

<table>
<thead>
<tr>
<th>Situation</th>
<th>Chance of Dozing or Sleeping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sitting and reading</td>
<td></td>
</tr>
<tr>
<td>Watching TV</td>
<td></td>
</tr>
<tr>
<td>Sitting inactive in a public place</td>
<td></td>
</tr>
<tr>
<td>Being a passenger in a motor vehicle for an hour or more</td>
<td></td>
</tr>
<tr>
<td>Lying down in the afternoon</td>
<td></td>
</tr>
<tr>
<td>Sitting and talking to someone</td>
<td></td>
</tr>
<tr>
<td>Sitting quietly after lunch (no alcohol)</td>
<td></td>
</tr>
<tr>
<td>Stopped for a few minutes in traffic while driving</td>
<td></td>
</tr>
<tr>
<td><strong>Total score</strong></td>
<td>223</td>
</tr>
</tbody>
</table>
Appendices

Standard Shift work Index

(N.B. Only those questions used to assess shift work duration and schedules are included in this appendix due to space constraints)

We are an independent research team engaged on a research programme looking at the problems which people may experience as a result of working shifts. We have no particular "axe to grind" within an organisation.

Throughout this questionnaire the terms "Morning", "Afternoon" and "Night" shifts are used. Please ignore the fact that these terms may differ from the ones used in your organisation. For example, you may call your "Morning" shift an "Early" one, while your "Afternoon" shift may be referred to as a "Late", "Evening" or "Swing" shift.

It is possible that completing this questionnaire may draw your attention to problems you experience as a result of shiftwork. If you are worried that these are serious we would advise you to contact your GP (see back page).

Thank you for your co-operation.

Developed by the:

Shiftwork Research Team,
MRC/ESRC Social and Applied Psychology Unit

Professor Emeritus Simon Folkard D.Sc.
1. Your General Biographical Information

Please answer the following questions as accurately as possible. Please note that the information you give will be treated in strictest confidence.

1.10 How long have you worked altogether? ________ years
1.11 How long have you worked in your present shift system? ________ years ________ months
1.12 How long altogether have you been working shifts? ________ years ________ months
1.13 On average, how many hours do you work each week excluding overtime? ________ hours ________ minutes
1.14 On average, how many hours paid overtime do you work each week? ________ hours ________ minutes
1.15 On average, how many hours unpaid overtime do you work each week, (e.g. over-run of shifts)? ________ hours ________ minutes
1.16 Do you have a second paid job in addition to your main one? (tick one) yes no
1.17 If you have taken a career break (or breaks), how long was this for in total? ________ years ________ months

Your Shift Details

1.18 For each of the shifts that you normally work, at what time do they start and finish? (Please use 24h time, e.g. 21:30 or clearly indicate "am" or "pm").

<table>
<thead>
<tr>
<th>Shift Type</th>
<th>START</th>
<th>FINISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Morning (or early) shift</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) Afternoon (or late, evening or swing) shift</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(c) Half-day shift</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(d) Night shift</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(e) Other</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.22 For each of the shifts that you normally work, on average how many successive shifts of the same kind do you normally work before changing to another shift or having some days off?

<table>
<thead>
<tr>
<th>Shift Type</th>
<th>NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Number of successive morning shifts</td>
<td></td>
</tr>
<tr>
<td>(b) Number of successive afternoon shifts</td>
<td></td>
</tr>
<tr>
<td>(c) Number of successive night shifts</td>
<td></td>
</tr>
</tbody>
</table>
Appendices

1.23 What is the maximum number of shifts of any kind you have worked between days off in the past month? __________

1.24 On average, how many days off in succession do you normally have? __________

1.25 In general, when changing from one type of shift to another, what type of shift is each shift or day off followed by?

(a) Morning shifts are normally followed by: __________
(b) Afternoon shifts are normally followed by: __________
(c) Night shifts are normally followed by: __________
(d) Other ......................... are normally followed by: __________
(e) Days off are normally followed by: __________

1.26 On average how many nights do you work per year? __________

1.27 How are these night shifts organised?
(please tick the one which best describes your night work)

(a) permanent nightshift __________
(b) a single block of night duty per year __________
(c) occasional blocks of night duty per year __________
(d) a block of nights each month __________
(e) one or two nights each week __________
(f) any other? (please specify) __________

1.28 On average how many weekends do you have off per 28 days? __________

1.29 How regular is the shift system you work?
(please tick one)

(a) REGULAR i.e. a fixed roster which is repeated when the cycle of shifts finishes, even if occasional variations occur to meet special requests. __________
(b) **IRREGULAR** i.e. the duty roster does not cycle or repeat in any regular manner and individual preferences are not taken into account.

(e) **FLEXIBLE** i.e. where the individuals concerned are consulted about their preferred duty hours before the duty roster is drawn up.

1.30 If your shift system is **regular**, over how many weeks does the cycle run before it is repeated?
## Appendix F Subject demographics

<table>
<thead>
<tr>
<th>subject ID</th>
<th>group</th>
<th>gender</th>
<th>age (years)</th>
<th>BMI (kg/m²)</th>
<th>waist (cm)</th>
<th>height (m)</th>
<th>weight (kg)</th>
<th>blood pressure</th>
<th>cholesterol (mmol/L)</th>
<th>serum HDL (mmol/L)</th>
<th>genotype</th>
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</thead>
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<td>C-2</td>
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<td>97</td>
<td>1.75</td>
<td>97.6</td>
<td>140</td>
<td>9.8</td>
<td>4.2</td>
<td>45</td>
</tr>
<tr>
<td>17</td>
<td>SW</td>
<td>female</td>
<td>0</td>
<td>23.9</td>
<td>97</td>
<td>1.75</td>
<td>91.6</td>
<td>140</td>
<td>9.8</td>
<td>4.2</td>
<td>45</td>
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<tr>
<td>18</td>
<td>SW</td>
<td>female</td>
<td>25</td>
<td>23.9</td>
<td>97</td>
<td>1.75</td>
<td>91.8</td>
<td>140</td>
<td>9.8</td>
<td>4.2</td>
<td>45</td>
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<tr>
<td>29</td>
<td>SW</td>
<td>female</td>
<td>0</td>
<td>23.9</td>
<td>97</td>
<td>1.75</td>
<td>82.9</td>
<td>140</td>
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<td>4.2</td>
<td>45</td>
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<tr>
<td>34</td>
<td>SW</td>
<td>female</td>
<td>0</td>
<td>23.9</td>
<td>97</td>
<td>1.75</td>
<td>97.6</td>
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<td>35</td>
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<td>0</td>
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<td>97</td>
<td>1.75</td>
<td>91.6</td>
<td>140</td>
<td>9.8</td>
<td>4.2</td>
<td>45</td>
</tr>
</tbody>
</table>

Superscripts indicate values from the second assessment prior to the study (see section 2.2)
<table>
<thead>
<tr>
<th>Current shift work</th>
<th>Previous shift work</th>
<th>Other paid job</th>
<th>Current shift pattern</th>
<th>Estimated number of nights per month</th>
<th>Time in current shift pattern (years)</th>
<th>Total shift work (years)</th>
<th>Time since last shift (months)</th>
<th>Career break (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSD-S-1 prison officer</td>
<td>n/a</td>
<td>no</td>
<td>M EE NNNN NNN xx*</td>
<td>24.5</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>TSD-S-2 IT</td>
<td>n/a</td>
<td>no</td>
<td>EEEEE xx MMMMM xx NNNN NNNN xxxxxx</td>
<td>7</td>
<td>5.6</td>
<td>5.6</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>TSD-S-3 fire fighter</td>
<td>n/a</td>
<td>no</td>
<td>DD NN xx</td>
<td>8.3</td>
<td>5.1</td>
<td>5.1</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>TSD-S-4 jailer</td>
<td>?</td>
<td>no</td>
<td>MMM EEEE xx NNNN NNNN **</td>
<td>8.3</td>
<td>3.3</td>
<td>5</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>TSD-S-5 unemployed</td>
<td>?</td>
<td>no</td>
<td>NNNN*</td>
<td>20</td>
<td>7</td>
<td>7</td>
<td>18</td>
<td>n/a</td>
</tr>
<tr>
<td>TSD-S-6 police officer</td>
<td>?</td>
<td>no</td>
<td>EEE(E) (O) MMM xxx(x) (for 7 wks) NNNN NNNN xxxxxx</td>
<td>3.8</td>
<td>6.1</td>
<td>7</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>TSD-17 fire fighter</td>
<td>n/a</td>
<td>yes</td>
<td>DD NN xx</td>
<td>7.7</td>
<td>18.5</td>
<td>18.5</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>TSD-18 nurse, long days</td>
<td>nurse; early and late shifts</td>
<td>no</td>
<td>LLL xx LLL xx LLL xx LLL xx LLL xx LLL xx NNN xx</td>
<td>3</td>
<td>3</td>
<td>6.3</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>TSD-19 care-assistant</td>
<td>n/a</td>
<td>yes</td>
<td>NNNN(N) xx</td>
<td>18.8</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>TSD-20 night duty</td>
<td>?</td>
<td>no</td>
<td>DD NN xxx</td>
<td>6.7</td>
<td>16.7</td>
<td>16.7</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>TSD-21 events bar manager</td>
<td>?</td>
<td>no</td>
<td>NNNN(NN) x*</td>
<td>22.8</td>
<td>10</td>
<td>15</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>11.9</strong></td>
<td><strong>8.2</strong></td>
<td><strong>8.7</strong></td>
<td><strong>1.6</strong></td>
<td><strong>0.8</strong></td>
</tr>
</tbody>
</table>

**SD**

7.9 5.3 5.2 5.4 2.1

M, morning / early shift; E, evening shift; N, night shift; x, day off; O, other shift; L, long shift > 12 hrs; *, no regular/well-defined shift pattern
Appendices

Appendix H Procedures 2 days prior to the laboratory session

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Steps to be taken on the 2 days prior to study (from waking up):

1. Do not take:
   - Caffeine (e.g. tea, coffee, coke, red bull, chewing gum and chocolate.) Drinks that do not contain caffeine and are allowed include: herbal tea, 7-up, ginger ale and sprite
   - Drinks and foods containing high levels of antioxidants (see table below for more details)
   - Bananas
   - Foods containing nitrate/nitrites listed below:
     - Green vegetables especially spinach, broccoli, green beans, peas and lettuce
     - Processed meats such as spam, bacon, ham, hot dogs, corn beef, burgers
     - Oysters
   - Alcohol
   - Tobacco
   - Recreational drugs

2. Do not take:
   a. Prescribed medication
   b. ‘Over the counter’ medication e.g. non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin, ibuprofen and antihistamines, medication for soar throats and colds or homeopathic medication such as St John’s Wort. An exception is Paracetamol which is allowed because it is NOT a NSAID
   c. Food supplements e.g. vitamins / minerals, fish oil, protein supplements, anabolic steroids, antioxidant tablets

3. Do not take part in any heavy exercise

4. Do not apply lip balm; plain vaseline is ok.

5. Continue keeping your event diary (which will be provided) recording all the foods and drinks that you consume that day. Also continue wearing your light and activity monitors and keeping your sleep and light diary.

6. 5 h before your scheduled study time (following lunch on the day you are scheduled to start the study) you must fast. This means no food or drink other than water. E.g. if you are scheduled for 6 pm you must eat your lunch between 12:00 and 13:00 and not eat anything after 13:00.
Table. Details on antioxidant contents of vegetables and fruits.
Please avoid taking vegetables and fruits listed in the ‘NO’ column, since they are high in antioxidants! If you do want to eat vegetables and fruits, please chose from those listed in the ‘YES’ column.

<table>
<thead>
<tr>
<th>NO</th>
<th>YES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh vegetables</td>
<td>Fresh vegetables</td>
</tr>
<tr>
<td>Red cabbage</td>
<td>White potato</td>
</tr>
<tr>
<td>Beets</td>
<td>Red potato</td>
</tr>
<tr>
<td>Mushrooms</td>
<td>Cauliflower</td>
</tr>
<tr>
<td>Broccoli flowers</td>
<td>Carrots</td>
</tr>
<tr>
<td>Spinach</td>
<td>Yellow onions</td>
</tr>
<tr>
<td>Brussels sprouts</td>
<td>Tomato</td>
</tr>
<tr>
<td>Sweet corn</td>
<td>Green cabbage</td>
</tr>
<tr>
<td>Peas</td>
<td>Cucumber</td>
</tr>
<tr>
<td>Green beans</td>
<td>Celery</td>
</tr>
<tr>
<td>Head lettuce</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fresh fruits</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red plum</td>
<td>Dried fruit</td>
</tr>
<tr>
<td>Black plum</td>
<td>Fruit juices</td>
</tr>
<tr>
<td>Red grape</td>
<td></td>
</tr>
<tr>
<td>Red apple</td>
<td></td>
</tr>
<tr>
<td>Green grape</td>
<td></td>
</tr>
<tr>
<td>Nectarine</td>
<td></td>
</tr>
<tr>
<td>Banana</td>
<td></td>
</tr>
<tr>
<td>Kiwi</td>
<td></td>
</tr>
<tr>
<td>Pineapple</td>
<td></td>
</tr>
<tr>
<td>Sweet cherry</td>
<td></td>
</tr>
<tr>
<td>Berries</td>
<td></td>
</tr>
</tbody>
</table>

If you are unsure about any of the restrictions, do not hesitate to contact us!
Appendix I Protocol PER3 genotyping in human buccal swabs

Methods are according to an established protocol (Ebisawa et al. 2001), with some modifications (Vandewalle et al. 2009)

Use only sterile pipette tips

TNES solution (in RNase free water)

- 10 mM Tris at pH 7.65
- 400 mM NaCl
- 100 mM EDTA (inactivates metal-dependent enzymes that can damage DNA)
- 0.6% SDS (opens up the cellular membrane)

Example for a 10 ml solution

- 100 μl 1M TRIS
- 800 μl 5M NaCl
- 2 ml 0.5M EDTA
- 600 μl 10% SDS
- Complete up to 10 ml with DNase free water

Isolation protocol

- Thoroughly dissolve tissue from the buccal swab in 400 μl TNES
- Add 23 μl Proteinase K (10 mg/ml) and incubate for 1 hour at 55 °C
  *This will digest the cells*
- Add 111 μl 5 M NaCl and turn to mix (do not shake; SDS will foam) and leave 2 min
- Spin down for 10 minutes at max speed and transfer supernatant to new tube
  *The salt has precipitated the peptides which are left in the pellet*
- Add 1 volume (= 400 + 111 μl) -20 °C 95% EtOH and tumble to mix until clear
- Spin down for 5 minutes (or up to 12 if a smaller centrifuge is used) at max speed and discard supernatant
  *The EtOH has precipitated the DNA*
- Wash the pellet in 180 μl -20 °C 70% EtOH; spin down at max speed for 5 minutes
  *This will clean up the sample (remove hydrophilic impurities)*
- Remove supernatant and dry to air for 5 - 10 minutes at 55 °C
- Resuspend in 200 μl DNase free water
- Vortex 2 x 20 seconds

Make sure to resuspend very thoroughly, the DNA is now attached to the tube

- Store at -20 °C until further processing or run PCR immediately

PCR protocol

Primers:
- Primer 1 = X18 AF
- Primer 2 = X18 AR

10 μM primer stocks:
- Add 20 μl primer to 180 μl nuclease free water (not frozen)
- Vortex and spin down
- Vortex GoTaq (gently heat to thaw and then keep on ice)
- Make PCR mix in 0.5 ml tube (N = # samples + 1):
Appendices

- Add N * 8 µl nuclease free water (from frozen)
- Add N * 15 µl GoTAQ (2 times conc. solution)
*GoTAQ is always 50% of the end volume including sample DNA*
- Add N * 2 µl first primer (10 µM stock)
- Add N * 2 µl second primer (10 µM stock)

- Mark PCR tubes **high on the side, not on the lid**
- Add 27 µl of PCR mix to each tube
- Add 3 µl purified sample DNA to each tube and **put on ice**
- Add 3 µl nuclease free water (frozen) to blank (X) tube and put on ice
- Spin down samples and blank (and then **keep on ice**)
- Run PCR (do not add tubes until 95°C is reached):
  - 3 min – 95°C
  - Repeat 40 times:
    - 1 min – 95°C
    - 1 min – 58°C
    - 1 min – 72°C
    - 5 min – 72°C
  - ∞ at 4°C

**Blotting protocol:**

*No sterile pipette tips needed*

- Prepare 2% agarose-TaE gel
  - 0.5 gram agarose in 25 ml TaE buffer + 1 µl ethidum bromide
  - or-
  - 2 grams agarose in 100 ml TaE buffer + 5 µl ethidum bromide

*Microwave to dissolve until clear and than cool so that it can be held in hand, then add ethidium bromide and swirl to suspend*

- Pour gel
- Place gel in electrophoresis chamber, submerge in TaE buffer and remove combs
  - Load the samples
  - Attach black (negative) pole to top (comb side) of setup
  - Attach red (positive) pole to bottom of setup
- Set voltage to 120 V (or 65 V for small gel)

*DNA is negatively charged and will move towards the positive pole
Voltage is always volt/cm²; too high voltage = too warm gel*

- Run gel for ~3 hours (until yellow dye has ran off the end of the gel)
- Visualize gel under UV light (do not look into the light)
- Photograph gel

*Use settings 2 and 8 on camera
Pull strip and than photo (gently and at constant speed) from camera
Wait one minute and pull photo from developer strip*
## Appendix J Meal composition for the laboratory session

<table>
<thead>
<tr>
<th>Meal</th>
<th>Quantity</th>
<th>Unit</th>
<th>Energy (Kcal)</th>
<th>Protein (g)</th>
<th>Fat (g)</th>
<th>Carbohydrates (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breakfast</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cornflakes (Kelloggs)</td>
<td>40</td>
<td>g</td>
<td>148.8</td>
<td>2.8</td>
<td>0.4</td>
<td>33.6</td>
</tr>
<tr>
<td>Semi skimmed milk (Tesco)</td>
<td>125</td>
<td>ml</td>
<td>58.8</td>
<td>4.3</td>
<td>2.0</td>
<td>6.1</td>
</tr>
<tr>
<td>Croissant (Tesco full butter, 8 per pack)</td>
<td>1</td>
<td>unit</td>
<td>225.0</td>
<td>6.2</td>
<td>12.7</td>
<td>21.5</td>
</tr>
<tr>
<td>Butter (Country Life spreadable)</td>
<td>10</td>
<td>g</td>
<td>68.9</td>
<td>0.0</td>
<td>7.6</td>
<td>0.0</td>
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<tr>
<td>Strawberry cream yogurt (Tesco finest) (150 g)</td>
<td>1</td>
<td>unit</td>
<td>206.0</td>
<td>5.0</td>
<td>10.4</td>
<td>25.2</td>
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<tr>
<td>Sugar (Silverspoon) in 100 ml water</td>
<td>20</td>
<td>g</td>
<td>80.0</td>
<td>0.0</td>
<td>0.0</td>
<td>20.0</td>
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<td>Double cream (Tesco)</td>
<td>20</td>
<td>ml</td>
<td>89.0</td>
<td>0.3</td>
<td>9.6</td>
<td>0.5</td>
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<tr>
<td>Orange squash (Robinsons High Juice Orange Squash Drink) + 200 ml water</td>
<td>50</td>
<td>ml</td>
<td>91.0</td>
<td>0.2</td>
<td>0.1</td>
<td>22.0</td>
</tr>
<tr>
<td><strong>Total breakfast</strong></td>
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<td></td>
<td>967.5</td>
<td>18.8</td>
<td>42.7</td>
<td>129.0</td>
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<td>%composition</td>
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<td></td>
<td>7.8</td>
<td>39.7</td>
<td>53.3</td>
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<td><strong>Lunch</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slices of white bread (Hovis Granary 2 = 88g)</td>
<td>2</td>
<td>slice</td>
<td>203.0</td>
<td>8.1</td>
<td>1.5</td>
<td>39.4</td>
</tr>
<tr>
<td>Cheese (Tesco value mild)</td>
<td>50</td>
<td>g</td>
<td>410.0</td>
<td>25.0</td>
<td>34.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Butter (Country Life spreadable)</td>
<td>15</td>
<td>g</td>
<td>103.4</td>
<td>0.0</td>
<td>11.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Crisps (Tesco Ready Salted)</td>
<td>34.5</td>
<td>g</td>
<td>184.5</td>
<td>1.8</td>
<td>11.9</td>
<td>17.5</td>
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<tr>
<td>Orange squash (Robinsons High Juice Orange Squash Drink) + 200 ml water</td>
<td>50 ml</td>
<td>96.0</td>
<td>0.2</td>
<td>0.1</td>
<td>23.2</td>
<td></td>
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<td><strong>Total lunch</strong></td>
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<td></td>
<td>996.9</td>
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<td>80.3</td>
</tr>
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<td></td>
<td>14.1</td>
<td>53.6</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tesco vegetarian lasagne (629 gm)</td>
<td>1</td>
<td>unit</td>
<td>629.0</td>
<td>27.0</td>
<td>34.5</td>
<td>52.2</td>
</tr>
<tr>
<td>Strawberry cream yogurt (Tesco finest) (150 g)</td>
<td>1</td>
<td>unit</td>
<td>225.0</td>
<td>4.4</td>
<td>11.7</td>
<td>25.5</td>
</tr>
<tr>
<td>Orange squash (Robinsons High Juice Orange Squash Drink) + 200 ml water</td>
<td>50 ml</td>
<td>96.0</td>
<td>0.2</td>
<td>0.1</td>
<td>23.2</td>
<td></td>
</tr>
<tr>
<td><strong>Total dinner</strong></td>
<td></td>
<td></td>
<td>950.0</td>
<td>31.6</td>
<td>46.3</td>
<td>100.9</td>
</tr>
<tr>
<td>%composition</td>
<td></td>
<td></td>
<td>13.3</td>
<td>43.9</td>
<td>42.5</td>
<td></td>
</tr>
<tr>
<td><strong>Snack</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frij (strawberry)</td>
<td>250</td>
<td>ml</td>
<td>160.0</td>
<td>9.0</td>
<td>2.3</td>
<td>25.8</td>
</tr>
<tr>
<td>Biscuits (McVitie's digestives, 15 g each )</td>
<td>2</td>
<td>unit</td>
<td>140.0</td>
<td>2.2</td>
<td>6.2</td>
<td>18.6</td>
</tr>
<tr>
<td><strong>Total snack</strong></td>
<td></td>
<td></td>
<td>300.0</td>
<td>11.2</td>
<td>8.5</td>
<td>44.4</td>
</tr>
<tr>
<td>%composition</td>
<td></td>
<td></td>
<td>14.9</td>
<td>25.4</td>
<td>59.1</td>
<td></td>
</tr>
<tr>
<td><strong>Overall Total</strong></td>
<td></td>
<td></td>
<td>3214.4</td>
<td>96.6</td>
<td>156.9</td>
<td>354.5</td>
</tr>
<tr>
<td><strong>Overall % composition</strong></td>
<td></td>
<td></td>
<td>12.0</td>
<td>43.9</td>
<td>44.1</td>
<td></td>
</tr>
</tbody>
</table>
Appendices

Appendix K Metabolite reaction equations

Glucose (Oxidase) (IL TestTM, catalogue number 0018250840, Instrumentation Laboratory, Warrington, UK)

Enzymatic end point analysis, Trinder methodology:
\[
\text{\(\beta\)-D-Glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{GOD}} \text{GOD Gluconic acid} + \text{H}_2\text{O}_2
\]
\[
2 \text{H}_2\text{O}_2 + \text{phenol} + 4\text{-aminoantipyrine} \xrightarrow{\text{POD}} \text{quinoneimine} + 4 \text{H}_2\text{O}
\]
Glucose oxidase (GOD) / Peroxidase (POD)

The increase in absorbance as generated by the red quinoneimine dye is proportional to the glucose concentration in the sample. Primary measurements are taken at 510 nm. On the ILab 600, a blanking reading is taken at 600 nm.

Triglycerides (IL TestTM, catalogue number 0018255640, Instrumentation Laboratory)

Enzymatic end point analysis:
\[
\text{triglycerides} \xrightarrow{\text{lipoprotein lipase}} \text{glycerol} + \text{fatty acids}
\]
\[
\text{glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol-3-phosphate} + \text{ADP}
\]
\[
\text{glycerol-3-phosphate} + \text{O}_2 \xrightarrow{\text{glycerophosphate oxidase}} \text{dihydroxyacetone phosphate} + \text{H}_2\text{O}_2
\]
\[
\text{H}_2\text{O}_2 + 4\text{-chlorophenol} + 4\text{-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + \text{H}_2\text{O}_2
\]

The concentration of red quinoneimine dye generated in the reaction is proportional to the concentration of triglyceride in the sample. Absorbance measurements are taken at a reading and a blanking wavelength: ILab 600/900/1800: 510/700 nm.

Cholesterol (IL TestTM, catalogue number 0018250540, Instrumentation Laboratory)

Enzymatic end point dichromatic analysis:
\[
\text{cholesterol ester} + \text{H}_2\text{O} \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{fatty acids}
\]
\[
\text{cholesterol} + \text{O}_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholestenone} + \text{H}_2\text{O}_2
\]
\[
2 \text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + 4 \text{H}_2\text{O}
\]
Red quinoneimine production is proportional to the cholesterol concentration in the sample. Absorption measurements are taken at a primary wavelength of 510 nm and a blanking wavelength of 700 nm.

**HDL-cholesterol** (catalogue number CH2652, Randox Laboratories Ltd., Crumlin, UK)

The assay consists of 2 distinct reaction steps:

1. Elimination of chylomicron, VLDL-cholesterol and LDL-cholesterol by cholesterol esterase, cholesterol oxidase and subsequently catalase.

\[
\text{cholesterol ester} \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{fatty acids}
\]

\[
\text{cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholestenone} + H_2O_2
\]

\[
2 \text{H}_2O_2 \xrightarrow{\text{catalase}} 2 \text{H}_2O + O_2
\]

2. Specific measurement of HDL-cholesterol after release of HDL-cholesterol by detergents in reagent 2.

\[
\text{cholesterol ester} \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{fatty acids}
\]

\[
\text{cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholestenone} + H_2O_2
\]

\[
2 \text{H}_2O_2 + 4\text{-aminoantipyrine} + N\cdot(2\text{- hydroxy} - 3\text{ sulfopropyl}) - 3,5\cdot \text{dimethoxyaniline} \\ \xrightarrow{\text{peroxidase}} \text{quinoneimine} + 4 \text{H}_2O
\]

The intensity of the red quinoneimine dye produces is directly proportional to the cholesterol concentration when measured at 600 nm.

In the second reaction catalase is inhibited by sodium azide in Enzyme reagent 2.

The assay uses a Rate method and a single point calibration.

**NEFA** (catalogue number FA115, Randox Laboratories Ltd.)

\[
\text{NEFA} + \text{ATP} + \text{CoA} \xrightarrow{\text{acyl CoA synthetase}} \text{Acyl CoA} + \text{AMP} + \text{PPi}
\]

\[
\text{Acyl CoA} + O_2 \xrightarrow{\text{acyl CoA oxidase}} 2,3\cdot\text{trans-enoyl-CoA} + H_2O_2
\]

\[
2 \text{H}_2O_2 + 4\text{-aminoantipyrine} + N\cdot\text{ethyl} - N\cdot(2\text{- hydroxy} - 3\text{ sulfopropyl}) \text{ m-toluidine} \\ \xrightarrow{\text{peroxidase}} \text{purple adduct} + 4 \text{H}_2O
\]
Appendix L Plasma insulin assay procedure

Kit protocol (catalogue number HI-14K, Millipore Ltd., Watford, UK)

For optimal results, accurate pipetting and adherence to the protocol are recommended.

Assay Set-Up, Day One
1. Pipet 300 μL of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4), 200 μL to Reference (Bo) tubes (5-6), and 100 μL to tubes 7 through the end of the assay.
2. Pipet 100 μL of Standards and Quality Controls in duplicate.
3. Pipet 100 μL of each Sample in duplicate. (NOTE: Smaller volumes of sample may be used when insulin concentrations are anticipated to be elevated or when sample size is limited. Additional Assay Buffer should be added to compensate for the difference so that the volume is equivalent to 100 μL, e.g., when using 50 μL of sample, add 50 μL of Assay Buffer). Refer to Section IX for calculation modification.
4. Pipet 100 μL of hydrated 125I-Insulin to all tubes. Important: For preparation, see Section III, Part C.
5. Pipet 100 μL of Human Insulin antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
6. Vortex, cover, and incubate overnight (20-24 hours) at room temperature (22-25°C).

Day Two
7. Add 1.0 mL of cold (4°C) Precipitating Reagent to all tubes (except Total Count tubes).
8. Vortex and incubate 20 minutes at 4°C.
9. Centrifuge, 4°C, all tubes [except Total Count tubes (1-2)] for 20 minutes at 2,000-3,000 xg. NOTE: If less than 2,000 xg is used or if slipped pellets have been observed in previous runs, the time of centrifugation must be increased to obtain a firm pellet (e.g., 40 minutes). Multiple centrifuge runs within an assay must be consistent.
Conversion of rpm to xg:
\[ xg = (1.12 \times 10^{-5}) \times (r)^2 \]
r = radial distance in cm (from axis of rotation to the bottom of the tube)
rpm = revolutions per minute
10. Immediately decant the supernate of all tubes except Total Count tubes (1-2), drain tubes for at least 15-60 seconds (be consistent between racks) and blot excess liquid from lip of tubes. NOTE: Invert tubes only one time. Pellets are fragile and slipping may occur.
11. Count all tubes in a gamma counter for 1 minute. Calculate the μU/mL of Human Insulin in unknown samples using automated data reduction procedures.

Conversion to SI units: 1 μU Insulin / mL = 6 pM
Appendix M Karolinska Sleepiness Scale

Identification code: _________________

Date: _________________

Time of rating: _________________

Please indicate your sleepiness during the 10 minutes before this rating.

<table>
<thead>
<tr>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very alert</td>
<td>1</td>
</tr>
<tr>
<td>Alert - normal level</td>
<td>2</td>
</tr>
<tr>
<td>Neither alert nor sleepy</td>
<td>3</td>
</tr>
<tr>
<td>Sleepy, but no effort to keep awake</td>
<td>4</td>
</tr>
<tr>
<td>Very sleepy, great effort to keep awake</td>
<td>5</td>
</tr>
</tbody>
</table>

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Date: _________________

Time of rating: _________________

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</tr>
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<td>5</td>
</tr>
</tbody>
</table>
Appendix N Case study subject S8

Subject S8 is 38 years of age, worked night shifts for 7 years, but quit shift work 1.5 years ago. Some of his screening results are: PER3^{45}, HÖ: 71, PSQI: 6, BDI: 17, ESS: 7 (see appendix F for more details).

The saliva melatonin profiles before and after baseline sleep suggest that subject S8 is phase advanced (Figure 1). However, the week prior to the study, get up time and actual bed time are fairly regular and correspond to the agreed times (Figure 2). Moreover, the subject has called the lab voicemail every day at the correct times, indicating he was awake at these times. The agreed bed time was 24:00 h and the subject's habitual bed time was 23:00. The agreed wake up time was 7:30 h although the subject says he often wakes up around 4:00 h. However, the subject reports he is 'unable to get 8 h' since he quit shift work and is keen on trying a regular sleep/wake cycle with bed time at 24:00 and a 7.5 h sleep opportunity. Despite his efforts to try and adhere to the agreed sleep/wake cycle, the subject indicates in his sleep diary the week prior to the laboratory session that he woke up almost every day around 5:30 h but did not get up until 7:30 h. In addition, on the majority of the days the subject wrote down he would like to have napped between 20:30 and 21:30 h but was unable to (probably because this was outside the designated 'napping-window'). This agrees with the low level of activity in the actograms around this period.

These observations indicate that subject S8 may be an 'early type' but does not have an habitual sleep/wake cycle according to this, either as he may not know what his optimal cycle is because of former shift work or due to social commitments.

Figure 1. Saliva melatonin profiles of subject S8. On the evening before baseline sleep (from 4 – 0.5 h before bed time) and in the morning following baseline sleep (from 0 – 4.75 h after waking).
Figure 2 A. Actogram from the wrist actiwatch from subject S8 the week prior to the laboratory session.
The actogram is double plotted with the time of day on the x-axis and the height of the vertical bars indicating the amount of activity (black) and light exposure (yellow) on the y-axis.

Figure 2 B. Actogram from the neck actiwatch from subject S8 the week prior to the laboratory session.
The actogram is double plotted with the time of day on the x-axis and the height of the vertical bars indicating the amount of activity (black) and light exposure (yellow) on the y-axis.